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Immunity surveillance of mumps and rubella: improved methods for the detection of virus-specific antibody

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Abstract

The aim of these studies was to improve laboratory methods for the detection of virus-specific antibody to mumps and rubella. The presence of virus-specific antibody is indicative of immunity to disease so simple and effective antibody detection allows for the planning and monitoring of immunisation programmes.

In facilitating antibody surveillance, oral fluid has advantages as a sample compared with blood. It is simple, safe and cheap to collect and being non-invasive encourages subject recruitment. In this study, an 'IgG' antibody capture enzyme-linked immunosorbent assay (GACELISA) was developed and evaluated for the detection of mump-specific IgG in oral fluid. Compared to an indirect commercial ELISA for the detection of mumps-specific IgG in serum, the oral fluid GACELISA was 100% sensitive and specific. The GACELISA should therefore be useful for future antibody prevalence studies.

The limitation of oral fluid samples compared with blood are that they contain lower antibody concentrations. Immuno-polymerase chain reaction (I-PCR) is an ultrasensitive method and in this study was adapted to detect antibodies to mumps virus. Though the method was shown to be feasible for antibody detection and quantification, its sensitivity and specificity did not exceed that of a conventional ELISA. Sensitivity was limited by non-specific binding of human IgG to the solid phase. It is necessary to further develop reagents and assay formats to fully exploit the potential of quantitative I-PCR, so that potential improvements in the sensitivity of viral-specific IgG detection can be realised.

Increasingly, recombinant antigens are being employed in ELISAs as cell culture antigens are difficult and expensive to produce, and are potentially infectious. In this study, the PinPoint^{TM} Xa-1 T-Vector system was used to produce recombinant rubella virus (RV) E1 fusion proteins in *Escherichia coli*. Their antigenicity was assessed by Western blotting and ELISA. One of these antigens may be a suitable reagent for immunity studies as it reacted with RV E1-specific monoclonal antibodies (MAb's) and a high percentage (80%) of RV antibody positive sera.

Publications arising from this thesis

M^{c}Kie, A., Samuel, D., Cohen, B. and Saunders, N.A. (2002) Development of a quantitative immuno-PCR assay and its use to detect mumps-specific IgG in serum. *Journal of Immunological Methods* **261**, 167-75.

M^{c}Kie, A., Samuel, D., Cohen, B. and Saunders, N. (2002) A quantitative immuno-PCR assay for the detection of mumps-specific IgG. *Journal of Immunological Methods* **270**, 135-41. Protocol.

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Abbreviations

ALP	Alkaline phosphatase
BCCP	Biotin carboxyl carrier protein
bp	Base pairs
BSA	Bovine serum albumin
C	Capsid protein
cDNA	Complementary DNA
CCDC	Consultant for Communicable Disease Control
CDSC	Communicable Disease Surveillance Centre
CF	Complement fixation
COVER	Cover of Vaccination Evaluated Rapidly
CI	Confidence interval
CNS	Central nervous system
CnBr	Cyanogen bromide
CPE	Cytopathic effect
CRS	Congenital Rubella Syndrome
CSF	Cerebrospinal fluid
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DMF	Dimethylformamide
DNA	Deoxyribose nucleic acid
dNTP	Deoxynucleotide triphosphate
ds	Double stranded
DTCS	Dye Terminator Cycle Sequencing
DTT	Dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
E1	E1 glycoprotein
E2	E2 glycoprotein
EBV	Epstein Barr virus
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
ERNVL	Enteric, Respiratory and Neurological Virus Laboratory

F	Fusion protein
FAM	Carboxyfluorescein
FCS	Foetal calf serum
FITC	Fluoroscein isothiocyanate
FRET	Fluorescence Resonance Energy Transfer
GACELISA	IgG antibody capture enzyme-linked immunosorbent assay
GACRIA	IgG antibody capture radioimmunoassay
GST	Glutathione-S-transferase
h	Hour
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCl	Hydrochloric acid
HPA	Health Protection Agency
HI	Haemagglutination inhibition
HIG	Haemolysis-in-gel
HIV	Human immunodeficient virus
HN	Haemaglutinin-neuraminidase
HRP	Horseradish peroxidase
IBD	Inflammatory Bowel Disease
Ig	Immunoglobulin
I-PCR	Immuno-PCR
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
IU	International units
JCD	Juvenile Crohn's Disease
kb	kilo base pairs
kDa	Kilodalton
L	Litre
LB	Luria-Bertani
LP	Large protein
log	Logarithmic
M	Matrix protein
MACRIA	IgM antibody capture radioimmunoassay
$MgCl_2$	Magnesium chloride
MT	Mean titre

min	Minute
MAb	Monoclonal antibody
ml	Millilitres
MMR	Measles, Mumps and Rubella
MSC	Mean serum control
MW	Molecular weight
MV	Mumps virus
nm	Nanometres
NaCl	Sodium chloride
NCP	Nitrocellulose paper
NGS	Normal Goat Serum
NP	Nucleocapsid-associated protein
NS	Nonstructural
NT	Neutralising
OD	Optical density
ON	Overnight
ONS	Office of National Statistics
ORF	Open reading frame
P	Phosphoprotein
PCR	Polymerase chain reaction
PHLS	Public Health Laboratory Service
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing 0.1% Tween 20
PRN	Plaque neutralisation
PV	PinPoint^{TM} Xa-1 T-vector
RBC	Red blood cell
RCGP	Royal College of General Practitioners
rpm	Revolutions per minute
RNA	Ribonucleic acid
rNP	Recombinant nucleocapisd-associated protein
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
RV	Rubella virus
S	Soluble protein

sec	Second
S4B	Sepharose 4B
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH	Small hydrophobic protein
SMCC	Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate
SPDP	N-succinimidyl-3- (2-pyridyldithio) propionate
SRMD	Specialist and Reference Microbiology Division
ss	Single stranded
Strep-ALP	Steptavidin-alkaline phosphatase conjugate
TCEP-HCl	Tris (2-carboxyethyl)phosphine hydrochloride
tDNA	Target DNA
T20	Tween 20
TBE	Tris-borate buffer
TCID	Tissue culture infectious dose
tDNA	Target deoxyribose nucleic acid
Taq	*Thermus aquaticus* DNA polymerase
T_m	Melting temperature
TMB	Tetramethylbenzidene
T/N	Test to negative ratio
Tris	Tris(hydroxymethyl) methylammonium chloride
U	Units
UK	United Kingdom
USA	United States of America
V	Viral protein
WHO	World Health Organisation
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1

General Introduction

1.1 Mumps

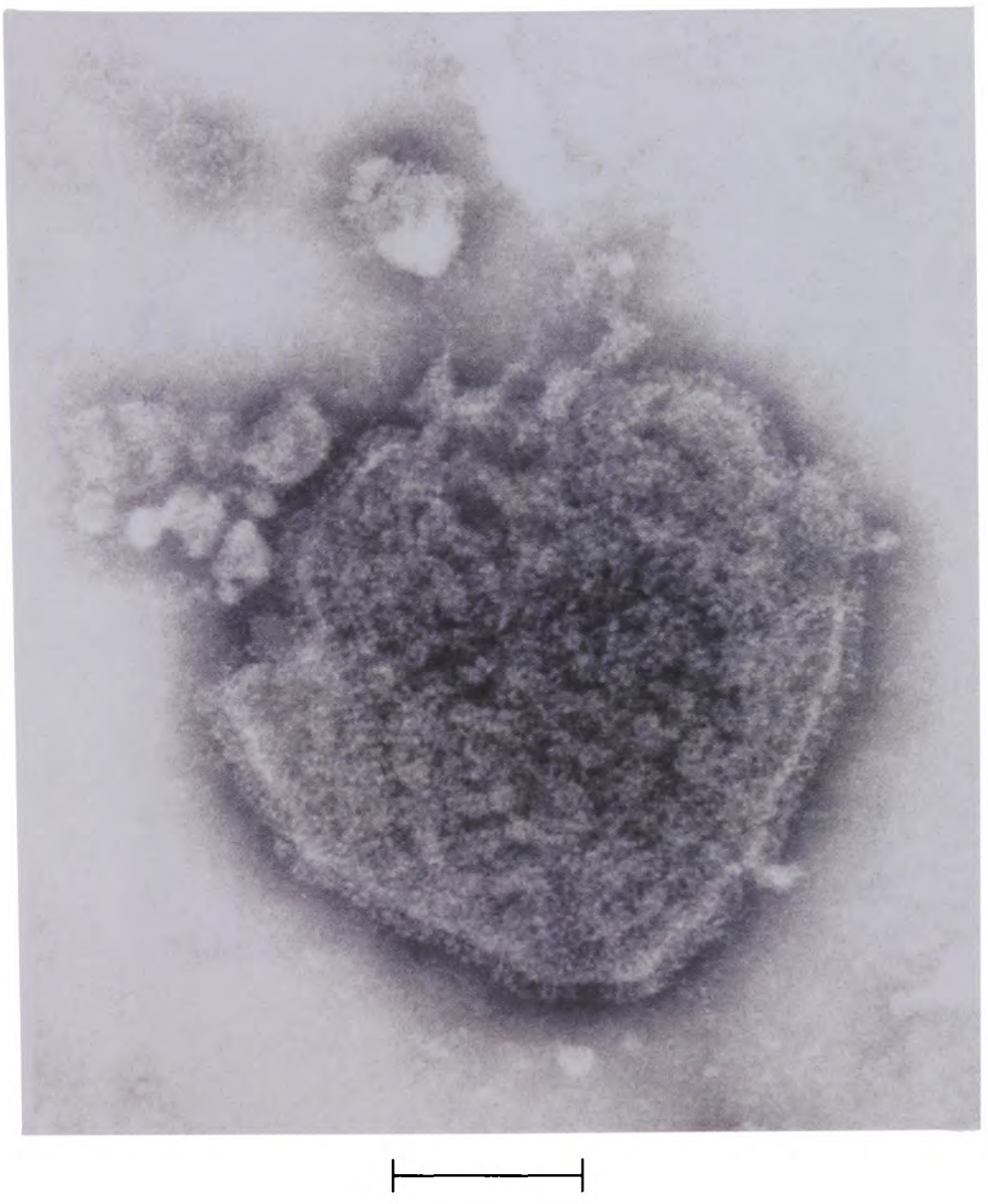
1.1.1 History

Mumps was first described in the fifth century B.C. by Hippocrates (in Book 1 of his *Book of Epidemics*). He noted the characteristic swelling of the salivary glands (parotitis) and the common complication of painful swelling in one or both testes (orchitis). The origin of the word *mumps* is unclear but may derive from the old English verb meaning "grimace, grin or mumble" (cited Carbone and Wolinsky, 2001). In 1790, the physician Hamilton was the first to describe infection of the central nervous system (CNS) in some cases of mumps. In 1934, Johnson and Goodpasture showed that the disease could be transmitted from infected patients to monkeys. They also demonstrated the filtrable nature of the causative agent. The virus was first isolated and propagated in embryonated eggs in 1945 by Habel, and in cell culture in 1955 by Henle and Deinhardt. This led to the development of live attenuated vaccines that have been used to control mumps (Buynak and Hilleman, 1966).

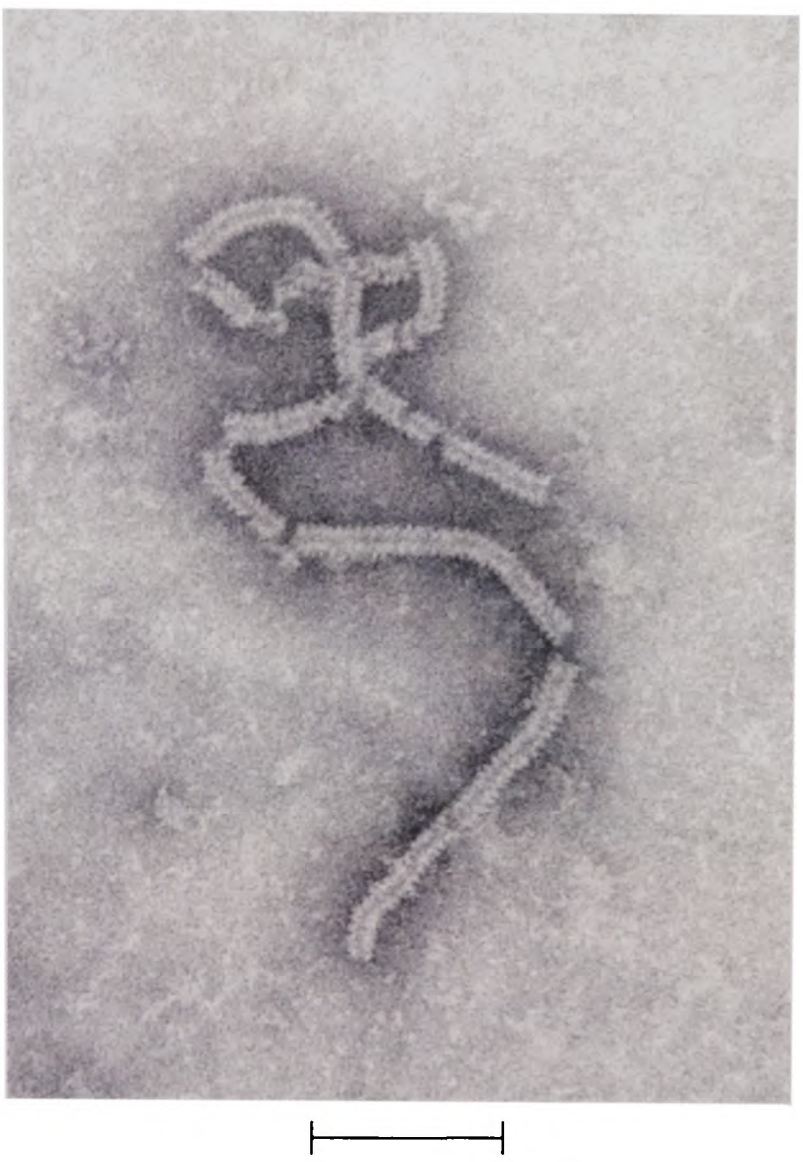
1.1.2 Mumps virus

Mumps virus (MV) is a member of the genus *Rubulavirus* in the family *Paramyxoviridae* (Rima *et al.*, 1995). The virion has an irregular spherical shape, with a diameter ranging from 90 to 300 nm and averaging approximately 200 nm. It consists of an internal ribonucleoprotein core (nucleocapsid) surrounded by a lipid bilayer membrane (Figure 1.1a). The nucleocapsid (Figure 1.1b) displays the characteristic herringbone pattern of *Paramyxoviridae* and contains the ribonucleic acid (RNA) genome covered with the nucleocapsid-associated protein (NP), the phosphoprotein (P) and the large protein (L) (Orvell, 1978). The lipid membrane is derived from the host cell. Embedded within this membrane is the matrix protein (M), which interacts with the NP protein and the viral glycoproteins, haemagglutinin-neuraminidase (HN) and fusion (F) protein (Jensik and Silver, 1976; Orvell, 1978). Spikes of 12 - 15 nm in length protrude from the membrane and these contain the viral glycoproteins in homo- or hetero oligomeric complexes.

Figure 1.1 Electron microscopy of Mumps virus (Magnification x 200, 000).



(a) Mumps virion studded with fine surface projections that represent the viral glycoprotein spikes. The membrane of the virion has partially broken allowing the escape of the nucleocapsid. Bar = 100nm.



(b) Mumps nucleocapsid showing the typical herringbone pattern. Bar = 100nm (Magnification x 200,000).
Courtesy of Dr Hazel Appleton (Enteric, Respiratory and Neurological Virus Laboratory, Central Public Health Laboratory, Colindale, London).

The RNA genome is an unsegmented, single stranded macromolecule of negative polarity that contains 15,384 nucleotides. It encodes seven genes, the sequence of which are known (Waxham *et al.*, 1987; Waxham *et al.*, 1988; Elango *et al.*, 1989; Elliott *et al.*, 1989; Kovamees *et al.*, 1989; Yamada *et al.*, 1989). The order of the genes is: 3'-NP-P-M-F-SH-HN-L-5' (Elango *et al.*, 1988). As well as the six structural proteins mentioned above the virus encodes a small hydrophobic (SH) protein (Elango *et al.*, 1989; Elliott *et al.*, 1989) and induces the synthesis of the nonstructural (NS) proteins, NS2 and NS1, from transcripts of the P gene (Takeuchi *et al.*, 1988; Elliott *et al.*, 1990; Paterson and Lamb, 1990). Many workers have contributed to our understanding of the structural polypeptides of MV (Jensik and Silver, 1976; Huppertz *et al.*, 1977; Orvell, 1978; Merz and Wolinsky, 1981; Naruse *et al.*, 1981; Herrler and Compans, 1982; Server *et al.*, 1982; Merz *et al.*, 1983), the findings of which are summarised in Table 1.1.

Table 1.1 Mumps virus proteins and their properties

Protein	Molecular mass in SDS-PAGE (kDa)	Biologic activity
Nucleocapsid-associated protein	68 - 73	Major nucleocapsid-associated protein; protects genome from cellular ribonucleases
Phosphoprotein	45 - 47	Phosphorylated protein associated with the nucleocapsid; forms part of the transcriptase complex
Matrix	39 - 42	Membrane-associated protein; involved in nucleocapsid alignment
Fusion	65 - 74	Surface glycoprotein with fusion activity; mature form is a disulphide-linked complex of F_1 (58-61 kd) and F_2 (10-16 kd)
Small hydrophobic	6	Hydrophobic membrane-associated protein
Haemagglutinin-neuraminidase	74 - 80	Surface glycoprotein with haemagglutinin and neuraminidase activity; disulphide-linked oligomer in native state
Large	180 - 200	Nucleocapsid-associated protein, contributes to RNA-dependent RNA-polymerase

Adapted from Carbone and Wolinsky (2001).
SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis
kDa, kilodalton

1.1.3 Epidemiology

Mumps is mainly a childhood disease that has been reported worldwide. Before widespread immunisation, immunity to mumps was mostly acquired between the ages of five and nine years, and the prevalence of immunity was higher in densely populated urban areas compared with rural communities (Bennett *et al.*, 1975; Anderson *et al.*, 1987). In the Northern Hemisphere most cases occurred in the late winter and early spring months. The clustering of cases presumably reflected increased contact rates among susceptible children attending school. Outbreaks of mumps were common among military personnel, where the potential for transmission to susceptible individuals was high due to overcrowding (Brooks, 1918).

MV needs a population of about 200,000 people to sustain it. The attack rate in virgin populations (reproduction number [number of secondary cases arising from a typical primary case] R = 5) suggests the virus is highly contagious, similar to influenza and rubella but less than measles (R = 16 – 450) and chickenpox. The infectious period is considered to be from three days before to approximately the fourth day of active disease (Brunell *et al.*, 1968). While persons with asymptomatic or nonclinical infection can transmit the virus, no carrier state is known to exist.

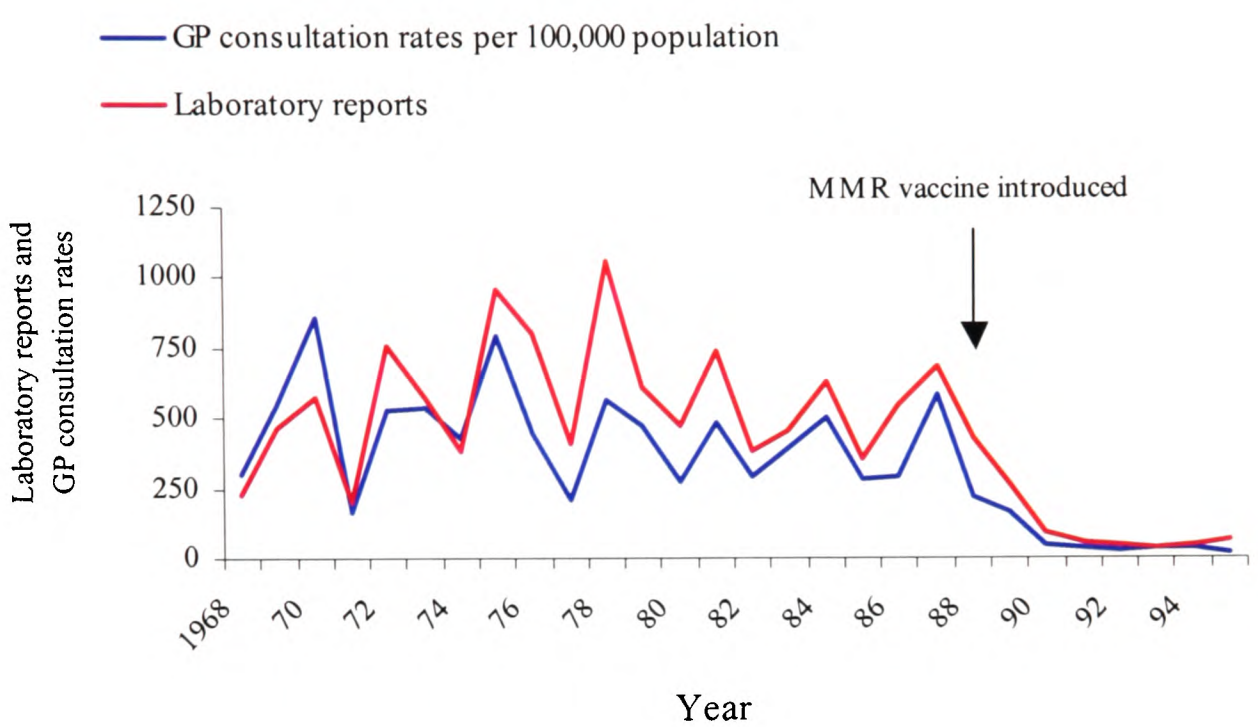
The incubation period of infection with MV is usually 16 to 18 days, but symptoms may occur as early as 14 or as late as 21 days after exposure (Reed *et al.*, 1967). Placental transfer of mumps antibody does occur, and probably provides protection in the early months of life. The major morbidity from mumps primarily results from complications of meningitis, encephalitis and orchitis. In the prevaccine era, mumps was the commonest identified cause of viral meningitis in children in England and Wales; there were approximately 1,200 hospital admissions a year associated with the disease (Anon, 1990a).

Humans are the only known natural hosts for the virus, although animal models have been described, including, mice (Tsurudome *et al.*, 1987), rats (Rubin *et al.*, 1998) and monkeys (Rubin *et al.*, 1999). Only one serotype of MV has been identified but the virus cross-reacts serologically with other members of the *Paramyxovirdae* family, particularly the human parainfluenza viruses (Ito *et al.*, 1987).

In industrialised areas of the world (e.g. the UK, USA and Scandinavia) mumps has been brought under control by the use of a safe and effective vaccine. Figure 1.2 shows the dramatic fall in the number of clinically diagnosed mumps cases reported to the Royal College of General Practitioners (RCGP) and the number of laboratory reports received by

the Communicable Disease Surveillance Centre (CDSC) following the introduction of the measles, mumps and rubella (MMR) vaccine in the UK.

Figure 1.2 Mumps incidence (RCGP) and number of confirmed cases reported to CDSC from 1968 to 1996.



Sources: data for 1968 – 1981 (Galbraith *et al.*, 1984); data for 1982 – 1996 CDSC, Colindale, UK.

Despite high uptake rates of vaccine, outbreaks are still reported, sometimes due to importations from areas where the virus is endemic. Cases in vaccinated people are thought to result from both primary (failure to seroconvert) and secondary (loss of antibody) vaccine failures (Briss *et al.*, 1994). Fortunately, vaccine failure is uncommon in individuals who receive two doses of vaccine (Hersh *et al.*, 1991); the policy that has been instituted in most industrialised countries. Factors known to adversely affect vaccine "take" include: pre-existing antibodies, either of maternal or passively transferred origin; the presence of interferon due to ongoing viral infection at the time of vaccination; and loss of vaccine potency due to improper handling or cold storage (Gluck, 1991).

1.1.4 Pathogenesis and clinical features

MV is transmitted by direct contact with saliva or droplets from the saliva of an infected person. During the incubation period the virus replicates in the nasopharynx, spreads to the draining lymph nodes and then disseminates to multiple organs via the bloodstream.

The severity of mumps infection is very variable. Approximately one third of all infections occur without producing noticeable symptoms, particularly in children (Philip *et al.*, 1959; Levitt *et al.*, 1970a). The prodromal symptoms are non-specific and include low-grade fever, headache, malaise, myalgia and anorexia. The most common manifestation of the disease is salivary gland swelling, particularly the parotid glands, which may be unilateral or bilateral. Gland enlargement tends to occur within the first two days and may be present for a week or more.

Various organs can be infected during mumps infection. CNS involvement is common, occurring asymptomatically in 40 – 60 % of cases (Bang and Bang, 1943; Holden, 1946; Levitt *et al.*, 1970b). Symptomatic meningitis occurs in up to 15% of cases and usually resolves without sequelae in three to ten days. Encephalitis is reported to occur in approximately 0.5% of cases (Bennet *et al.*, 1975). Orchitis (inflammation of the testes) occurs in 20 to 35% of males, especially after puberty, (Philip *et al.*, 1959; Reed *et al.*, 1967) and may lead to testicular atrophy but sterility is rare (Shulman *et al.*, 1992). In females, oophritis (inflammation of the ovaries) may occur but is much less common (5%) than orchitis in males (Reed *et al.*, 1967). It is accompanied by pain and tenderness in the abdomen and may mimic appendicitis. There is no relationship to impaired fertility. About 15% of females also complain of mastitis (breast swelling and tenderness) (Philip *et al.*, 1959). Other organs that may be clinically involved during mumps infection include, the eyes, heart, kidneys, pancreas, prostate, liver, spleen, thyroid, lungs, bone marrow and joints. All organs are generally affected after parotitis, but their involvement may be present before, during or even in the absence of parotitis.

1.1.3 Immune response

The host immune response to MV infection consists of cell-mediated and humoral responses. The relative importance of these responses for the clearance of MV from the infected host is unclear. Virus-specific IgM responses can be demonstrated early in the infection and usually wane within two to six months (Bjorvatn, 1974; Ukkonen *et al.*, 1981). The detection of viral-specific IgM antibody is the most sensitive serological indicator of acute or very recent infection. Virus-specific IgG responses reach peak levels

about the third week of clinical disease and are thought to persist indefinitely (Ukkonen *et al.*, 1981). The major IgG response is composed of the IgG1 subclass, with a minor contribution from IgG3 (Sarnesto *et al.*, 1985a). Virus-specific secretory IgA can be detected five days after disease onset (Chiba *et al.*, 1973) and most likely terminates viral excretion in saliva.

Both cytotoxic T cells and lymphoproliferative responses have been detected after MV infection. Kreth *et al.* (1982) demonstrated the presence of cytotoxic T cells reactive with MV in the blood and the CSF of patients with mumps meningitis. Ilonen (1979) using the blast transformation test detected *in vitro* lymphoproliferative responses to MV antigens in seropositive individuals.

Re-infection has been reported but is uncommon. Symptomatic reinfection may reflect waning vaccine induced immunity (Briss *et al.*, 1994) or patients may be infected with a different strain of virus. Nojd *et al.* (2001) described a chronic case of mumps where despite having a good neutralisation titre against genotype D, the patient was not protected against infection with genotype A. Alternatively, putative symptomatic reinfection in individuals with pre-existent antibody may reflect the lack of specificity of mumps tests due to known cross-reactivities of antibodies to other paramyxoviruses with MV antigens (Meurman *et al.*, 1982; Orvell *et al.*, 1986).

1.1.6 Diagnosis

The clinical diagnosis of mumps is based on presenting symptoms, particularly parotitis. Laboratory confirmation is often sought, which may involve isolating virus; detecting viral genome by reverse transcriptase polymerase chain reaction (RT-PCR); demonstrating a diagnostic rise in specific IgG antibodies; or detecting specific IgM antibodies.

Virus is most often isolated from respiratory secretions or urine. In patients with meningitis, virus can be isolated from the CSF. A variety of primary and continuous cell lines may be used for virus isolation, including primary monkey kidney cells, human embryonic kidney cells, HeLa cells, HEp-2 cells and B95a cells (Carbone and Wolinsky, 2001; Knowles and Cohen, 2001). However, not all mumps strains produce cytopathic effects (CPEs) *in vitro* so haemadsorption using guinea-pig red blood cells (RBCs) and/or immunofluorescence may be necessary to confirm the presence of mumps. Haemadsorption occurs because the infection of cells in culture results in the expression of viral proteins on the cell surface that bind to erythrocyte membranes. Immunofluorescence detects mumps antigens by staining infected cells with virus-specific monoclonal antibody (MAb) labelled with a fluorescent tag such as fluorescein isothiocyanate (FITC). Virus

isolation is advantageous as it provides an isolate of viable virus that can be further characterised and stored for future studies. However, as a diagnostic method it is slow and expensive; it requires specialised facilities and expertise.

RT-PCR can be used to detect virus isolated in cell culture or directly from clinical specimens (Boriskin Yu *et al.*, 1993). Sequencing of the amplified fragments can provide useful epidemiological data and virus strain identification. The SH gene has been shown to be highly variable between strains and comparison of the nucleotide sequences of this gene from mumps strains collected worldwide has shown the existence of ten genotypes, namely A - J (Afzal *et al.*, 1997; Wu *et al.*, 1998; Jin *et al.*, 1999; Kim *et al.*, 2000; Tecle *et al.*, 2001).

A wide range of assays are available to demonstrate a diagnostic (four-fold) rise in antibodies in serum taken at the onset of clinical disease (acute-phase) and 2 to 3 weeks later (convalescent-phase). These include plaque reduction neutralisation (PRN), haemagglutination inhibition (HI), haemolysis-in-gel (HIG), complement fixation (CF), and ELISA (Grillner and Blomberg, 1976; Freeman and Hambling, 1980). PRN, HI, HIG and CF are functional assays as they measure a biological function of the virus-specific antibody. PRN measures the capacity of mumps antibody in serum to block the infection of cells in culture. HI is based on the ability of MV to haemagglutinate RBCs. If specific antibodies are incubated with MV before the addition of RBCs, they will prevent haemagglutination. HIG, like the HI assay, relies on the presence of antibody to the MV haemagglutanin. Antibody detection is based on complement-mediated lysis of MV antigen-sensitised RBCs suspended in an agarose gel. CF measures the ability of mumps antibodies to fix complement, preventing the complement from lysing indicator RBCs. Two different antigens are routinely used in CF tests: the viral (V) and the soluble (S) which correspond to the HN and NP of mumps respectively (Jensik and Silver, 1976).

Although the techniques described above have been widely used in the past, they are increasingly being replaced by the ELISA. ELISA is a relatively sensitive, specific, rapid, and cost effective method that can be automated. Also, it allows for isotype determination (i.e. IgG, IgM or IgA). Various ELISA formats have been described including the indirect, competitive, sandwich and antibody capture. They all rely on the binding of virus-specific antibodies to viral antigens. ELISA for virus-specific IgM antibodies is important as it allows a specific diagnosis to be made on the basis of a single serum specimen collected during the acute-phase of illness.

1.1.7 Control

In developing countries mumps is not considered a major public health problem. In these countries, children usually are immune by the time they enter school because of subclinical infections acquired early in life. By contrast, extensive cost-benefit analysis favours the routine vaccination of children against mumps in affluent developed nations (Koplan and Preblud, 1982; Galazka *et al.*, 1999).

(i) Mumps vaccines

The first mumps vaccine developed was killed vaccine, which was licensed for use in 1950. However it produced only short-lived immunity and required periodic booster injections, so when the more effective live attenuated vaccines were developed, killed vaccine was no longer used (Plotkin and Wharton, 1999).

Several different strains of live attenuated MV have been developed for vaccine use, including Jeryl Lynn, Urabe, and Rubini. The Jeryl Lynn was the first vaccine strain to be developed (by Merck Sharpe and Dohme) and was attenuated by serial passage in embryonated hens' eggs and chick embryo cell cultures (Buynak and Hilleman, 1966). Over 100 million doses of vaccine manufactured with this strain have been administered worldwide since it was first licenced for use in 1967. More recently, the Jeryl Lynn vaccine has been shown to contain a mixture of at least two closely related strains, JL5 and JL2, at a ratio of five to one respectively (Afzal *et al.*, 1993). A new vaccine (RIT 4385) derived from the JL5 component has recently been licenced in Europe (cited Carbone and Wolinsky, 2001)

The Urabe vaccine was developed in Japan in 1982 by the Biken Institute and has been used in Japan, North America and Europe. However, it has been removed from many markets, as its use has been associated with an increased incidence of meningitis (Hockin and Furesz, 1988; Furesz and Contreras, 1990; Sugiura and Yamada, 1991). For example, in the UK, Miller *et al.* (1993) reported 23 cases of aseptic meningitis (estimated at 1 in 11,000 doses) associated with Urabe vaccine compared with no cases after vaccination with the Jeryl Lynn vaccine. These findings led to the withdrawal of mumps vaccine containing the Urabe strain from immunisation schedules in the UK. More recently, Brown *et al.* (1996) showed that the Urabe vaccine was a mixture of viruses differing at amino acid 335 of the HN gene, possessing either wild type Lys335 or mutant Glu335, while clinical isolates were homogeneous and possessed the wild type Lys335. They suggested that post-vaccine meningitis was associated with virus containing the wild type Lys335.

Comparative immunity studies have shown the Urabe vaccine strain is more efficacious than the Jeryl Lynn vaccine strain. Poppw-Kraupp *et al.* (1986) measured the antibody responses of 400 children by ELISA and showed 96.9% of children seroconverted when they received the Urabe vaccine compared to 90% of the Jeryl Lynn vaccine recipients. Miller *et al.* (1995) showed children given vaccine containing the Urabe vaccine strain were less likely to be antibody negative than those given the Jeryl Lynn vaccine strain. Boulianne *et al.* (1995) showed the prevalence of children without detectable antibody levels were 14.9 and 7% for the Jeryl Lynn and Urabe vaccine strains respectively. These results are consistent with the idea that the Urabe vaccine strain disseminates more widely in the host than the Jeryl Lynn vaccine strain.

The Rubini vaccine was developed in Switzerland in 1986 and is the only vaccine produced on human diploid cells. Although it was initially shown to be highly immunogenic, the clinical efficacy of the Rubini strain has been questioned following outbreaks in Switzerland (Schlegel *et al.*, 1999), Portugal (Afzal *et al.*, 1997) and Singapore (Goh, 1999). As a result of all the above findings, in the USA, Canada, the UK and several European countries, mumps vaccines contain the Jeryl Lynn vaccine strain.

Mumps vaccines have been produced as monovalent, bivalent measles-mumps and trivalent measles-mumps-rubella (MMR) preparations as health authorities recommend all three vaccines to be given to the same age group. In clinical studies to assess the efficacy of the trivalent vaccine, it was found that simultaneous administration of all three components at the same dosage levels [1000 Tissue culture infective dose ($TCID_{50}$) = highest dilution of virus suspension which produces a CPE in 50% of the cell cultures inoculated] as for the monovalent preparations resulted in a substantial reduced seroconversion rate for mumps, but not for measles or rubella. To address this, the number of viable mumps viruses in the trivalent preparation was increased to 5000 $TCID_{50}$ (Andre and Peetermans, 1986; Berger *et al.*, 1988). Despite this, the results of immunity studies suggest the mumps strain is still the weakest immunogenic part of the combined vaccine (Christenson and Bottiger, 1991; Gay *et al.*, 1997). In the UK there are two licensed MMR vaccines: Priorix (Smithkline Beecham) and MMR II (Aventis Pasteur). Both contain the Jeryl Lynn strain of mumps. There is no single antigen mumps vaccine licensed for use in the UK.

Post vaccination antibody titres develop more slowly and are lower than those following natural infection. Weibel *et al.* (1967) found the mean neutralisation antibody titres were 1:9 and 1:6 following vaccination and natural disease respectively. It is unclear whether a

single dose of properly administered vaccine provides life-long immunity. Neutralising antibody has been shown to persist for more than 19 years (Plotkin and Wharton, 1999) but these subjects may have been exposed to mumps during this time resulting in a boosting of their antibody levels.

(ii) Adverse reactions

Mumps vaccines, alone or in combination, are generally considered to be safe. Adverse reactions are uncommon and mild. The most common adverse reactions are transient fever and mild parotitis and orchitis.

(iii) Vaccination programmes

In the UK, immunisation against mumps was first introduced in October 1988 as one component of the MMR vaccine offered to children aged 12 to 15 months with the aim of eliminating the disease. In addition, all preschool children were offered the vaccine in a three-year catch up programme; some districts extended the programme to children aged 5 to 10 years. In the first two years of the vaccine programme, there was a 79% reduction in notified cases in England and Wales and the vaccination coverage exceeded 90% in some areas (Jones *et al.*, 1991). Despite this, Miller *et al.* (1995) showed that between 15 and 19% of children vaccinated at age 12 to 18 months were seronegative 4 years after vaccination. Concerns raised by this and other studies led to the adoption of a two-dose regimen in October 1996, where the first dose is administered at 12 to 15 months of age, and the second dose is given before school entry at 4 to 5 years of age. Other industrialised countries have adopted a similar immunisation policy, although some administer the second dose at 11 to 13 years of age (Johnson *et al.*, 1996). In developing countries with no previous vaccination programme, the World Health Organsiation (WHO) recommends that the introduction of mumps vaccine be considered only if the country is in the process of establishing adequate programmes for the elimination of measles and rubella.

1.1.8 Mumps outbreaks

Despite the overall reduction in the incidence of mumps, outbreaks of mumps have been reported in the UK (van den Bosch *et al.*, 2000; Reaney *et al.*, 2001) and elsewhere (Briss *et al.*, 1994; Strohle *et al.*, 1997). Between July 1998 and April 1999, 144 cases of mumps were notified in North London; all but two were members of a religious community (van den Bosch *et al.*, 2000). The median age of cases was 10 years and approximately half of

both the clinical and laboratory confirmed cases had received one dose of MMR vaccine, while only three cases had received two doses. The immunisation coverage for the first and second doses of MMR vaccine at the time was between 67% and 86% in this particular community. Around the same time as this outbreak there were outbreaks in similar religious groups in Israel, New York and Russia. Members of these communities often travel between these centres but do not mix much outside there own community. This may explain why the outbreak spread to similar communities in different countries but not outside the communities locally.

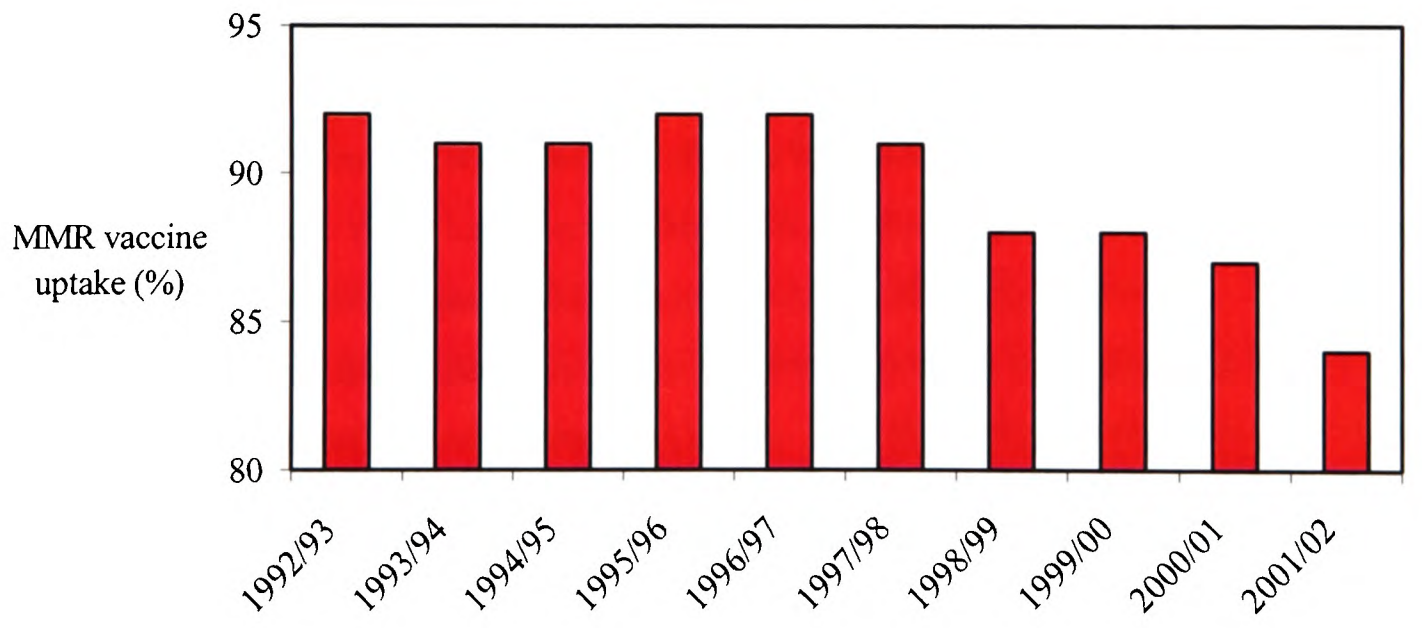
An outbreak of mumps was also been recently reported in Northern Ireland (Reaney *et al.*, 2001). Between 1st November 1999 and 31st August 2000, 729 cases of mumps were notified. Of those who underwent confirmatory laboratory testing, infection was found in 77% (332/450). Three hundred and sixteen (95.2%) of these confirmed cases were in the age range 9 to 19, with a median of 10 years. One hundred and eighty-four (55.4%) confirmed cases had received one dose of MMR but only 3 (0.9%) confirmed cases had received 2 doses of MMR. Schools appeared to be the focus for the spread of infection, especially secondary schools where the children travelled longer distances to school, usually in crowded buses providing an opportunity for spread.

Detailed analysis suggested that these outbreaks were mainly a reflection of the pool of susceptible children, teenagers, and young adults who either missed or failed to respond to vaccine during the first years following the introduction of the one-dose policy. Using susceptibility data and mixture modelling, Gay *et al.* (1997) predicted that the proportion of children aged 11 to 15 years with no detectable antibody to mumps was expected to peak at 19% in 1997, and suggested that this increase in susceptibility was unlikely to allow a large resurgence of mumps in the short term but school outbreaks would become more common. Their predictions were correct. The outbreaks question the efficacy of the mumps vaccine and clearly show that one dose of vaccine does not give adequate protection against mumps. Many young people received measles and rubella vaccine in a mass vaccination campaign in 1994 but no second dose of mumps vaccine. If mumps had been included in this vaccination campaign the outbreaks detailed above would have been prevented.

In recent years, uptake rates of MMR have fallen (Figure 1.3) following adverse publicity about the vaccine. This stemmed from the suggestion that measles and MMR live attenuated vaccines may cause juvenile Crohn's disease (JCD) and autism (Thompson *et al.*, 1995; Wakefield *et al.*, 1998; Wakefield *et al.*, 1999). The proposed link initially emerged from epidemiological and case control studies that claimed a temporal relationship

between the rising numbers of JCD cases and the introduction of live attenuated measles vaccine in the UK (Thompson *et al.*, 1995; Wakefield *et al.*, 1999). However, other reports suggested that the observed rise in the number of inflammatory bowel disease (IBD) cases started much earlier than the introduction of the measles vaccination campaign (Hermon-Taylor *et al.*, 1995; Miller *et al.*, 1998; Taylor *et al.*, 1999). As well as the epidemiological evidence, the presence of measles virus in clinical tissues obtained from cases of JCD and ulcerative colitis has been reported (Wakefield *et al.*, 1993). However the specificity of the techniques used to detect measles has been questioned. Further studies using more specific techniques (gene-specific RT-PCR and nested RT-PCR) were used by a number of independent groups however none were able to demonstrate the presence of measles virus genome in gut tissues taken from IBD cases (Iizuka *et al.*, 1995; Haga *et al.*, 1996; Afzal *et al.*, 1998a; Afzal *et al.*, 1998b; Chadwick *et al.*, 1998). Also, more recent serological and epidemiological studies show a lack of association between measles or MMR vaccines and the development of IBD and autism (Feeney *et al.*, 1997; Peltola *et al.*, 1998). As a result the government continues to recommend MMR.

Figure 1.3 Completed Primary Courses of MMR vaccine at Two Years of Age in England and Wales, 1992 – 2002.



Source: Department of Health Statistics Division

1.2 Rubella

1.2.1 History

For many centuries rubella was confused with measles and scarlet fever as it is characterised by a rash. It was first described in the mid 18th century by two German physicians, de Bergen and Orlow and was known by the German name Rötheln (Wesselhoeft, 1947); it was due to the early interest of the German physicians and its cause of a measles-like illness (albeit a milder form) that the disease subsequently became known as "german measles". The name rubella, derived from the Latin meaning "little red", was proposed by the English physician Henry Veale in 1866 as he found the original name "harsh and foreign to our ears".

In 1914, Hess proposed the viral aetiology of rubella based on his work with monkeys. Hiro and Tasaka (1938) confirmed the viral aetiology of the disease by transmission of rubella to susceptible children using filtered throat washings from acute cases (cited Forbes, 1969).

Until relatively recently, rubella was considered to be a mild illness. However, following a widespread epidemic of rubella infection in Australia in 1940, Norman McAlister Gregg, an Australian ophthalmologist noted a striking increase in the number of infants with congenital cataracts (Gregg, 1941). He proposed that the cataracts and often associated cardiac defects were the result of maternal infection during pregnancy. Although at the time he was doubted, studies by other investigators supported his findings (Greenberg *et al.*, 1957; Lundstrom, 1962; Pitt and Keir, 1965) and further revealed a wide range of abnormalities associated with intra-uterine rubella infection.

The virus was first isolated in cell culture independently by Weller and Neva (1962) and by Parkman, Buescher and Artenstein (1962). This led to the development and subsequent licencing of live, attenuated virus vaccines in the US in 1969 and in Britain in 1970.

1.2.2 Rubella virus

Rubella virus (RV) is the only member of the genus *Rubivirus* in the family *Togaviridae*. The RV virion contains an RNA genome enclosed in an icosahedral capsid, which is surrounded by a lipid bilayer. The nucleotide sequence and organisation of the RV genome have been resolved (Dominguez *et al.*, 1990; Frey, 1994). It consists of a single stranded 40S RNA of positive polarity (about 11,000 bp in length) which contains two long open reading frames (ORFs): a 5' ORF that encodes two non-structural proteins

(p150 and p90) that function primarily in viral replication, and a 3' ORF that encodes the virion structural proteins (C, E2 and E1) (Oker-Blom *et al.*, 1984; Marr *et al.*, 1994). In RV infected cells, the 5' ORF is translated from the genomic RNA while the 3' ORF is translated from a 24S subgenomic RNA. The translation of the 24S subgenomic RNA produces a 110 kDa precursor polyprotein (p110) that is proteolytically processed to yield the structural proteins in the order: NH_2-C-E2-E1-COOH (Kalkkinen *et al.*, 1984; Oker-Blom, 1984). The capsid protein C (33 kDa) is a nonglycosylated protein that is associated with the 40S genomic RNA (Oker Blom *et al.* 1983; Liu *et al.*, 1996). E1 (58 kDa) and E2 (42 – 47 kDa) are envelope glycoproteins that form heterodimeric complexes (Baron and Forsell, 1991) and are found on the virion surface as viral spikes. The region between amino acids 81 and 109 of the E1 glycoprotein is thought to be involved in interaction with the E2 glycoprotein (Yang *et al.*, 1998). E1 consists of 481 amino acids and has three potential N-linked glycosylation sites (asn-X-Ser/Thr) (Hobman *et al.*, 1991). E2 is smaller than E1, consisting of 282 amino acids and the number of N-linked glycosylation sites appears to vary depending on the strain. Oker-Blom *et al.* (1983) showed the E2 glycoprotein migrates as two different species in SDS-PAGE, designated E2a (47 kDa) and E2b (42 kDa). The heterogeneity is thought to be due to post-translational modifications of a single gene product as tryptic maps of E2a and E2b were identical and intrinsic labelling experiments with [^{3}H] mannose showed that mannose was more efficiently incorporated into E2b than into E2a. E1 has been suggested to be the immunodominant glycoprotein as it bears the majority of MAb-defined erythrocyte binding and neutralising epitopes (Ho-Terry *et al.*, 1984; Trudel *et al.*, 1985; Umino *et al.*, 1985; Waxham and Wolinsky, 1985; Green and Dorsett, 1986; Gerna *et al.*, 1987). Although a neutralising epitope has been identified on the E2 glycoprotein (Green and Dorsett, 1986) it has been less well characterised. It is thought E2 is largely based beneath E1 in the virus particle and is inaccessible to the action of enzymes (i.e. glycosidase) and the immune response.

1.2.3 Epidemiology

Rubella has a worldwide distribution (Cockburn, 1969). In temperate climates, prior to rubella vaccination, immunity to rubella was mostly acquired between the ages of five and nine years, with the disease being less common among pre-school aged children (Kono *et al.*, 1985). Epidemics of rubella usually occurred in the spring months at intervals of three to nine years, depending on geographic location (Assaad and Ljungars-Esteves, 1985). Infection rates during the course of an epidemic were highest among closely housed susceptible individuals, such as student populations and military establishments.

In developing countries without a national rubella vaccination policy, infection is common in both pre-school aged children and in five to nine year olds (Gomwalk and Ahmad, 1989; Cutts *et al.*, 1997). Despite this a substantial proportion of women of childbearing age are susceptible (10 - 25%), and epidemiological studies conducted in South America (De Azevedo Neto *et al.*, 1994) and India (Kishore *et al.*, 1990; Eckstein *et al.*, 1996) suggest that the burden of congenitally acquired rubella may be considerable; 1 to 2 per 1000 live births (Cutts *et al.*, 1997).

Rubella is a human disease and has no known animal reservoir. The incubation period ranges from 12 to 23 days and the period of infectiousness in acquired rubella is believed to be seven days before the rash to about five days afterwards; patients who do not develop a rash are also infectious (Davis *et al.*, 1971). Infants with congenital infection may shed RV for an extended period but no true carrier state has been described.

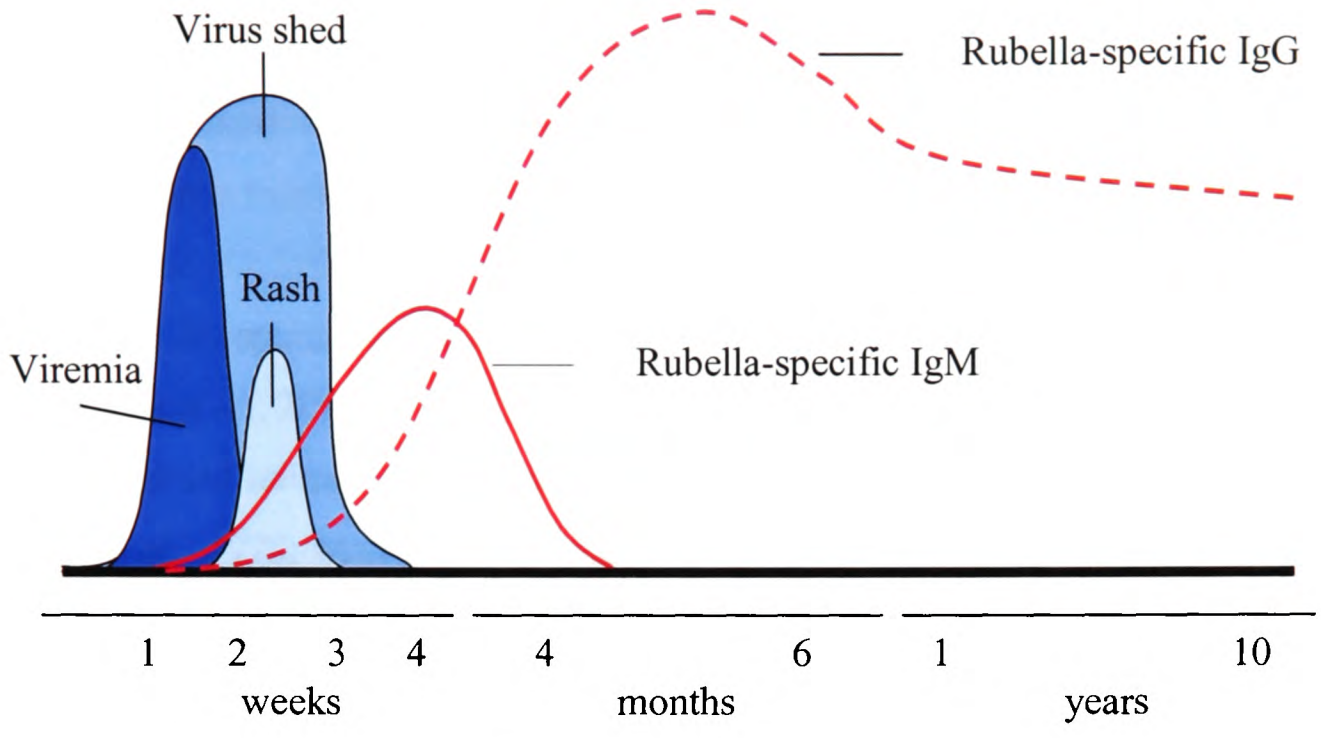
In developed countries that have achieved high uptake rates for rubella vaccine, acquired and congenital rubella is now uncommon. Unlike for mumps, outbreaks of rubella have not been reported recently in the UK.

1.2.4 Pathogenesis and clinical features

Rubella virus is transmitted by direct contact or droplet spread from both acquired and congenital cases. The virus initially replicates in the nasopharynx and the upper respiratory tract, followed by spread to the regional lymph nodes. A viremia occurs five to seven days after exposure, with spread of the virus throughout the body. Transplacental infection of the foetus occurs during viremia, which causes destruction of foetal tissues.

Rubella infection in early childhood or adult life is usually mild, with 20 to 50% of cases passing as subclinical or unrecognised events (Green *et al.*, 1965). Clinically apparent rubella is characterised by any combinations of symptoms that include maculopapular rash, enlarged lymph nodes, mild fever, and sore throat. The most common complication is transient joint involvement such as arthralgia and arthritis. Less frequent complications are encephalitis and thrombocytopenia. The course of the acquired infection and the accompanying immune response is shown in Figure 1.4.

Figure 1.4 Schematic diagram representing the course of postnatally acquired rubella infection.



Adapted from Chantler *et al.* (2001).

The major risk of rubella virus is infection during early pregnancy (the first 12 weeks) resulting in virus passage across the placenta and replication in the foetus, causing severe birth defects, such as deafness, cataract, cardiac abnormalities, microcephaly and sometimes in foetal death. These congenital abnormalities are referred to as congenital rubella syndrome (CRS).

1.2.5 Immune response

Rubella-specific IgM response develops quickly after the onset of symptoms and persists for four to twelve weeks, although it can be detected for up to one year after both naturally acquired infection and rubella immunisation (Al-Nakib *et al.,* 1975). The IgG response can be also detected early and is thought to persist for life. Sarnesto *et al.* (1985b) showed the IgG response is predominantly IgG1. O'Shea *et al.* (1985) demonstrated that serum IgA was detectable in 90% of vaccinees one year after vaccination but in only 45.5% of vaccinees 10 - 12 years after vaccination, while nasopharyngeal IgA was detectable for up to five years after vaccination with RA27/3 or natural infection. Rubella specific IgD and IgE antibodies increase rapidly after the onset of infection, remain at a high level for at least 2 months, and decline slightly by 6 months (Salonen *et al.*, 1985).

Most of the humoral response appears to be directed at the E1 glycoprotein with proportionally lesser amounts of the response directed at E2 and C (Katow and Sugiura, 1985; Chaye *et al.*, 1992b). Using a mouse model, Cusi *et al.* (1995) showed that the E1 was essential for protection and that mice immunized with recombinant E2 and C proteins alone were not sufficient to protect the animals against RV infection. The avidity of IgG antibody to E1 matures during the two years following rubella infection (most pronounced in the first three months), but IgG anti-E2 and anti-C show minimal avidity maturation (Mauracher *et al.*, 1992; Bottiger and Jensen, 1997; Hedman *et al.*, 1989). IgG avidity development is reported to be slower in vaccinees than in patients with natural infection (Nedeljkovic *et al.*, 2001).

A generalised immunosuppression occurs following natural and vaccine-related rubella (Maller *et al.*, 1978; Maller and Soren, 1978; Buimovici-Klein and Cooper, 1979; Niwa and Kanoh, 1979). Niwa and Kanoh (1979) detected a decrease in total leucocytes, neutrophils and T cells, and a marked insensitivity to dinitrochlorobenzen and purified protein derivative in many patients. However, RV-specific cell-mediated immune responses develop and can be measured *in vitro* within one or two weeks of onset of clinical illness, and may persist for many years.

Reinfection can occur (Horstmann *et al.*, 1970; Vesikari, 1972). Horstmann *et al.* 1970 reported asymptomatic reinfection in five of 149 recruits exposed to rubella during an epidemic. These individuals all had HI antibodies from previous natural infection. The risk of reinfection was greater in persons with low HI antibody titres. Reinfection during pregnancy resulting in viral spread to the foetus has been documented but is rare (Best *et al.*, 1989; Weber *et al.*, 1993).

1.2.6 Diagnosis

Clinical diagnosis of rubella is unreliable so laboratory confirmation is needed to confirm suspected cases. Accurate diagnosis is especially essential in pregnant women as termination is an accepted means of preventing congenital rubella. The methods used are similar to those used to confirm mumps infection.

Postnatally acquired rubella is usually diagnosed serologically by the detection of RV-specific IgM or a fourfold rise in specific IgG between acute and convalescent specimens by ELISA. PRN, HI, HIG have also been employed to detect RV-specific IgG but these are now used infrequently as they are time consuming and automated methods are not available. ELISA for the detection of RV-specific IgM is sensitive but may be compromised by cross-reactivity with other viruses, such as parvovirus B19 or Epstein Barr

virus (EBV), or by the presence of rheumatoid factor, which can lead to false positive results. More recently, tests for specific IgG avidity have been developed and used as an alternative or additional test for recent infection (Thomas and Morgan-Capner, 1988; Thomas and Morgan-Capner, 1991). This has been possible as the avidity of specific IgG from cases of primary infection is low compared with that from people with past infection or reinfection. Two approaches, the shift value and index methods have been described. The shift value method compares absorbances with and without a protein denaturant (e.g. diethylamine or urea) in the serum diluent over a range of serum dilutions and the difference between the dilution curves is measured. While the index method compares absorbance's at a single serum dilution, with and without denaturant in the wash fluid used after the antigen/serum incubation (Thomas and Morgan-Capner, 1991).

Virus isolation is rarely used to diagnose acquired rubella, as it is labour intensive and slow. However, it is sometimes of use for determining the duration of excretion in congenitally infected infants as they might transmit infection to susceptible contacts. RV can be isolated from nasopharyngeal secretions, faeces, urine, and the CSF of infants with CRS, as well as from cord blood or placental tissue of the mother at the time of birth. RV can be identified by the production of CPE in rabbit kidney (RK13) or Vero cells, by interference in primary vervet monkey kidney cells or by immunofluorescence for the detection of antigen in RK13, BHK21 or Vero cells (Banatvala and Best, 1998).

RT-PCR can be used to detect virus isolated in cell culture or directly from clinical specimens. Amplified fragments can be sequenced to confirm the correct RV genome fragment has been amplified and to provide useful epidemiological information.

1.2.7 Control

Rubella is a public health problem because primary infection in the first trimester of pregnancy gives rise to foetal damage. In some developing countries, due to large families and overcrowding most children experience a rubella infection, which is often subclinical, early in life. Therefore very few women of childbearing age are susceptible to infection, and CRS is too rare to warrant large-scale immunisation efforts. In other countries with moderate transmission rates (where rubella vaccine has not been introduced) a significant proportion of women of childbearing age are still susceptible to rubella and CRS is an important public health problem. In contrast, many industrialised countries have practically eliminated rubella through the widespread use of live attenuated vaccines.

(i) Rubella vaccines

Several live attenuated RV strains have been used for the production of rubella vaccines, including, Cendehill (grown in rabbit kidney cells), HPV-77 (grown in duck embryo cells) and RA27/3 (propagated in human diploid fibroblast cells) (Plotkin, 1999). The vaccine containing the RA27/3 strain is the most widely used as it is highly immunogenic and has a low rate of side effects; it is attenuated in human cells and is therefore not subject to possible side effects associated with vaccines grown in non-human cells (Plotkin *et al.*, 1973). It is manufactured by Merck, Sharpe and Dohme and is available in monovalent, bivalent (measles-rubella) or trivalent (MMR) vaccines. Antibody responses to rubella as part of the triple vaccine are the same as those seen after rubella vaccination as a single antigen and are produced in 95% of vaccine recipients older than 12 months of age (Weibel *et al.*, 1980). Although antibody titres are lower than those following natural infection, protection is believed to endure for more than 12 years in most of those immunised (Herrmann *et al.*, 1982; O'Shea *et al.*, 1984; Horstmann *et al.*, 1985; O'Shea *et al.*, 1988). Reinfection has been reported in a small number of individuals following vaccination. Cusi *et al* (1993) found subclinical reinfection (defined by a fourfold rise in preexisting antibody titre) in about 10% of girls immunised 5 years previously with the RA27/3 vaccine after reexposure of the virus during an epidemic.

(ii) Adverse reactions

Adverse reactions to rubella vaccines include fever, lymphadenopathy, arthralgia and arthritis. Mostly these reactions are less severe than following natural infection. Tingle *et al* (1986) showed wild type rubella infection in adult populations is associated with a higher incidence, increased severity, and more prolonged duration of joint manifestations than is seen after RA 27/3 rubella immunisation. Joint manifestations appear to be age related, being uncommon in children of both sexes but occurring more frequently in postpubertal females.

(iii) Vaccination programmes

Several rubella vaccination policies have been developed in different countries with markedly varied initial results. In the USA and Finland a policy of universal childhood immunisation was introduced, supplemented by vaccine administration to susceptible postpubertal women. This policy resulted in a marked decrease in the incidence of

postnatally acquired and congenitally acquired rubella in these countries (Anon, 1990b). In contrast, in the UK and other European countries, a selective vaccination programme was adopted where teenage girls aged 11 to 14 years were immunised, allowing natural rubella to continue to circulate (Hinman, 1985; Tobin *et al.*, 1985); this was due to concerns that vaccination would not provide long-lasting protection. However, after a rubella epidemic in the UK in 1986, the vaccination programme was augmented by offering rubella as a component of the MMR vaccine programme aimed at eliminating circulating rubella (Best *et al.*, 1987). Other countries have also switched to universal vaccination of infants, supplemented with the vaccination of seronegative adult women (Sullivan *et al.*, 1999).

Since the introduction of MMR in the UK, rubella and CRS has declined sharply and has remained at a low level. Table 1.2 shows the number of cases reported to the National Congenital Rubella Programme from 1983 to 2001.

Table 1.2 Cases reported to the National Congenital Rubella Programme

Year of birth	Congenital Rubella Syndrome
1983	49
1984	35
1985	17
1986	24
1987	29
1988	18
1989	8
1990	9
1991	3
1992	7
1993	1
1994	7
1995	1
1996	12
1997	0
1998	0
1999	1
2000	4
2001	1

Source: CDSC, Colindale, London.

Despite the fall in the number of rubella and CRS cases, serological surveillance studies showed that a high percentage (~15 - 20%) of young men were susceptible to rubella and that continuing outbreaks among young men posed a risk of transmission of rubella to pregnant women. This led to the inclusion of rubella in the national immunisation campaign carried out in 1994 in the UK, primarily to prevent an epidemic of measles, where all children aged 5 to 16 years were offered measles-rubella vaccine. Following the campaign, in children aged 5 to 10 years, the proportion without rubella antibody fell from 17.5% to 3.0% (there was no difference in susceptibility between the sexes) and in 11 to 16 year olds the proportion fell from 6.2% to 1% in females and 24.5% to 6.9% in males; the lower susceptibility in the females reflected the effect of the selective vaccination up to 1994 (Miller *et al.*, 1997). Following this it was agreed to discontinue vaccination of schoolgirls but to continue to ensure that susceptible pregnant women were vaccinated postpartum.

Due to the success of rubella vaccination in virtually eliminating CRS from the industrialised world, there has been a recent drive to eradicate rubella worldwide. The WHO now recommends the addition of rubella vaccine to the measles vaccine when it is already being administered.

1.3 Surveillance

Vaccination is one of the most successful and cost-effective disease prevention interventions in public health. The use of an effective vaccine enabled the global eradication of smallpox (Fenner, 1980) and has greatly reduced the incidence of many vaccine-preventable diseases in industrialised countries. The WHO Expanded Program on Immunisation, which was established in 1974, has extended these benefits to developing countries (Henderson, 1984).

Critical to the success of any immunisation programme is a comprehensive surveillance system that allows measurement of health impact, definition of target populations for vaccination, evaluation of the impact of the immunisation programme and detection of problems requiring alterations in immunisation strategy.

The CDSC in the UK and Centre for Disease Control (CDC) in the US carry out continual surveillance on a variety of infectious diseases, including mumps and rubella. The WHO performs a similar role but at an international level. At a national level surveillance information comes from a variety of sources, including health care providers, laboratories, hospital records and death certificates (Orenstein and Bernier, 1990).

In England and Wales, data on disease incidence mostly relies on statutory notifications by doctors to the proper officer of the local authority, who is usually the Consultant for Communicable Disease Control (CCDC). The proper officer sends a weekly return to the Office of National Statistics (ONS). Statutory notifications are important to detect short-term trends in disease incidence and outbreaks of disease so that immediate action can be taken to prevent the spread of infection; mumps and rubella were made notifiable diseases in the UK in October 1988.

Recently, MMR disease surveillance was enhanced to provide more accurate data as it was demonstrated that the predictive value of clinical notifications declines with decreasing incidence of disease; Brown *et al.* (1994) found only 85 of 236 (36%) clinically diagnosed cases of measles could be confirmed in the laboratory. Since 1994 the PHLS (now HPA) has offered confirmatory testing of clinical notifications. Oral fluid-testing kits have been distributed to CCDC's in all health authorities, so when a case is notified, a kit is sent to the reporting doctor requesting a sample of oral fluid. The sample is taken by the practice nurse, doctor, parent or patient themselves and posted in a reply-paid box to the Specialist and Reference Microbiology Division (SRMD) at Colindale, London. Samples are tested for virus specific IgM by antibody capture radioimmunoassays (Perry *et al.* 1993) to confirm recent infection. Results are returned to the CCDC and the doctor. Confirmed cases are reported to the CDSC. In 2001, 63% (1774/2806) of the notified cases of clinically diagnosed mumps were tested for mumps-specific IgM antibody, and antibody was detected in 574 (32%).

As well as disease incidence, adverse events following vaccination, vaccine efficacy and coverage should be monitored in a comprehensive surveillance programme. For example, vaccination coverage is important to enable changes in uptake to be detected quickly. This can be achieved by monitoring vaccine distribution and by monitoring the doses of vaccine administered to children in target age groups. In England and Wales, CDSC carry out surveillance of childhood vaccination uptake. The programme is known as COVER (Cover of Vaccination Evaluated Rapidly) and was established to: improve vaccination cover by providing programme coordinators with timely comparative information; detect rapidly local and national changes in vaccination cover; encourage the development and dissemination of quarterly reporting within districts; and identifiy measures and remedies for poor performance (Begg *et al.*, 1989).

Interpretation of surveillance data and summary statistics are disseminated regularly in a variety of ways, including local and national newsletters, the HPA website, letters from the

Chief Medical Officer and journals, such as the Communicable Disease Report and the Mortality and Morbidity Weekly Report so that the medical community is made aware of trends in patterns of disease and action can be taken quickly and efficiently.

1.4 Serological surveillance

Another important component of any immunisation programme is serological surveillance, especially age stratified antibody prevalence studies. Since the presence of antibody can be used to indicate immunity (or exposure to vaccination or past infection), serological surveys can identify those age groups at risk of infection. This information, especially when combined with mixture modelling, is particularly useful for evaluating the need for and the planning of new vaccination programmes and supplementary measures. Identifying cohorts with low immunity enables vaccination strategy to be updated accordingly.

In the UK, a serological surveillance programme was introduced just before the introduction of MMR in 1988 and has been running every year since. Serum aliquots, residues remaining from microbiological or biochemical testing, are collected by participating PHLS and National Health Service laboratories and are sent to Preston PHL where they are tested for IgG antibodies to MMR. Results are then forwarded to CDSC where the information is entered into a central database and analysed.

The serological surveillance programme has directly influenced national vaccination policy. The first age-stratified survey was conducted in 1986/7 (Morgan-Capner *et al.*, 1988). The results showed that 60% of 1 - 2 year olds did not have measles antibody (despite the measles vaccination programme at that time) and over 80% did not have antibody to mumps or rubella, whereas 17%, 55% and 73% of children aged 3 - 4 years were susccceptible to MMR respectively. These findings suggested that vaccinating children early in life would be necessary to eliminate disease and was a central factor in the decision to offer MMR to infants 12 to 15 months old.

The decision to carry out the national measles and rubella (MR) immunisation campaign in 1994 was also based on the findings of the serological surveillance programme. Results of sera collected in 1989 - 91 revealed that the proportion of school age children who were susceptible to measles was increasing as a result of reduced exposure to infection. Mathematical models, used to interpret the data, demonstrated that the increasing susceptibility of schoolchildren provided the potential for a major epidemic in the mid 1990's (Gay *et al.*, 1995). So all children aged 5 to 16 years of age were offered a single dose of MR vaccine. The rubella component of the vaccine was included in order to reduce

the high level of susceptibility to rubella in young adult males and thus reduce the risk of transmission from this group to pregnant women. Ninety two percent of children in the target cohort (5 to 16 years) were vaccinated.

Another example of the influence of the serological surveillance programme on vaccination policy was the adoption of a routine second dose of MMR. Although the vaccination campaign had a significant impact on the prevalence of measles and rubella antibody in the target cohort (5 - 16 year olds), a high proportion (~15%) of 2 - 4 year olds had low levels (<100 mIU/ml) of antibody to measles both before and after the campaign. Also, Gay *et al.* (1997) showed that up to 15% of 2 to 6 year olds had no detectable antibody to mumps. Thus a second dose was added to the schedule providing another opportunity for children to be immunised before they start school.

Other countries have adopted similar serological surveillance systems. For example in the Netherlands, every five years blood samples donated from a random sample of the population are used for surveillance purposes (Esveld, 1994). As well as on-going surveillance programmes, periodic serosurveys are beneficial for defining the immunisation status of a community, especially in developing countries with meagre resources.

To harmonise and co-ordinate the serological surveillance of communicable diseases in Europe, a European Sero-Epidemiological Network has been established (Osborne *et al.*, 1997). The project has involved serosurveys in eight European countries to measure the age-specific prevalence of antibodies to five diseases, including to mumps and rubella (Andrews *et al.*, 2000). Thousands of age and sex stratified sera have been collected and tested for virus-specific antibodies by ELISA in a national laboratory of each country. Although different kits were used in different countries a standardisation procedure was developed to allow direct comparison between assay results, and ultimately between the epidemiology of these infections under different vaccination programmes.

1.5 Laboratory techniques

A variety of laboratory techniques have been used to demonstrate mumps and rubella immunity (Grillner and Bloomberg, 1976; Sato *et al.*, 1978; Morgan-Capner *et al.,* 1979; Vaananen and Vaheri, 1979; Kurtz *et al.,* 1980; Popow-Kraupp, 1981; Meurman *et al.*, 1982; Enders *et al.*, 1985; and Grillner *et al.*, 1985). PRN is generally considered to be the most reliable method as it detects neutralising (NT) antibodies. NT antibodies are believed to correlate well with immunity in most viral infections. PRN, however, is not suitable for testing large numbers of samples as it depends on cell culture so is time consuming and relatively expensive. Modified versions of PRN have been developed but these are still

dependent on cell culture and take several days to complete (Harmsen *et al.*, 1992). HI or HIG techniques are also believed to correlate well with immunity as they measure a biological function of the virus-specific antibody, but require considerably less time and effort to perform than PRN and have been reported to be more sensitive (Albrecht and Klutch, 1981). HI, however, can give false positive results due to the incomplete removal of inhibitors of haemagglutination. HIG was initially used to measure mumps and rubella antibodies for the UK serological surveillance programme, however the ELISA has recently replaced it (Osborne *et al.*, 2000). ELISAs are quick and easy to perform, benefit from automation and validated kits and reagents are commercially available so are ideal for testing large number of samples. Moreover, ELISAs can be used to detect viral antibodies in non-invasive specimens, such as oral fluid, which are much easier and painless to collect compared with blood. This factor greatly facilitates subject recruitment for seroepidemiological studies. In Chapter 2 of this thesis, the development and evaluation of an 'IgG' antibody capture ELISA (GACELISA) for the detection of mumps-specific IgG in oral fluid is described.

An oral fluid GACELISA was recently developed and evaluated for rubella-specific IgG (Vyse *et al.*, 1999). They demonstrated the sensitivity of the assay was age-dependent: compared with serum in those aged 3.5 - 4 years the assay was 94.8% sensitive but only 60.8% sensitive in those aged 17 - 34 years. The lack of sensitivity in the older cohort may have reflected waning antibody levels, and highlights the need for more sensitive detection systems for the detection of antibodies in non-invasive samples. I-PCR has recently been described as a highly sensitive technique for the detection of antigens. It is a modification of the ELISA, where the enzyme conjugate is replaced by a DNA molecule that can be amplified by PCR. The method offers the potential for highly sensitive assays. In Chapter 3 of this thesis, the development of an I-PCR assay is described and its use for the detection of antibodies to MV is assessed.

As well as sensitivity, another limitation of the ELISA is the difficulty in obtaining antigens of reliable quality. Most assays use antigens prepared by concentrating virus particles from the maintenance medium of infected cell cultures. Different batches of antigens may contain different amounts of viral proteins so careful standardisation is required. Moreover, they are potentially infectious and do not distinguish antibodies that are of functional significance. NT and HIG assays measure antibodies against surface proteins, such as the HN of the MV, which are important for protection. The antigens usually employed by ELISAs are from infected cell cultures, so may measure antibodies to

the surface proteins but also to internal proteins, which are not thought to correlate with protection.

To address the problems of using cell culture derived viral antigens, recombinant and synthetic proteins have been produced and used as antigens in ELISAs. The advantages of using such antigens is that they are non-infectious and can be more easily produced and standardised. Moreover they may represent functional epitopes, such as putative NT or HI determinants so offer the potential for assay improvements. In Chapter 4 of this thesis, the production of recombinant RV fusion proteins using a prokaryotic expression system is described and the suitability of these proteins as antigens in ELISA is assessed.

Chapter 2

Development and evaluation of an antibody capture ELISA for the detection of mumps-specific IgG in oral fluid

2.1 Introduction

2.1.1 Oral fluid

Traditionally, serum has been the specimen of choice for detecting antibody to diagnose infection or determine immune status. Blood collection, however, is invasive, risky and relatively expensive. It requires trained staff and sterile equipment. Oral fluid, by contrast, can be collected safely and cheaply, and is more acceptable, especially for population immunity studies where large numbers of specimens need to be easily and economically obtained, often from children (Figure 2.1) (Mortimer and Parry, 1991).

Figure 2.1 Collection of oral fluid from an infant with an Oracol sponge swab.

Oral fluid is defined as the fluid obtained by the insertion of absorptive collectors into the mouth (Atkinson *et al.*, 1993). Depending on the material it is composed of and the location in the oral cavity where the collector is used, the fluid collected is enriched in specific components of whole saliva. Fluid found in the oral cavity consists of secretions from the salivary glands (mainly the parotid, submandibular and sublingual glands) and transudate from the capillary bed, in particular the crevicular fluid that constantly flows from the crevice between the gum margin and the teeth. Crevicular fluid is the component in saliva that contains the highest concentrations of IgG and IgM. Oral fluid samples in which crevicular fluid is well represented are most suitable for antibody detection.

The mean immunoglobulin concentrations found in several salivary components and plasma is shown in Table 2.1. It should be noted that the salivary concentrations of plasma-derived immunoglobulins vary between individuals depending upon the collection methods used and levels of oral health and hygiene.

A drawback of using oral fluid samples is that while specific antibody levels reflect those in plasma, concentrations are considerably lower and more variable. This means that sensitive assays are required to detect antibody in oral fluid. The assay format most widely used for antibody detection in oral fluid is the antibody capture assay.

Table 2.1 Mean immunoglobulin concentrations (mg/L) in plasma and salivary components

Specimen	IgG	IgM	IgA
Plasma	14730	1280	2860
Parotid saliva	0.36	0.43	39.5
Crevicular fluid	3500	250	1110
Whole saliva	14.4	2.1	19.4

Sources: Brandtzaeg *et al.*, 1970; Roitt and Lehner, 1983.

2.1.2 Antibody Capture Assays

The basis of the 'antibody capture' technique is that a single class of human immunoglobulin (i.e. IgG, IgM or IgA) in the specimen is captured by an immobilised, class-specific anti-immunoglobulin. Specific antibody is then detected by the addition of an antigen and, for example, a mouse monoclonal antibody to the antigen and a labelled anti-mouse immunoglobulin. The label may be an enzyme that produces a colour signal in the presence of substrate or a radioisotope such as I^{125}.

The antibody capture assay differs from other assay configurations (i.e. indirect, sandwich and competitive) in that the strength of the signal is not dependent on the concentration of specific antibody in the sample, but on the proportion of the captured immunoglobulin that is specific for the antigen. Since oral fluid includes plasma transudate as the predominant source of immunoglobulin, the ratio of specific to total immunoglobulin is similar in both serum and oral fluid. Hence, as long as the test specimen contains sufficient IgG to saturate the anti-IgG binding sites, the signal obtained should be similar whichever sample is used (Mortimer and Parry, 1988; Parry, 1993; Nishanian *et al.*, 1998).

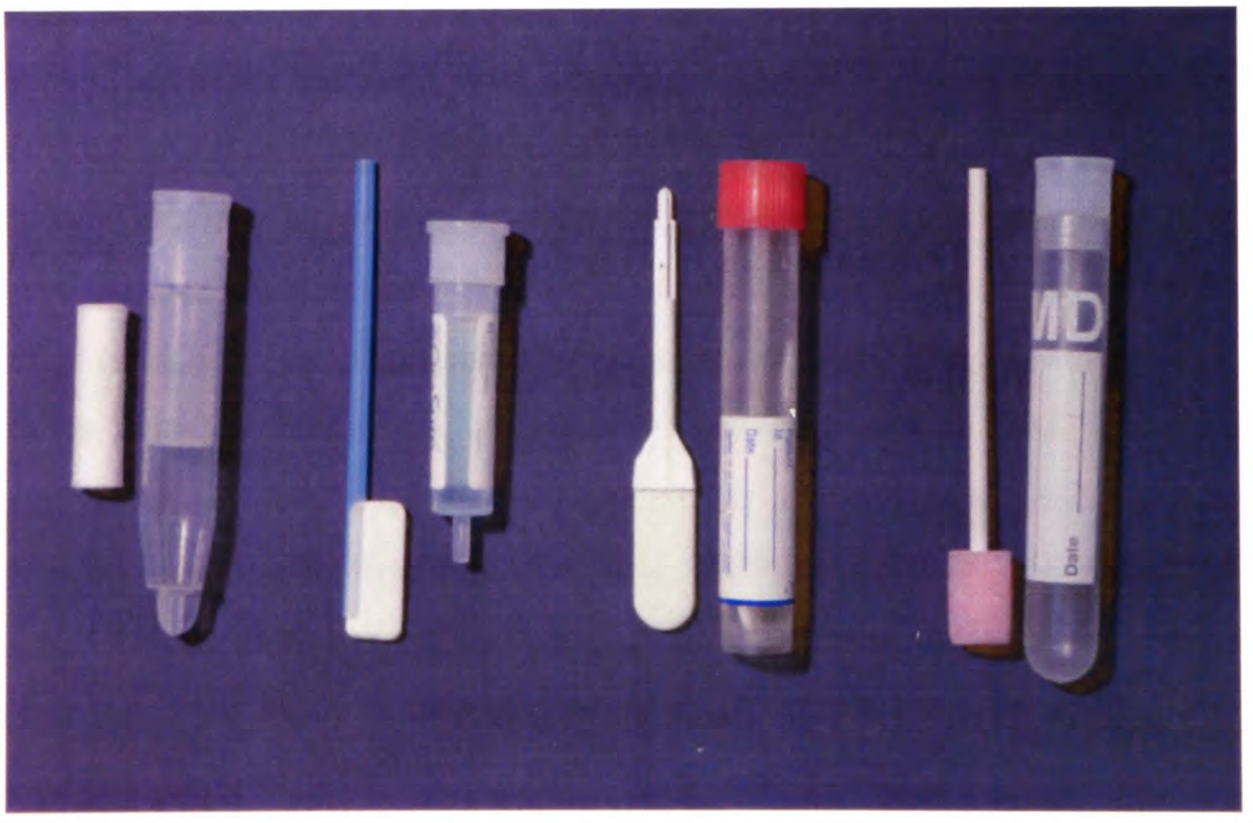
Antibody capture assays were first described by Flehmig *et al.* (1979) and Duermeyer *et al.* (1979) for the detection of IgM antibodies against hepatitis A virus (HAV). They are now widely used mainly in the diagnosis of acute virus infection but also in the determination of immune status. Originally, these assays were intended for use with serum. However, when the advantages of oral fluid sampling were realised, assay protocols were modified appropriately. Parry *et al.* (1987) were the first to describe IgG antibody capture radioimmunoassays (GACRIAs) and GACELISAs for the detection of specific antibodies to human immunodeficiency virus (HIV), RV, HAV and the core antigen of hepatitis B virus (HBV) in saliva. It is worth noting that most of the early developmental work used whole saliva that was collected by being dribbled into a sterile, wide mouthed container. However, subjects found dribbling distasteful and not infrequently either sputum specimens or samples with insufficient volume were received. Also, pipetting untreated saliva was difficult. This led to the development of the proprietary oral fluid collection devices featured in Table 2.2 and Figure 2.2.

Table 2.2 Characteristics of four oral fluid collection devices

Collection Device	Description	Manufacturer	Unit Cost[a]
Salivette®	Chew cotton wool for 30 to 45 seconds or place under tongue for 1 minute	Sarstedt	£0·36
OraSure®	Move pad gently along gum twice. Place against lower gum on 1 side and keep stationary for 2 minutes	Epitope	£2·50
Omni-SAL®	Place pad under tongue and keep until indicator of device turns blue	Saliva Diagnostic Systems	£2·20
Oracol	Rub sponge firmly along the base of the gums (inside and out) of the upper and lower jaw, for 1 minute, using an action similar to tooth brushing	Malvern Medical Developments	£0·50

[a]Unit costs as of November 2001

Figure 2.2 Four oral fluid collection devices



From left to right: Salivette®, OraSure®, OminSal®, and Oracol.

Antibody capture assays for oral fluid have also been developed for the detection of virus specific IgG to measles virus (Perry *et al.*, 1993), EBV (Vyse *et al.*, 1997) and parvovirus B19 (Rice and Cohen, 1996) and for specific IgM in patients with recent hepatitis A or B (Parry *et al.*, 1989), measles, mumps, rubella (Perry *et al.*, 1993) and parvovirus B19 infection (Cubel *et al.*, 1996). Many of these assays were developed as radioimmunoassays (RIAs), which have the advantages of a wide dynamic range, quantification and sensitive detection of radioactivity by a gamma counter. However, there are practical disadvantages associated with RIA. Not only does radiolabelling produce hazardous radioactive waste, but the technique is not easily transferable between laboratories. Unfortunately, however, the substitution of an enzyme-labelled conjugate for a ^{125}I-labelled conjugate has not in many cases resulted in an ELISA with the same sensitivity as RIA when testing oral fluid (Parry *et al.*, 1987).

2.1.3 Oral fluid testing

Oral fluid testing has been most useful for epidemiological surveillance. RIAs that reliably detect measles, mumps and rubella-specific IgM in oral fluid are used to confirm disease notifications in the UK (Brown *et al.*, 1994). In the context of virus specific-IgG, most success has been with HIV tests. Several oral fluid screening assays for IgG antibodies to HIV-1 have been developed and commercial test kits have become available that have been reported to be sensitive and specific (Malamud, 1997). The development of oral fluid IgG screening assays for other viral infections has been slow in part due to the lack of demand (i.e. for commercial reasons) but also due to the lack of availability of assays that are sensitive enough to replace serum assays. However, the findings of recent studies have been promising and moved us closer to realising the wider use of oral fluid surveys of viral specific IgG. For example, novel oral fluid GACELISA assays that are sensitive and specific enough to substitute for serum assays have been developed and evaluated for measles (Nigatu *et al.*, 1999), rubella (Vyse *et al.*, 1999) and EBV (Sheppard *et al.*, 2001). These assays employ a FITC/anti-FITC amplification step which compensates for the less sensitive spectrophotometric detection in ELISA compared to radioactive counting and the smaller surface area available for binding on the microtitre ELISA plate compared to that on the polystyrene bead used in RIA.

2.1.4 Detection of mumps antibodies in oral fluid

To date, there have been only three reports of assays to detect mumps antibodies in saliva/oral fluid. Frankova and Sixtova (1987) used indirect immunofluorescence and indirect ELISA to detect mumps-specific IgM in saliva but they were unable to identify mumps cases reliably. Perry *et al.* (1993) developed a saliva-based IgM capture radioimmunoassay (MACRIA) and a GACRIA. The sensitivity of the MACRIA was 100% but for the mumps GACRIA it was only ~50%, compared to the equivalent serum assay. Thieme *et al.* (1994) used a modified indirect commercial ELISA to detect mumps-specific IgG in oral fluid collected using the OraSure® device and found the assay was 94.2% sensitive for subjects over 18 years old, however only 75% sensitive for subjects younger than 18 years.

In this study, a GACELISA employing recombinant mumps nucleoprotein-associated antigen (rNP) and a mouse anti-rNP MAb conjugate was developed for the detection of mumps-specific IgG in oral fluid. This is the first time this method has been developed for mumps-specific IgG. The assay was evaluated by comparison of results from testing serum-oral fluid paired samples, as well as the more recently described statistical approach, mixture modelling (Parker *et al.*, 1990). Mixture modelling treats results from a sample of individuals as a mixture of different populations. Models are used to fit the distribution of results into seronegative and seropositive populations without first having to identify an uninfected population, and optimum cut-off values can be assigned that give the required balance between sensitivity and specificity. These models have been particularly useful for estimating antibody prevalence in age-related serological surveys where the assay gives results with two overlapping distributions for the negatives and positives (Gay, 1996).

2.2 Material and Methods

2.2.1 Oral fluid collection and processing

Oral fluid was collected using the Oracol (Malvern Medical Developments, Barbourne, Worcester, UK) and OraSure® (Epitope, Inc., Beaverton, USA) collection devices, according to the manufacturers instructions (Table 2.2). The Oracol consists of a 2 x 1.5cm polystyrene sponge on a 10 cm plastic stick, whereas the OraSure® consists of a 3 x 1cm flat pad of absorbent material supported by a 10 cm plastic stick (Figure 2.2). Both devices are provided with a stoppered tube that, in the case of OraSure®, contains transport medium with preservative.

After the collection of oral fluid, the devices were placed into the appropriate transport container and labelled with a unique identification number. Collected samples were transported to the laboratory where the oral fluid was extracted as shown in Table 2.3. Samples were stored in labelled screw capped microtubes at – 20°C until required for testing.

Table 2.3 Extraction of oral fluid

Collection Device	Extraction method[a]
Oracol	One millilitre of transport medium[b] was added to the collection tube. The swab was agitated manually until foaming of transport medium and then removed from the tube using a twisting motion to extract as much liquid as possible. The swab was inverted so that the sponge was at the top and the cap was replaced. The tube containing the swab was centrifuged at 2000 x g for 5 min. The sponge was discarded and the extracted fluid was pipetted into a labelled screw-capped storage tube.
OraSure®	The small plastic tip at the base of the OraSure® tube was broken off and discarded. The OraSure® tube was centrifuged at 2500 x g for 10 min within a test tube. The extracted fluid was pipetted into a labelled screw-capped storage tube.

[a] Oracol extracted as described by Brown *et al.* (1994); OraSure® extracted according to the manufacturers instructions.
[b] See Appendix A.

2.2.2 Samples

(i) Serum/oral fluid pairs

1. Six pairs were provided by Enteric, Respiratory and Neurological Virus Laboratory (ERNVL) staff for optimisation of the GACELISA.
2. One hundred and twenty two pairs were collected from healthy members of staff at the SRMD and CDSC at Colindale. The PHLS ethics committee approved the study procedures and the consent forms (Appendix B). The study was anonymised. Subjects were over 18 years of age and gave written consent. Five millilitres of venous blood was collected from each volunteer on day one by medical personnel using the Vacutainer system. Six oral fluid samples (five Oracol and one OraSure®) were self-collected from each volunteer: three Oracol swabs were self-collected on day one in the presence of laboratory personnel (swab 1 was collected from one side of the mouth, swab 2 from the other side of the mouth and swab 3 from both sides of the mouth); and two Oracol and the OraSure® were self-collected on day two in the absence of laboratory personnel (swab 4 was collected from one side of the mouth, swab 5 from the other side of the mouth and swab 6 (OraSure®) from either side of the mouth). The swabs were labelled 1 - 6 as taken. Upon receipt in the laboratory the oral fluid was processed as described above and the blood was allowed to clot and then centrifuged for 10 min at 3000 x g. The serum was collected and frozen at – 20 °C until use.

(ii) Oral fluid

Oral fluid samples were selected at random from those submitted to ERNVL for MMR testing. A total of 923 samples from persons aged 0.7 – 99 years were tested, and individual results were aggregated into four age groups: group 1, 0.7 - 1.4 years (n = 210); group 2, 1.41 – 8.99 years (n = 159); group 3, 9 – 19.99 years (n = 196); and group 4, over 20 years (n = 358).

2.2.3 Total IgG assay

The total IgG content of oral fluid specimens was measured using an indirect ELISA as previously described (Connell *et al.*, 1990). Oral fluid samples were screened at a dilution of 1/10 and 1/100.

2.2.4 Preparation of mouse immunoglobulin column

Mouse immunoglobulin (Ig) was prepared by precipitation with an equal volume of ammonium sulphate [$(NH_4)_2SO_4$] for 1 h, followed by centrifugation at 4000 x g for 15 min. The precipitate was dissolved in 0.1 M sodium bicarbonate buffer, pH 8.3 (Appendix A) and excess salts were removed by gel filtration through a PD-10 column containing Sephadex G-25 (Amersham Pharmacia Biotech, High Wycombe, U.K.). The crude Ig fraction containing approximately 60 mg of protein in 0.1 M sodium bicarbonate buffer, pH 8.3 was added to 0.73 g of swollen cyanogen bromide-activiated sepharose 4B (CNBr-S4B; Amersham Pharmacia Biotech), which had been washed with 10 ml of 1 mM HCl to remove preservatives as recommended by the manufacturers. After 2 h at 37°C on a circular rotator, the mix was added to an empty PD-10 column (Amersham Pharmacia Biotech). The column was centrifuged at 2500 rpm for 5 min and the optical density (OD) at 280 nm of the eluate was measured to estimate the percentage of bound antibody (~50mg). The column contents were then washed with 0.1 M sodium bicarbonate buffer, added to 20 ml of 0.1 M Tris-HCL, pH 8.0 (Appendix A) and allowed to mix for 1 h at 37°C on a circular rotator and then overnight (ON) at 4°C to block any remaining active groups. The column was packed by centrifugation at 2500 rpm and washed with 0.1 M Tris-HCl, pH 8.0 containing 0.5 M NaCl.

2.2.5 Purification of anti-human IgG on a mouse Ig column

One millilitre of rabbit anti-human IgG (Dako) was slowly applied to a mouse Ig column equilibrated with PBS azide (Appendix A). The column was incubated for 1 h at RT to allow cross-reacting antibodies to adsorb and then washed to remove unbound material until no protein was detected in the eluent, as determined by UV absorbance at 280 nm. The column was eluted with 10 column volumes of 0.1 M glycine-HCl, pH 2.5 (Appendix A) and re-equilibrated with PBS azide.

2.2.6 Mumps IgG antibody capture ELISA (GACELISA)

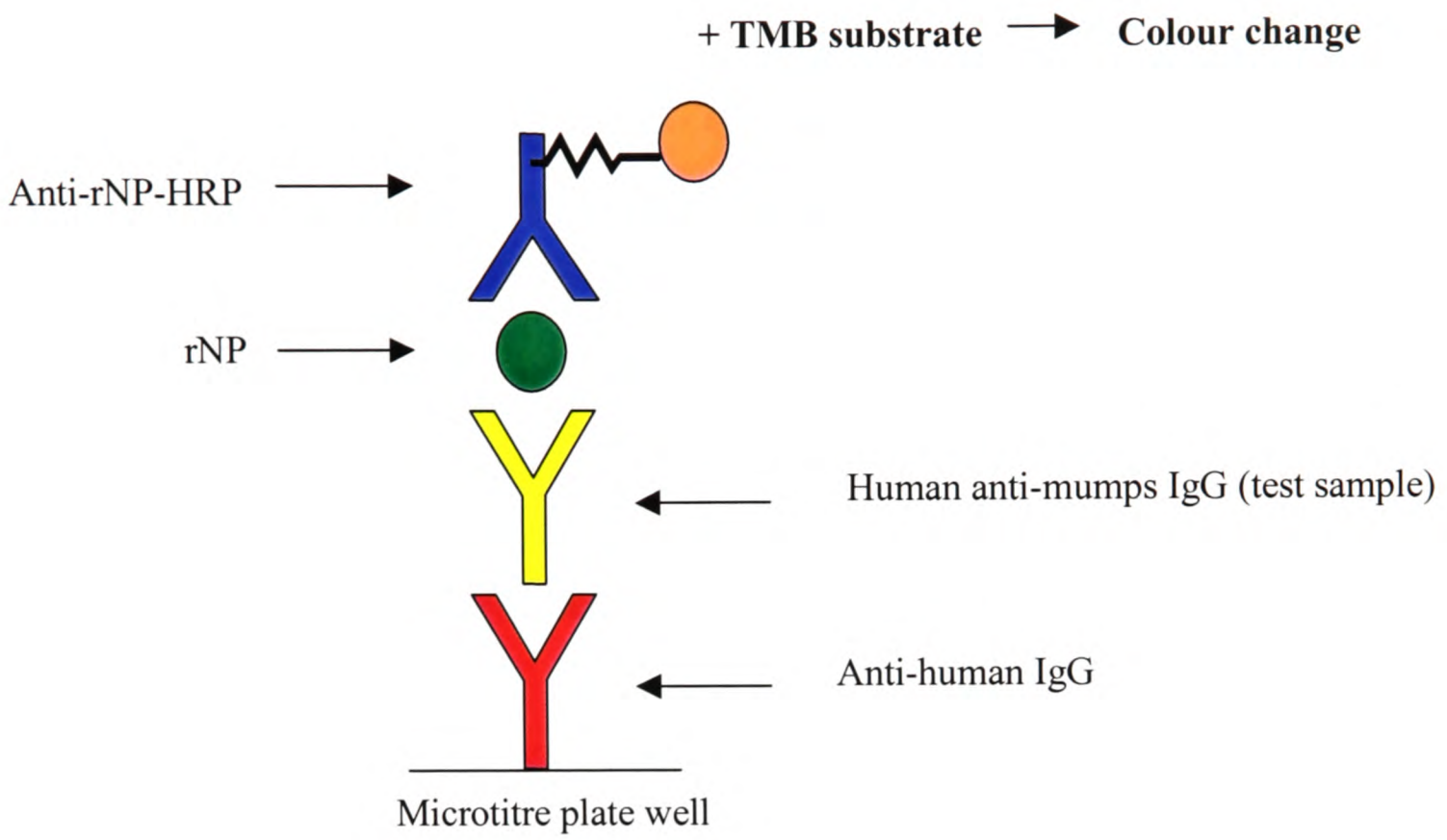
After a series of experiments to optimise the assay conditions the following procedure was used: microtitre plate wells (Nunc MaxiSorp™, Life Technologies, Paisley, U.K.) were incubated with 10 μg/ml of rabbit anti-human IgG (Dako, Ely, U.K.), pre-absorbed on a mouse Ig column, in 0.05 M sodium carbonate buffer, pH 9.6 (Appendix A) overnight at 4 °C and then for 2 h at 37 °C in a moist container. The wells were aspirated and blocked

with 5 % SoluPro (Dynagel Inc., Calumet City, IL, USA) for 2 h at 37 °C in a moist container. After aspirating, the wells were left to dry ON at 37 °C.

Coated wells were incubated at 37°C sequentially with 100 µl of the following: undiluted oral fluid or a 1/200 dilution of serum in diluent A (Appendix A) for 30 min; 50 ng/ml of rNP (Microimmune Ltd., Brentford, U.K.) in diluent A for 1 h; and 0.2 µg/ml of mouse anti-rNP horseradish peroxidase-labelled monoclonal antibody (anti-rNP-HRP; Microimmune Ltd.) in diluent A for 30 min. During the incubation steps the wells were agitated on an iEMS Incubator/Shaker (Thermo Labsystems, Helsinki, Finland) and between each stage of the assay the wells were washed four times with PBS containing 0.05% T20 (PBST; Appendix A). Finally, 100 µl of 3, 3', 5', 5' – tetramethylbenzidine (TMB) substrate solution (Microimmune Ltd.) was added to each well and the plate was incubated at room temperature (RT) for 30 min. The reaction was stopped by the addition of 100 µl of 0.5 M hydrochloric acid (HCl) and the OD was read at 450 nm with 620 nm reference on an ELISA plate reader (Thermo Labsystems iEMS-MF), unless otherwise stated. Duplicate negative and positive serum controls were run in parallel with test samples.

For the optimisation experiments, test OD results were divided by the mean OD of the negative serum control to give a test to negative ratio (T/N), unless stated otherwise. The use of T/N is conventional in the analysis of ELISA data (Tijssen, 1985). For the final protocol, test OD results were converted to logT/N (as logarithmic transformation made the data more normally distributed). A schematic diagram of the mumps GACELISA is shown in Figure 2.3.

Figure 2.3 Schematic diagram of the mumps GACELISA developed in this study.



Immobilised anti-human IgG was used to capture human IgG antibodies in oral fluid. Mumps-specific IgG was detected by the sequential addition of rNP, anti-rNP-HRP and TMB substrate. Spectrophometric readings were taken after the reaction was stopped with 0.5 M hydrochloric acid.

2.2.6 Statistical Methods

Mean concentrations of total IgG in oral fluid were compared using analysis of variance and paired t-tests. Paired t-tests were also used to determine if there was a significant difference between the T/N values of samples analysed in the optimisation experiments.

The 95% confidence intervals for the differences between duplicate serum control results of five representative assays were calculated to establish an acceptable difference between the duplicate controls in order to validate further assay runs.

The relationship between oral fluid and and serum OD values was measured using the Pearsons correlation co-efficient.

Mixture models using normal distributions were fitted to the GACELISA results from oral fluids submitted for MMR testing to estimate optimal cut-off values and assay sensitivity and specificity. The mean, standard deviation (SD) and prevalence of results in the different populations were obtained using the multinominal maximum likelihood method (Parker *et al.* 1990).

2.3 Results

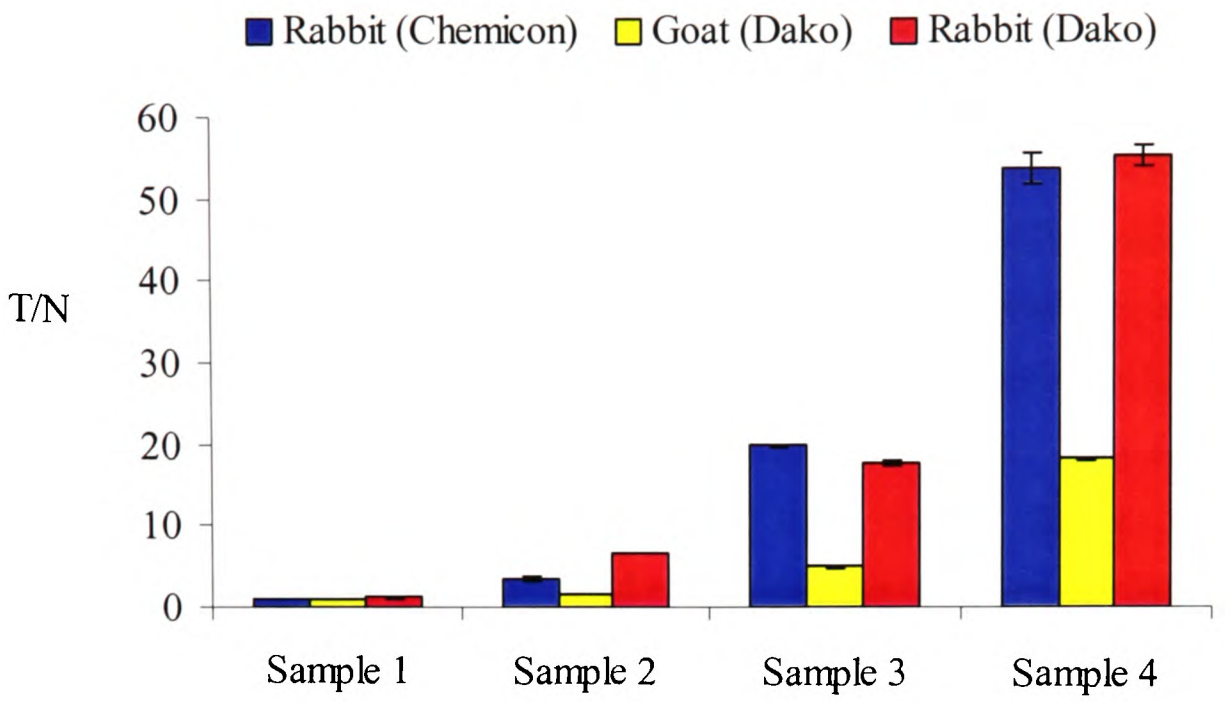
2.3.1 Optimisation of assay conditions

A series of experiments were performed to determine the optimal reagents and conditions for the GACELISA. These are described below.

(i) Anti-human IgG

Three commercial preparations, rabbit and goat anti-human IgG from Dako (Ely, Cambridge, U.K.) and rabbit anti-human IgG from Chemicon International Inc. (Temecula, California, U.S.A.), were compared using oral fluid samples collected from staff who were negative (n = 1) or positive (n = 3) for mumps-specific IgG in serum. As shown in Figure 2.4, the best T/N values were obtained when either of the rabbit anti-human IgG preparations were used; no significant difference ($p = 0.41$) in the T/N values of samples was observed. In further experiments, the rabbit anti-human IgG preparation from Dako was employed as this was being used for other assays in the laboratory.

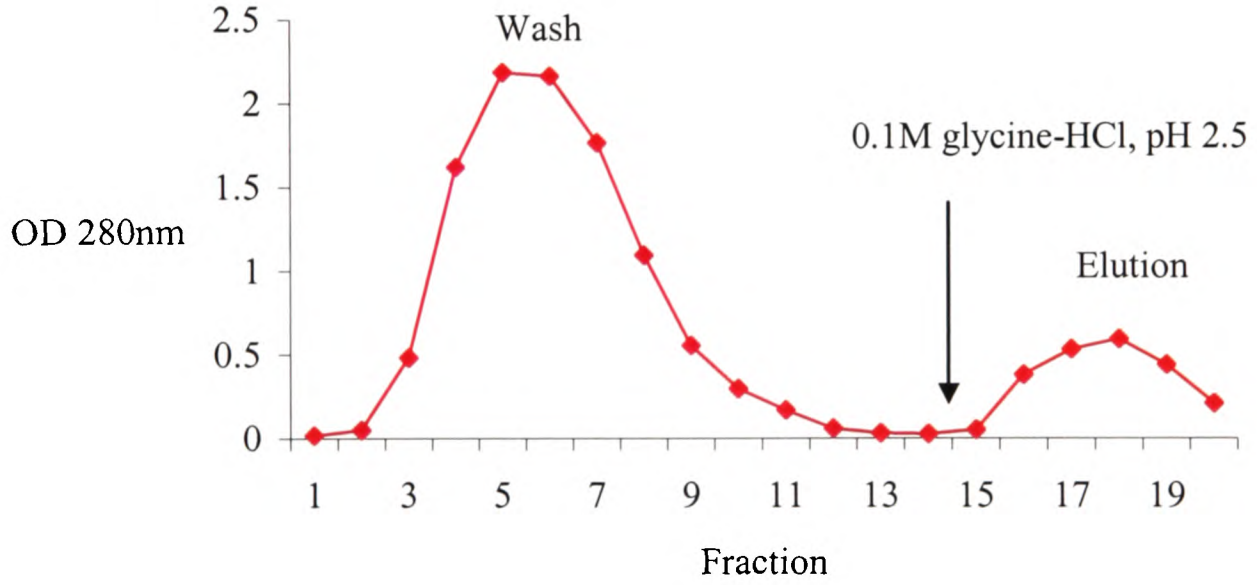
Figure 2.4 Effect of three commercial anti-human IgG preparations on T/N values in mumps GACELISA with four oral fluid samples collected from staff.



Microtitre plate wells coated with 1 μg/ml of anti-human IgG in 0.05 M sodium carbonate buffer (pH 9.6) were blocked with 5% SoluPro and incubated at 37 °C with 100 μl of the following: neat oral fluid or 1/200 dilution of serum in diluent B (Appendix A) for 30 min; 0.1 μg/ml of rNP in diluent B for 1 h; and 0.2 μg/ml of anti-rNP-HRP in diluent B for 30 min. Wells were washed four times with PBST between incubations. Bound conjugate was detected by incubating wells with 100 μl of TMB for 30 min at RT and the reaction was stopped with 100 μl of 0.5 M HCl. Samples were tested in duplicate and T/N values were calculated by dividing test OD results by the OD results of the negative control serum. Sample 1 was from a subject negative for mumps-specific IgG in serum, while samples 2 – 4 were from mumps-specific IgG positive subjects. Error bars represent one SD.

Initial experiments revealed false positive reactions in anti-human IgG coated wells that did not receive serum, which were not detected in wells that received negative serum. This was due to the binding of the mouse anti-rNP-HRP conjugate to the rabbit anti-human IgG coated on the solid phase ('no antigen' control wells). To overcome this problem, the commercial rabbit anti-human IgG preparation was applied to a mouse Ig column to remove cross-reacting antibodies (see 2.2.5). The elution profile of the absorption is shown in Figure 2.5. The first peak (wash) represented antibody that did not adsorb to the column, while the second peak (elution) represented antibody that cross-reacted with mouse Ig. After the adsorption, the 'wash' and 'elution' fractions were separately pooled, coated onto mictrotitre plate wells and reacted with anti-rNP-HRP conjugate. Only background signals were detected in wells coated with the pooled wash fraction whereas high signals were observed in wells coated with the pooled elution fraction (data not shown).

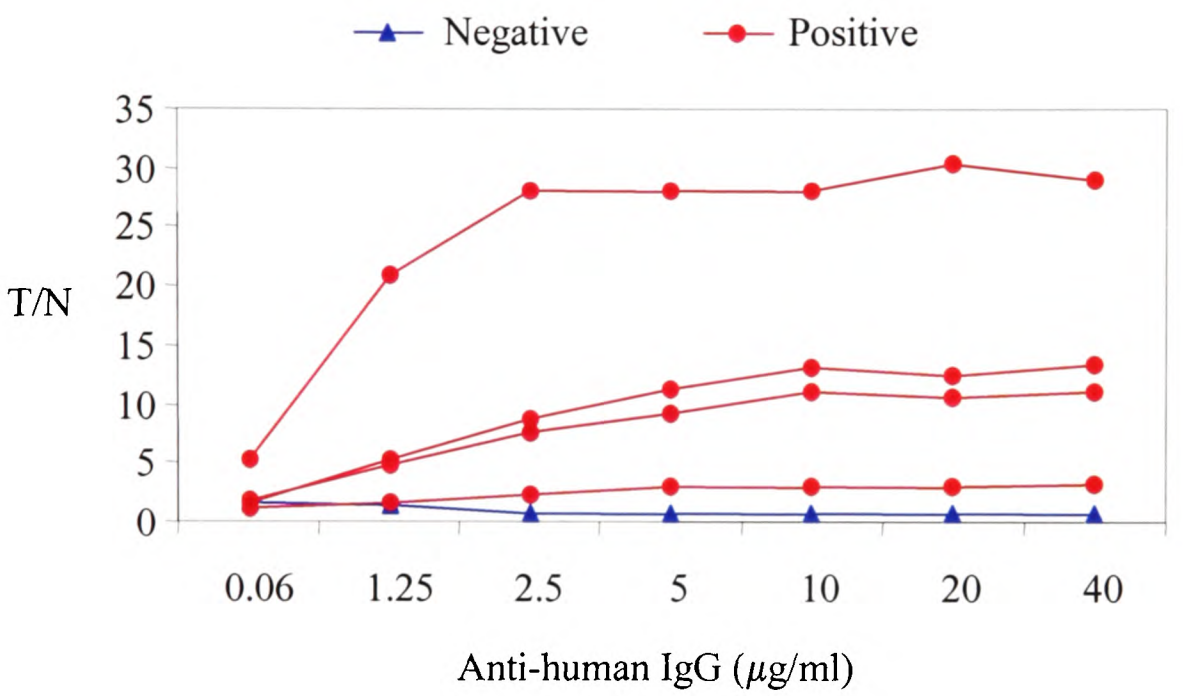
Figure 2.5 Purification of anti-human IgG on a mouse Ig column.



One millilitre (1.5 mg) of rabbit anti-human IgG (Dako) was applied to a mouse Ig column. The column was incubated for 1 h at RT to allow cross-reacting antibodies to adsorb and then washed to remove unbound material. The column was eluted with 0.1 M glycine-HCl, pH 2.5. One millilitre wash and elution fractions were collected and their UV absorbance measured at 280 nm.

The optimal concentration of absorbed anti-human IgG for coating microtire plate wells was determined using oral fluid samples from staff that were negative or positive for mumps-specific IgG in serum. As shown in Figure 2.6, the T/N values for the positive oral fluid samples, especially the weak positive samples, gradually increased up to 10 μg/ml of immobilised anti-human IgG, then plateaued. Thus, in further experiments anti-human IgG was coated on microtire plate wells at a concentration of 10 μg/ml.

Figure 2.6 Effect of different anti-human IgG concentrations on T/N values in mumps GACELISA with five oral fluid samples from laboratory staff.



Microtitre plate wells coated with 0.06 μg/ml to 40 μg/ml of absorbed anti-human IgG in 0.05 M sodium carbonate buffer (pH 9.6) were blocked and then incubated with 100 μl of neat oral fluid collected from staff negative (n = 1) and positive (n = 4) for mumps-specific IgG in serum. Other assay conditions were as those described in Figure 2.4. T/N values were calculated by dividing the test OD of single samples by the negative control ODs.

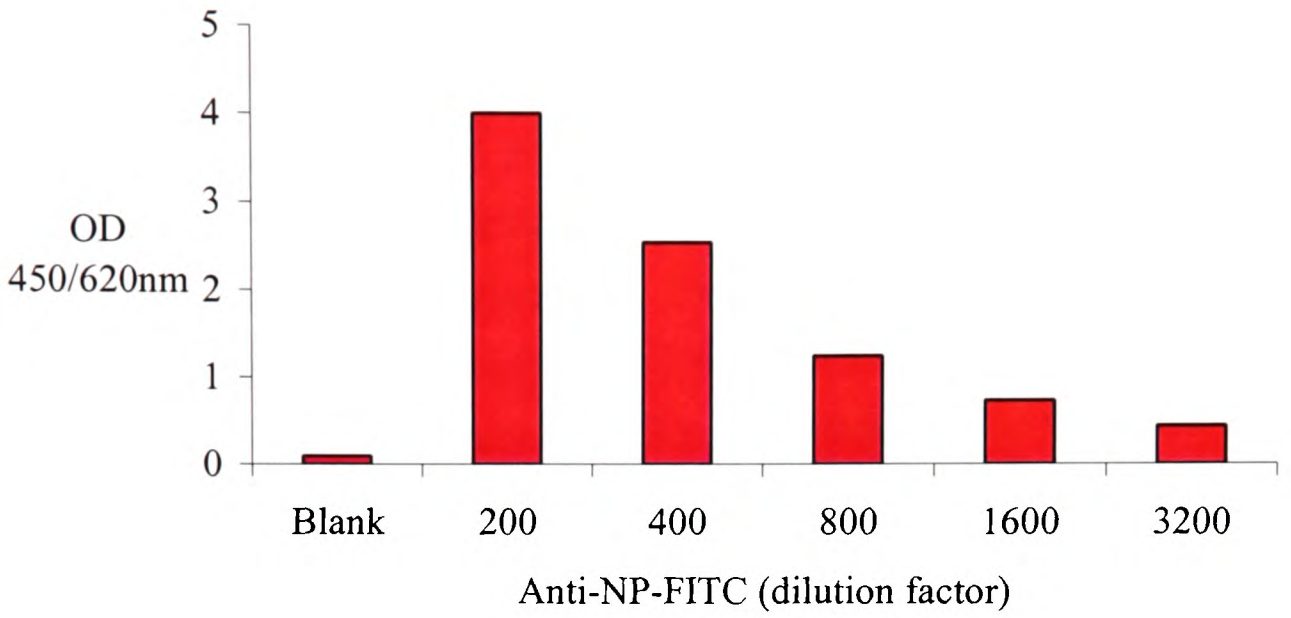
(ii) Assay Diluent

Initially BSA was used as a blocking agent in the assay diluent. However relatively high levels of non-specific binding were observed in the presence of samples negative for mumps-specific IgG (i.e. mean serum negative OD = 0.11; mean oral fluid negative OD = 0.19). To overcome this problem, 10% FCS was added to the assay diluent. In the presence of FCS, the binding with negative samples was greatly reduced (i.e. mean serum negative OD = 0.05; mean oral fluid negative OD = 0.07) whereas a relatively lower reduction in binding with positive samples was observed (i.e. mean oral fluid positive OD = 0.87 without FCS versus 0.64 with FCS), thus giving higher T/N values for positive samples (T/N = 12.8 versus 7.9 respectively).

(iii) FITC/anti-FITC detection system

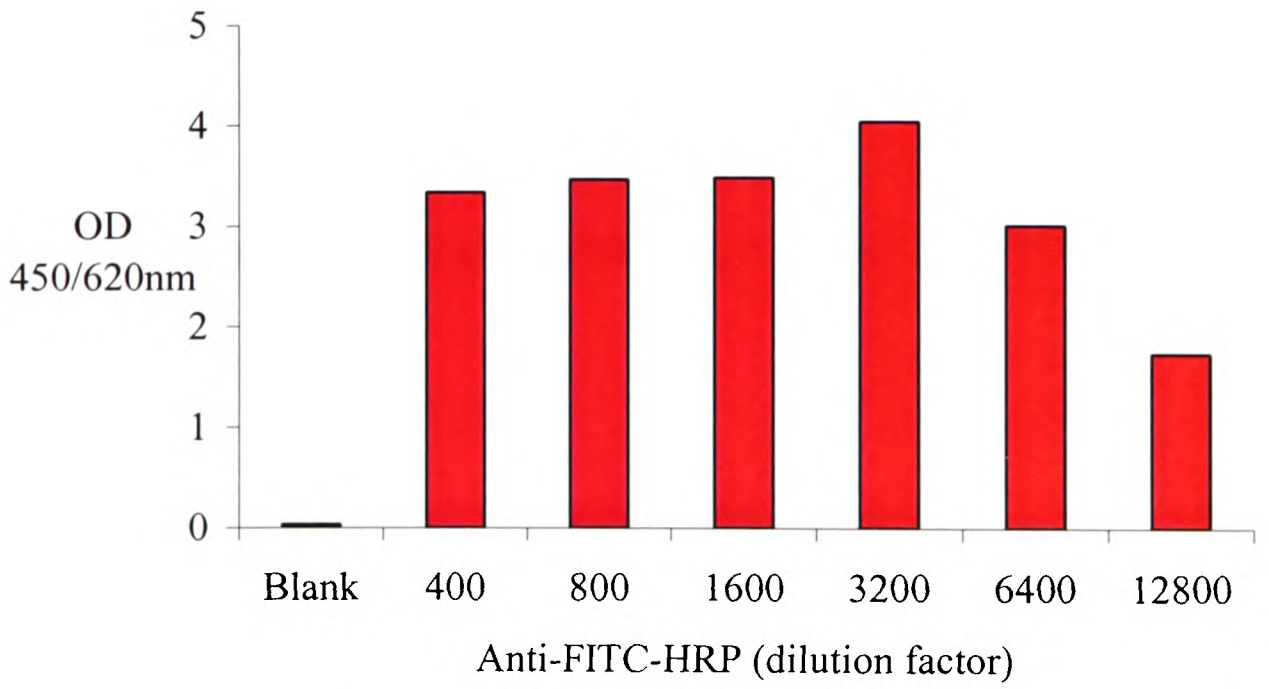
As mentioned in the introduction to this chapter, GACELISA's employing the FITC/anti-FITC amplification system have been developed for the detection of rubella and measles-specific IgG in oral fluid. The availabilty of a FITC-labelled MAb directed against mumps NP (anti-NP-FITC) and an anti-FITC-HRP conjugate enabled an anti-NP-FITC/anti-FITC-HRP detection system to be investigated as an alternative to using the anti-rNP-HRP conjugate in the GACELISA. Wells coated with rNP were reacted with doubling dilutions of anti-NP-FITC conjugate, which was detected using anti-FITC-HRP. As shown in Figure 2.7, the dilution of conjugate required to produce an OD of 1 - 2 was relatively low (1/400 - 1/800). Under similar conditions, the anti-rNP-HRP conjugate produced much higher OD values (data not shown). To establish whether the low OD values were a reflection of the low reactivity of the anti-FITC-HRP conjugate, doubling dilutions of anti-FITC-HRP were reacted with immobilised BSA-FITC (Microimmune Ltd.), and BSA as a control. As shown in Figure 2.8, even at very high dilutions (1/12800) the anti-FITC-HRP produced high OD values of 1 - 2. This suggested that the low OD values were a result of the low reactivity of the anti-NP-FITC conjugate. This conjugate was not used in further experiments.

Figure 2.7 Reactivity of anti-NP-FITC/anti-FITC-HRP detection system.



Microtire plate wells coated with 1 μg/ml of rNP in PBS azide were blocked and incubated with 100 μl of doubling dilutions of anti-NP-FITC in diluent B for 30 min at 37 °C. After washing, wells were incubated with 100 μl of anti-FITC-HRP in diluent B for 30 min at 37°C. Other conditions were as those described in Figure 2.4. OD results of single samples were plotted against dilution of anti-NP-FITC.

Figure 2.8 Reactivity of anti-FITC-HRP with immobilised BSA-FITC.



Microtire plate wells were incubated with 1 μg/ml BSA-FITC (Microimmune Ltd.) for 18 hr at 37 °C and then for 2 h at 37 °C. Wells were blocked and incubated with 100 μl of doubling dilutions of anti-FITC-HRP for 30 min at 37°C. Bound conjugate was detected as described in Figure 2.4. OD results of single samples were plotted against dilution of anti-FITC-HRP.

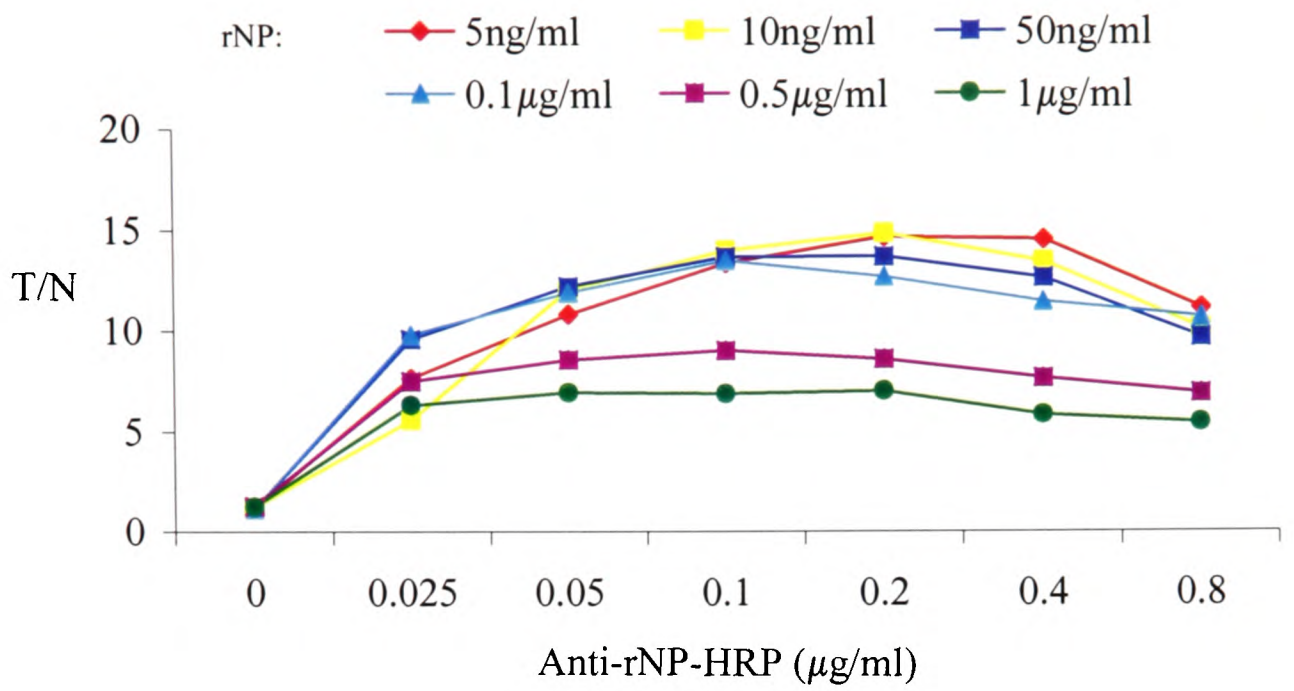
(iv) Determination of optimal antigen and conjugate concentrations for the mumps GACELISA with serum and oral fluid.

Mumps serum and oral fluid GACELISA 'checkerboard' titrations of immobilised rNP and anti-rNP-HRP conjugate were performed using negative and low positive serum samples, and a pool of negative and positive oral fluid. A pool of oral fluid was used to obtain sufficient material for the experiment. The concentrations of antigen investigated for both the serum and oral fluid assay were 5 ng/ml to 1 μg/ml. The concentrations of conjugate investigated for the serum GACELISA were 0.025 μg/ml to 0.8 μg/ml, and 0.025 μg/ml to 0.4 μg/ml for the oral fluid GACELISA. As shown in Figure 2.9(a), for the serum GACELISA the highest T/N values were observed at an antigen concentration of 10 ng/ml and a conjugate concentration of 0.2 μg/ml, although there was no significant difference in the T/N values between 5 ng/ml – 0.1μg/ml of antigen ($p = 0.22$) and 0.1 - 0.4 μg/ml of conjugate ($p = 0.42$). In Figure 2.9b, the highest T/N values in the oral fluid GACELISA were observed at an antigen concentration of 50 ng/ml and conjugate concentration of 0.4 μg/ml, although there was no significant difference in T/N values between 0.2 and 0.4 μg/ml of conjugate ($p = 0.56$). As a result of these findings, in further experiments the concentrations of antigen and conjugate used for the serum and oral fluid GACELISA were 50 ng/ml and 0.2 μg/ml respectively.

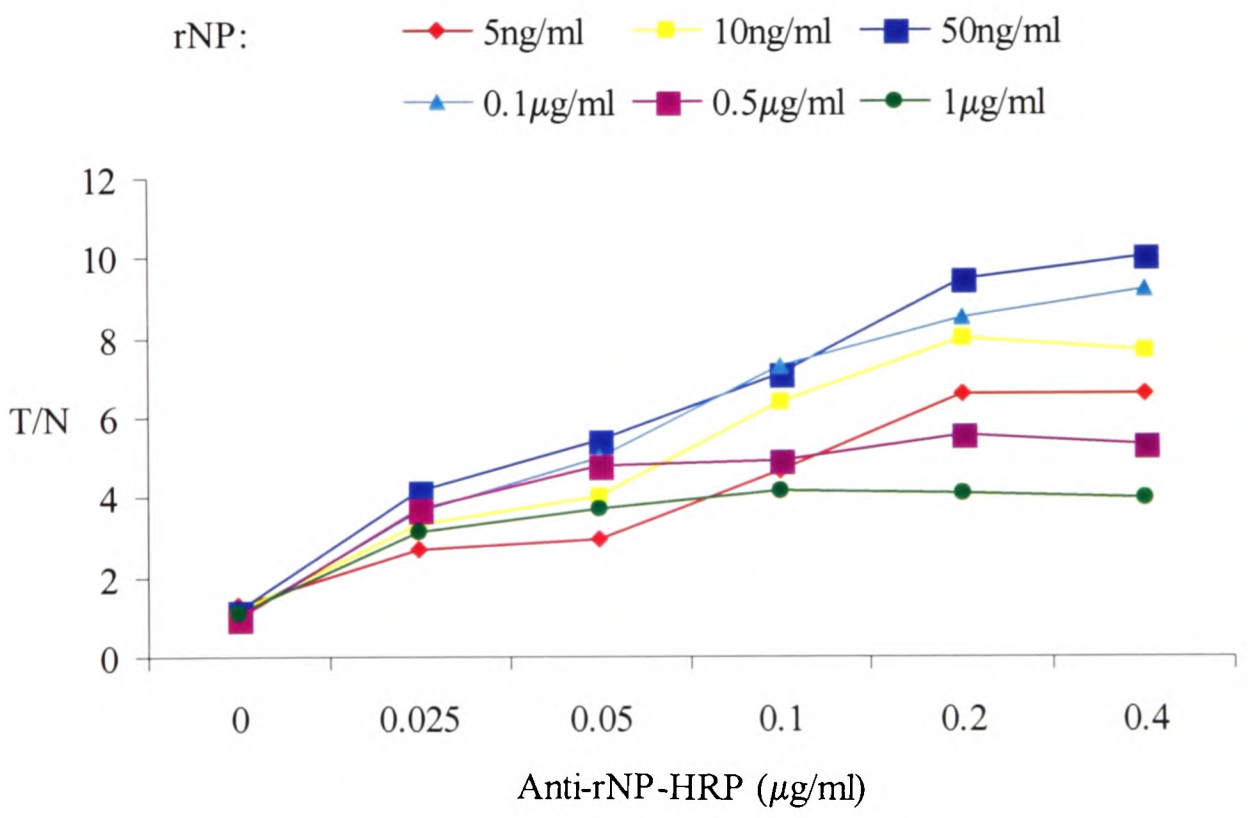
The effect of shaking wells during the incubation steps was then investigated. Although only marginally higher T/N values were observed when the wells were shaken during the sample, antigen and conjugate incubation steps (i.e. serum T/N = 11.8 versus 10.5; oral fluid T/N = 6.3 versus 5.5) shaking was employed in subsequent experiments.

Figure 2.9 Determination of the optimal working concentrations of rNP and anti-rNP-HRP for the mumps GACELISA with (a) serum and (b) oral fluid.

(a) serum



(b) oral fluid



Microtitre plate wells coated with 10 μg/ml of absorbed rabbit anti-human IgG in 0.05M sodium carbonate buffer (pH 9.6) were incubated with 100 μl of 1/200 dilution of negative and positive serum or 100 μl of neat, pooled negative and positive oral fluid in diluent B for 30 min at 37°C. After washing wells were incubated with titrations of rNP and anti-rNP-HRP as indicated. Other conditions were as described in Figure 2.4. The negative oral fluid samples were from unvaccinated infants and the positive oral fluid samples were from >30 year olds. T/N values were calculated by dividing the test ODs of single samples by the negative control ODs.

2.3.2 Quality of oral fluid

The total IgG concentrations of 300 oral fluid samples self-collected by 50 volunteers (6 swabs/subject) were used for a comparative analysis of sample quality (Table 2.4). The statistical comparisons showed that the mean titre (MT) of total IgG in oral fluid did not significantly differ between different sides of the mouth, whether collected on day 1 ($p = 0.605$) or day 2 ($p = 0.874$). Similarly, the MT of total IgG did not significantly differ whether oral fluid was collected from one side or both (side 1 + side 2) sides of the mouth (swab 2 v swab 3; $p = 0.983$). However, significantly higher ($p = <0.001$) MT were observed on day 2 (unsupervised collection) compared with day 1 (supervised collection). Also, the MT of total IgG in oral fluid was significantly higher (swab 5 v swab 6; $p = 0.033$) using the Oracol compared with the OraSure® device. From these findings, the oral fluid collected using the Oracol device was used in further experiments. Since no significant difference was observed between samples taken from different sides of the mouth it was not necessary to pool the samples prior to testing.

Table 2.4 Assessment of oral fluid quality collected by the Oracol and OraSure® devices

Oral Fluid (n=300)	Collection Device	Day of collection	Collected from one or both sides of the	Supervised	Total IgG mean mg/L (range)	p value
Swab 1	Oracol	1	Side 1	Yes	19.9 (6.4 – 81.2)	p = 0.605[a]
Swab 2	Oracol	1	Side 2	Yes	24.4 (8.3 – 101.2)	p = 0.98[b] p = <0.001[c]
Swab 3	Oracol	1	Side 1 + Side 2	Yes	24.4 (6.2 – 144.9)	
Swab 4	Oracol	2	Side 1	No	36.5 (7.3 – 145.7)	p = 0.874[d]
Swab 5	Oracol	2	Side 2	No	35.7 (5.4 – 133.6)	p = 0.033[e]
Swab 6	OraSure®	2	Side 1 or Side 2	No	26.7 (2.4 – 145.0)	

[a] Comparison of total IgG titres from swab 1 with swab 2.
[b] Comparison of total IgG titres from swab 2 with swab 3.
[c] Comparison of total IgG titres from swab 2 with swab 5.
[d] Comparison of total IgG titres from swab 4 with swab 5.
[e] Comparison of total IgG titres from Oracol (swab 5) with OraSure® (swab 6).

2.3.3 Assay Reproducibility

To determine the reproducibility of the GACELISA four serum and eight oral fluid samples were assayed six times on the same test plate (intra-assay variation), and on different plates on five separate occasions (inter-assay variation). The samples were arranged in the 96-well plates in a systematic way to avoid samples appearing in the same position in the different columns. Table 2.5 shows the mean OD values, SDs and 95% confidence intervals for the replicates. As expected the inter-assay variation was greater than the intra-assay variation; 95% of results were within 27% of the true mean between wells on different plates and within 11% of the true mean between wells on the same plate. These low values indicate that the assay was highly reproducible.

Table 2.5 Determination of GACELISA intra- and inter-assay variation

	Intra-assay variation				Inter-assay variation			
Sample No[a]	**Geometric mean OD (n = 6)**	**Geometric mean log OD (n = 6)**	**SD[b] logOD**	**95% CI[c] (+/-)**	**Geometric mean OD (n = 6)**	**Geometric mean log OD (n = 6)**	**SD[b] logOD**	**95% CI[c] (+/-)**
1	0.051	-1.29	0.032	15.9	0.042	-1.376	0.056	29.6
2	0.59	-0.23	0.021	0.3	0.50	-0.305	0.060	31.7
3	0.025	-1.61	0.047	24.0	0.024	-1.621	0.042	21.3
4	3.238	0.51	0.008	3.8	2.835	0.451	0.044	22.4
5	0.175	-0.76	0.017	8.3	0.147	-0.837	0.065	35.1
6	1.077	0.03	0.022	10.5	0.878	-0.059	0.057	29.9
7	0.486	-0.31	0.012	5.8	0.424	-0.374	0.040	20.1
8	2.229	0.35	0.013	6.1	1.937	0.286	0.037	18.6
9	0.419	-0.38	0.022	10.5	0.368	-0.439	0.070	38.0
10	0.524.	-0.28	0.006	2.6	0.452	-0.348	0.059	31.4
11	0.058	-1.24	0.045	23.1	0.055	-1.261	0.043	21.6
12	0.0469	-0.33	0.020	9.5	0.419	-0.380	0.052	26.8
			Average	11%			Average	27%

[a] Sample numbers 1 - 4 and 5 – 12 were serum and oral fluid samples respectively.
[b] SD denotes standard deviation.
[c] 95% CI denotes the 95% confidence interval around the mean OD.

2.3.4 GACELISA validation

An experiment was performed to determine a way of evaluating the validity of each assay run. The OD results (log scale) of four serum controls in five different assay runs were used to determine a 95% confidence interval for the differences between duplicates (Table 2.6). For the 20 sets of results, the SD of the differences was 0.05 (log scale). Thus, 95% of acceptable results were expected to fall within 0.103 (log scale) of one another, which was a 27% difference (i.e.$10^{0.103}$ = 1.27). Assays with differences in the serum controls of less than 27% were accepted.

Table 2.6 Differences in results (log OD) from duplicate control samples

Test No.	Serum Control	OD 1	OD 2	log OD1	log OD 2	Difference[a]
1	1	0.042	0.049	-1.377	-1.310	-0.067
	2	0.453	0.462	-0.344	-0.335	-0.009
	3	0.027	0.031	-1.569	-1.509	-0.060
	4	2.423	2.276	0.384	0.357	0.027
2	1	0.045	0.048	-1.347	-1.319	-0.028
	2	0.462	0.448	-0.335	-0.349	0.013
	3	0.027	0.027	-1.569	-1.569	0.000
	4	2.399	2.667	0.380	0.426	-0.046
3	1	0.048	0.048	-1.319	-1.319	0.000
	2	0.448	0.448	-0.349	-0.349	0.000
	3	0.027	0.027	-1.569	-1.569	0.000
	4	2.667	2.667	0.426	0.426	0.000
4	1	0.046	0.064	-1.337	-1.194	-0.143
	2	0.5	0.554	-0.301	-0.256	-0.045
	3	0.028	0.038	-1.553	-1.420	-0.133
	4	2.838	2.784	0.453	0.445	0.008
5	1	0.039	0.038	-1.409	-1.420	0.011
	2	0.42	0.428	-0.377	-0.369	-0.008
	3	0.026	0.023	-1.585	-1.638	0.053
	4	2.35	2.101	0.371	0.322	0.049

[a] Difference was log OD 1 – log OD 2.

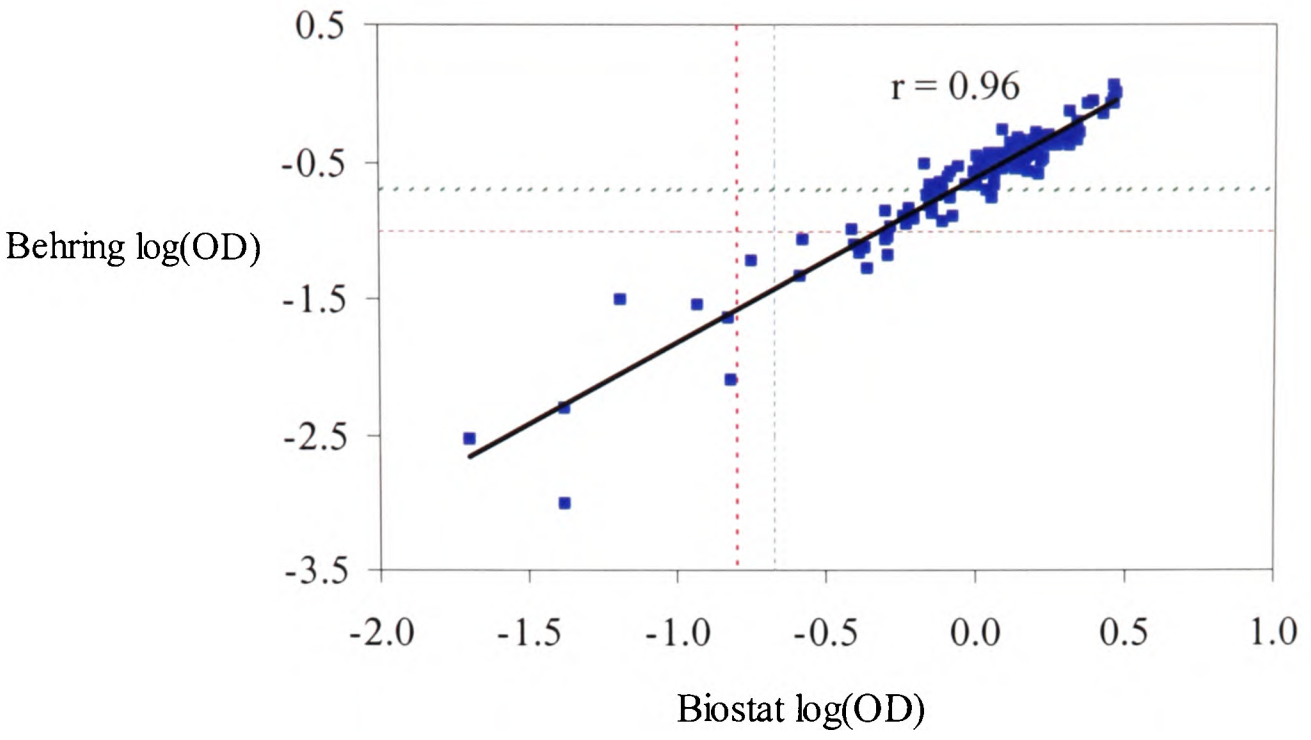
2.3.5 Performance of two commercial assays for the detection of mumps-specific IgG in serum

The Biostat (Bio-Stat Diagnostic Systems, Stockport, UK) and Behring (Behring Enzygnost, Marburg, Germany) commercial ELISAs for the detection of antibodies to mumps were investigated. The cut-off values used were as those recommended by the manufacturers. One hundred and twenty-two samples were tested and the results are shown in Table 2.7. Of the 122 samples, 87 were positive and 7 were negative by both assays. Eighteen samples that were Behring equivocal were positive by the Biostat assay and one Biostat equivocal was Behring negative. Also, nine positive Biostat samples were Behring negative. These findings suggest that the Biostat assay was either more sensitive or less specific than the Behring assay. A scatter plot of the results showed the assays correlated well (r = 0.96), particularly for positive samples (Figure 2.10).

Table 2.7 Comparison of Biostat and Behring ELISAs for the detection of mumps-specific IgG in 122 serum samples from staff

	BEHRING			
BIOSTAT	**Equivocal**	**Negative**	**Positive**	**Total**
Equivocal	-	1	-	1
Negative	-	7	-	7
Positive	18	9	87	114
Total	18	17	87	122

Figure 2.10 Scatter graph of Biostat and Behring results for 122 serum samples.



The Behring OD results were plotted against the Biostat OD results (log scale). The dotted lines represent the equivocal (red lines) and positive (green lines) cut-off values for the assays.

2.3.6 Evaluation of mumps GACELISA with serum and oral fluid using serum-oral fluid pairs

The mumps GACELISA with serum and oral fluid was initially evaluated using the 122 serum-oral fluid pairs, where the sera had been previously tested using the commercial assays described above. For both evaluations, the GACELISA results were compared with the serum results obtained using the Biostat assay (as this appeared to be more sensitive than the Behring assay). Table 2.8 shows how the cut-off values were calculated.

Table 2.8 Determination of serum and oral fluid GACELISA cut-off values using Biostat negative samples

Samples	GACELISA	
	Serum	Oral fluid
1	0.051	0.043
2	0.036	0.026
3	0.124	0.053
4	0.033	0.024
5	0.033	0.024
6	0.076	0.055
7	0.041	0.032
Standard deviation	0.0335	0.0135
Mean serum control	0.028	0.027
Cut-off[a]	0.128	0.068

[a]Cut-off = mean serum or oral fluid control + 3 SD

For the serum GACELISA, 118 of the 122 samples gave the same results as the Biostat ELISA (Table 2.9). One sample was equivocal by the Biostat assay and negative using the GACELISA, and three samples were Biostat positive and GACELISA negative, giving an overall sensitivity and specificity for the GACELISA of 97% and 100% respectively. A scatter plot of the results (Figure 2.11a) showed the assays correlated well ($r = 0.93$). For the oral fluid GACELISA, 121 of the 122 oral fluids gave the same results as the Biostat assay based on serum resulting in 100% sensitivity and specificity. One sample that was Biostat equivocal and GACELISA negative was not included in the analysis. A scatter plot of the results (Figure 2.11b) showed relatively good correlation ($r = 0.90$) between the oral fluid and serum mumps IgG levels.

Table 2.9 Comparison of Biostat assay and GACELISA with (a) serum and (b) oral fluid using 122 serum-oral fluid pairs collected from staff

(a) Serum GACELISA

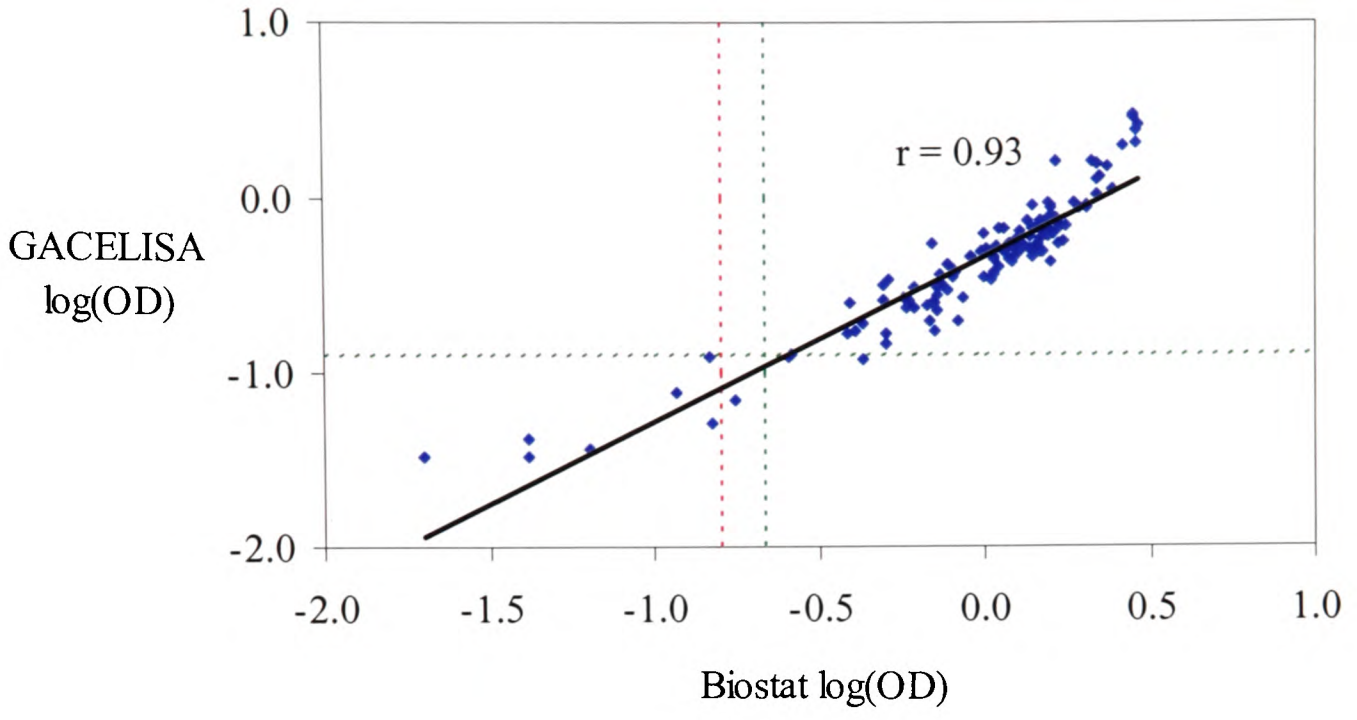
	GACELISA		
BIOSTAT	**Negative**	**Positive**	**Total**
Equivocal	1	-	1
Negative	7	-	7
Positive	3	111	114
Total	11	111	122

(b) Oral fluid GACELISA

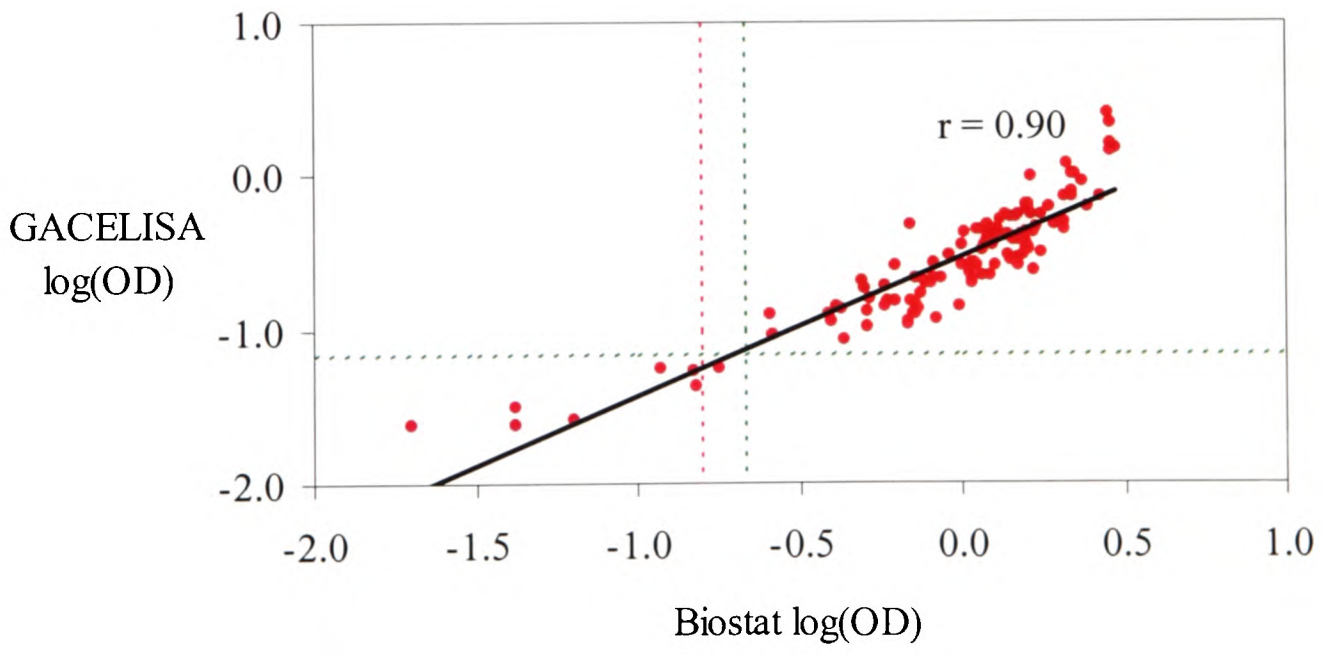
	GACELISA		
BIOSTAT	**Negative**	**Positive**	**Total**
Equivocal	1	-	1
Negative	7	-	7
Positive	-	114	114
Total	8	114	122

Figure 2.11 Scatter graph of Biostat and GACELISA (a) serum and (b) oral fluid results for 122 serum-oral fluid pairs collected from staff.

(a) Serum



(b) Oral fluid



The GACELISA (a) serum and (b) oral fluid OD results were plotted against the Biostat OD results (log scale). The dotted lines represent the equivocal (red lines) and positive (green lines) cut-off values for the assays.

2.3.7 Evaluation of mumps GACELISA with oral fluid using mixture modelling

In the previous evaluation using serum-oral fluid pairs, only seven of the 122 subjects were negative for mumps-specific IgG. This was not surprising as the samples were obtained from staff volunteers who were over 18 years of age. One would expect them to have been exposed to mumps. However, the low numbers of negatives means the specificity estimate was not reliable. To further investigate the performance characteristics of the assay, oral fluid samples from 923 subjects aged 0.7 – 99 years were tested by the GACELISA and the results were analysed using mixture modelling. Mixture modelling was used as it does not require the antibody status of specimens to be known. Models were fitted to the distribution of results and optimal cut-off values that maximised assay sensitivity and specificity were estimated. The prevalence of antibody in each category of the study population was also estimated. It should be noted, however, that the samples used for the study were not a random sample of the population. They were selected at random from oral fluids submitted to ERNVL for MMR testing.

For the analysis, the GACELISA results were categorised by age (group 1, 0.7 -1.4 years; group 2, 1.41 - 8.99 years, group 3, 9 - 19.9 years, and group 4, > 20 years), vaccination and mumps-specific IgM status (Table 2.10). Samples from infants aged 0.7 – 1.4 years were chosen to provide a negative population as they were unlikely to have been vaccinated against mumps and would have lost their maternal antibodies. Children aged 1.41 - 8.99 years may have received two doses of MMR (as the two-dose schedule was introduced in 1996) whereas those aged 9 - 19.99 years most likely only received one dose. Those greater than 20 years would not have routinely been offered vaccination but would most likely have been exposed to natural mumps infection.

Of the study population, 127 subjects were mumps-specific IgM positive; 37 (29%) had received one dose and ten (8%) had received two doses of MMR vaccine (Table 2.10). Only one of the IgM positive subjects was under 9 years. Of the IgM negative subjects, 196 were under 20 years and vaccinated; 249 were under 20 years and unvaccinated (most of these were under 1.4 years); 294 were over 20 years and unvaccinated; 16 were over 20 years and vaccinated; and for 41 subjects it was unclear if they had been vaccinated.

Table 2.10 Age, vaccination and IgM status of subjects

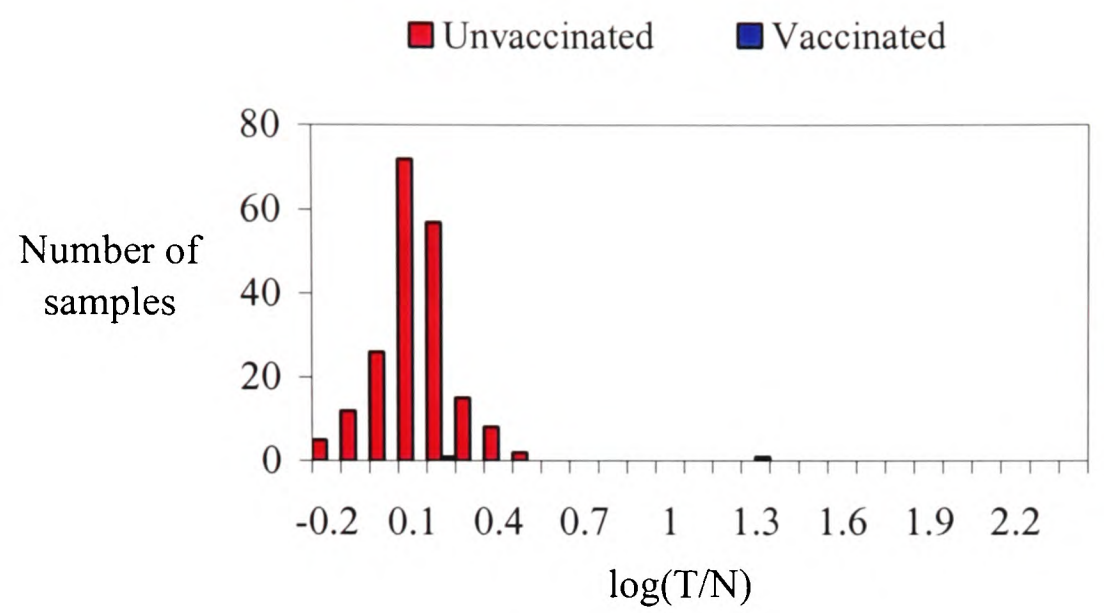
	Age Group (range)				
Status[a]	1 (0-1.4 years)	2 (1.41-8.99 years)	3 (8.99-19.99 years)	4 (> 20 years)	Total
IgM –ve + unvaccinated	197	30	22	294	584
IgM –ve + vaccinated (2 doses)	2	120 (67)	74	16 (6)	212 (73)
IgM +ve + unvaccinated	0	1	34	45	80
IgM +ve + vaccinated (2 doses)	0	0	44 (8)	3 (2)	47 (10)
Unknown	11	8	22	-	41
Total	210	159	196	358	923

[a]IgM status was determined by using a Mumps-specific IgM Antibody Capture Radioimmunoassay (Perry *et al.* 1993).

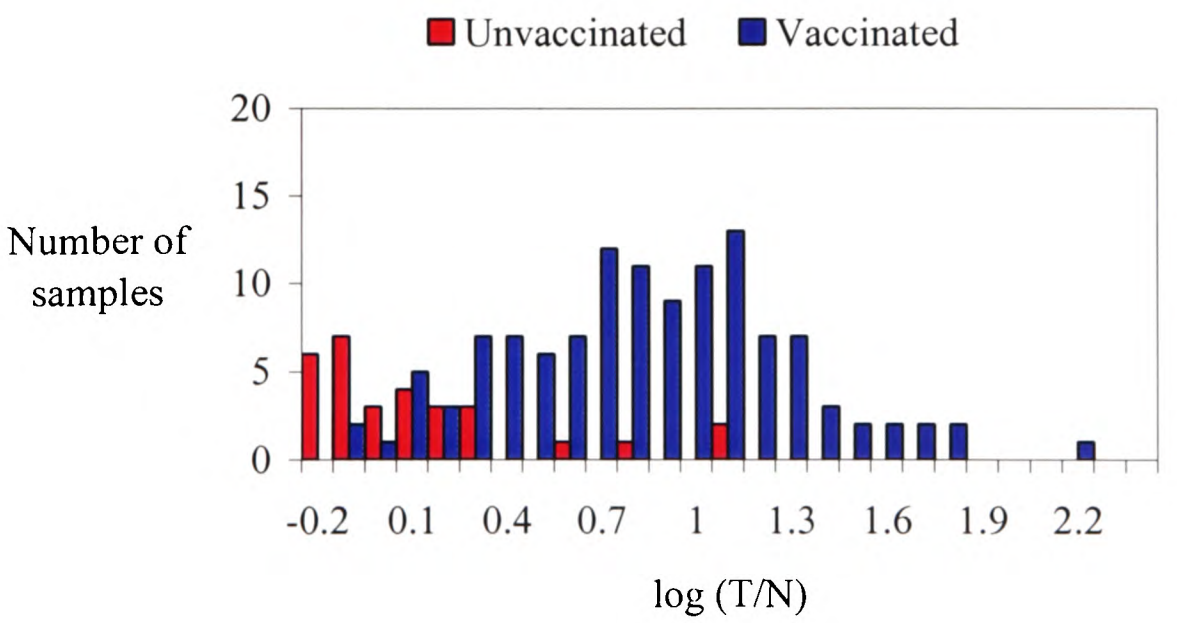
Figure 2.12 shows the distribution of GACELISA results in each age cohort. As expected, unvaccinated subjects in group 1 had very low log(T/N) values. Similarly, most of the unvaccinated subjects in group 2 had very low log (T/N) values, whereas in group 3, a wider spread of log(T/N) values was observed in those unvaccinated. Most likely some of the subjects in this cohort (8.99-19.99 years) had been exposed to natural mumps infection. In group 4, most of those unvaccinated had relatively high log(T/N) values due to natural mumps infection. The highest log(T/N) values were observed in those that were also IgM positive, most of which were in group 3 (teenagers). Most vaccinated subjects were in groups 2 and 3 and the distribution of results in these two age groups appeared very similar, despite many (56%) in group 2 having had received two doses of vaccine.

Figure 2.12 Distribution of log(T/N) values in the GACELISA by age group, vaccination and mumps IgM status in 882 oral fluids.

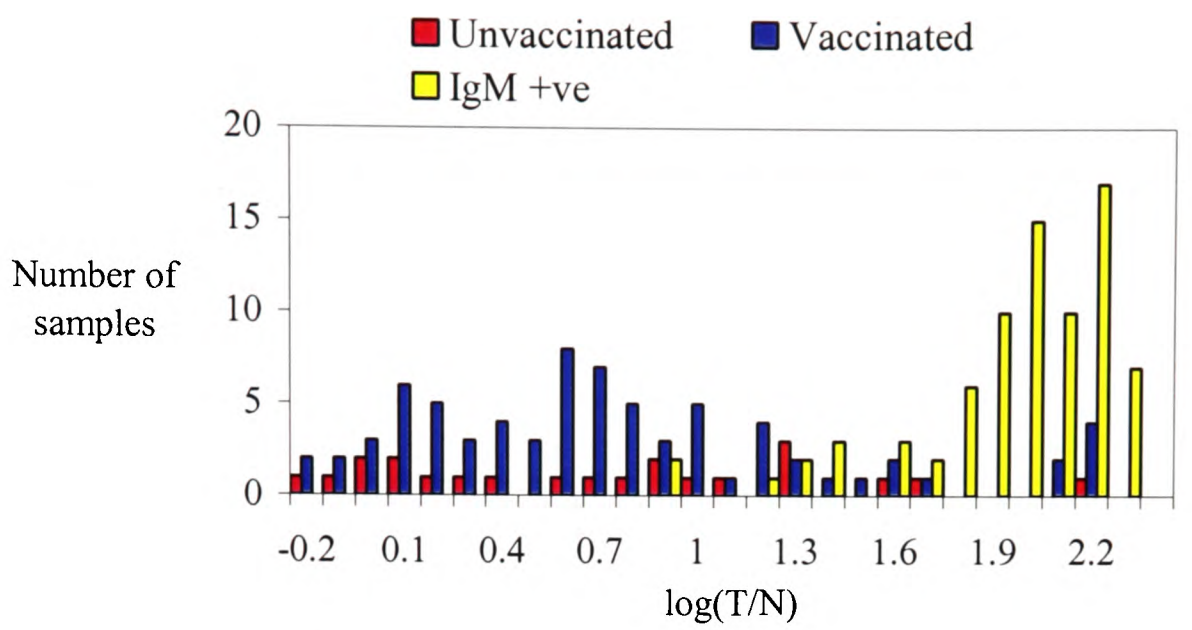
(a) Group 1 (0 - 1.4 years)



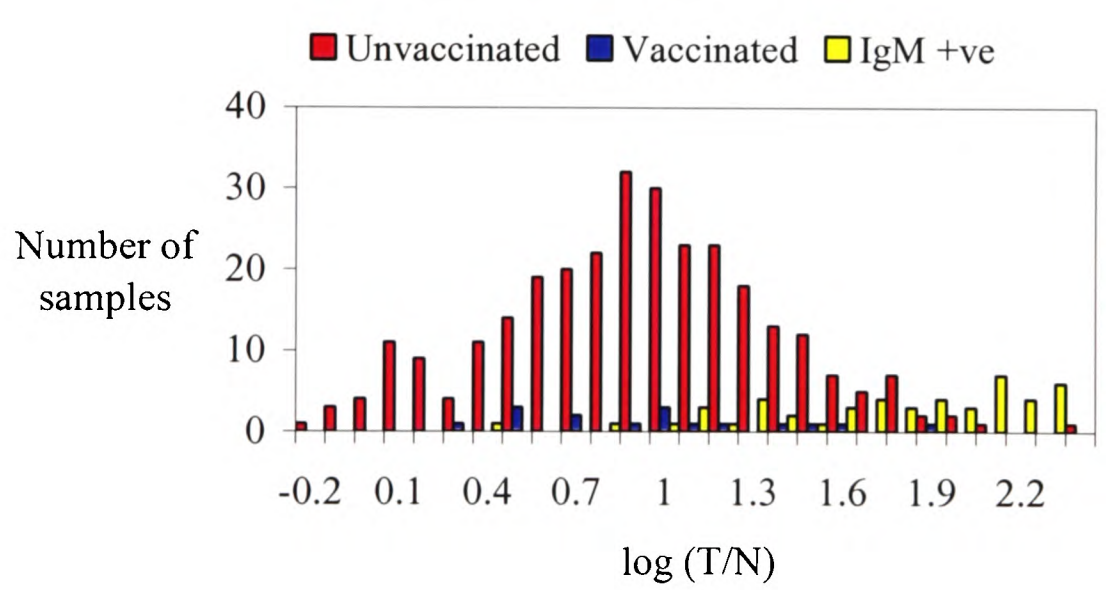
(b) Group 2 (1.44 - 8.99 years)



(c) Group 3 (9 – 19.99 years)

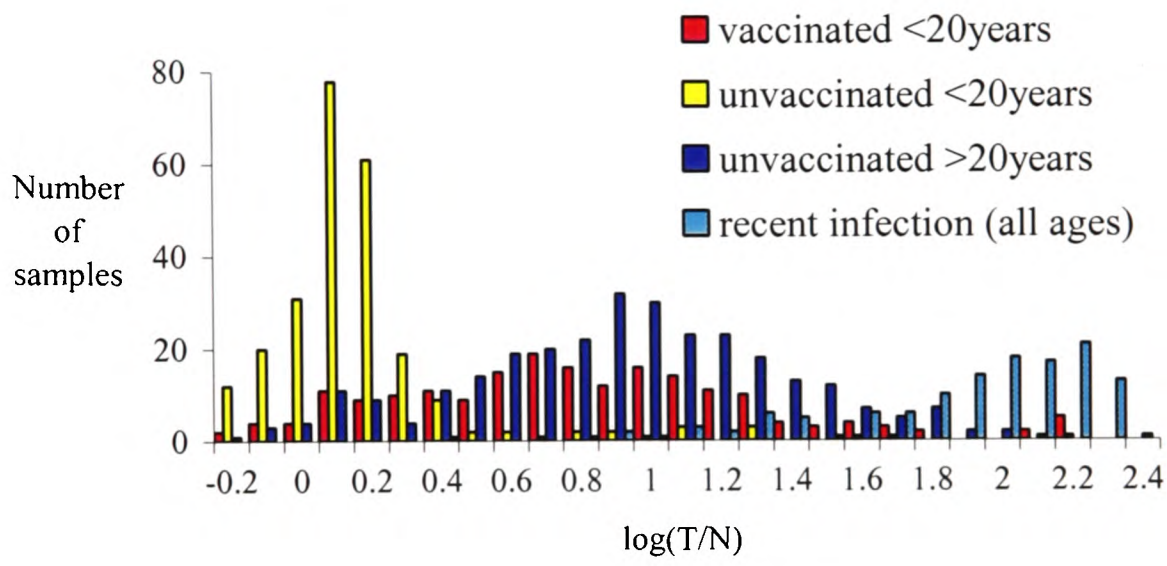


(d) Group 4 (> 20 years)



Further inspection of the distribution of the GACELISA results (Figure 2.13) revealed the log(T/N) values appeared to fall into four overlapping populations: a negative population (mostly unvaccinated infants) and three positive populations (vaccinated, past infection and recently infected). Since the distribution of results of those protected by vaccination (< 20 years [red]) appeared to be different from those protected by natural mumps infection (> 20 years [dark blue]), mixture models with negative, positive and recently infected components were fitted to the distribution of GACELISA results stratified by age (< 20 years and > 20 years). Table 2.11 summarises the mixture modelling results.

Figure 2.13 Distribution of log(T/N) values in the GACELISA by age, vaccination and mumps IgM status in 866 oral fluids.



T/N values were calculated by dividing the absorbance of the test oral fluid by the mean absorbance of a negative control serum. The log(T/N) values were categorised by age (< 20 years and > 20 years), vaccination and IgM status. The unknowns and vaccinated over 20 year old results were not included in the histogram due to the low number of samples in these categories. The categorised log(T/N) values were separated into 27 reactivity compartments, each of size 0.1 log OD and the number in each compartment plotted in a histogram.

Table 2.11 Results of fitting mixture models to GACELISA results stratified by age

(a) < 20 years

Distribution	Mean log(T/N) [SD[a]]	Optimal cut-off[b] log(T/N)	Sensitivity %	Specificity %	Prevalence %
Negative	0.07 [0.12]	-	-	-	39.3
Positive	0.70 [0.55]	0.35	73.7	99.2	48.5
Recently infected	2.03 [0.15]	1.65	99.3	96.0	12.2

(b) > 20 years

Distribution	Mean log(T/N) [SD[a]]	Optimal Cut-off[b] log(T/N)	Sensitivity %	Specificity %	Prevalence %
Negative	0.07 [0.12]	-	-	-	4.8
Positive	0.96 [0.43]	0.35	92.3	99.2	87.8
Recently infected	2.03 [0.15)]	1.65	99.3	94.8	7.4

[a] SD denotes standard deviation

[b] Optimal cut-off values for the positive and recently infected distributions were determined by using the mixture model to estimate sensitivity and specificity at various cut-off values, and the value that maximised both sensitivity and specificity was selected.

Due to insufficient sample numbers in the > 20 year old category, the negative and recently infected distributions were assumed to be the same for the two age groups. This explains why the means and SDs of these distributions were the same in both age groups. It is worth noting, however, that the distribution of results for both the negative and the recently infected individuals in the unvaccinated > 20 year old group appeared very similar to those of the unvaccinated < 20 years old group [i.e. in Figure 2.12 (a) and (d) the distribution of results for the negative individuals peaked at 0.1 log(T/N) and in Figure 2.12 (c) and (d) the distribution of results for the recently infected individuals peaked at 2.2 log(T/N)].

The distribution of positive results in the older group had a higher mean log(T/N) and lower SD reflecting the narrower spread of log(T/N) values, compared with the younger age group. Consequently there was less overlap between the positive and negative distributions resulting in the model estimating the assay sensitivity to be higher for the older age group. The specificity was the same for both age groups as the negative distributions were assumed to be the same. The cut-off value that maximised assay sensitivity and specificity was 0.35 log(T/N) for both age groups.

The distribution of results for those recently infected (IgM positive) in both age groups had a relatively high mean log(T/N) and low SD. The estimated assay sensitivity for recently infected subjects was the same for both age groups (as the distributions were assumed the same) however the specificity was marginally higher for the younger group when using the optimal cut-off 0.65 log(T/N).

Overall for the younger group, the model estimated that results below 0.35 log(T/N) included all negatives (>99% specificity) and about 26% (73.7% sensitivity) of those who had been vaccinated; results between 0.35 log(T/N) and 1.65 log(T/N) were from those that had been vaccinated; while results over 1.65 log(T/N) were for all recently infected subjects and for 4% who had not been vaccinated.

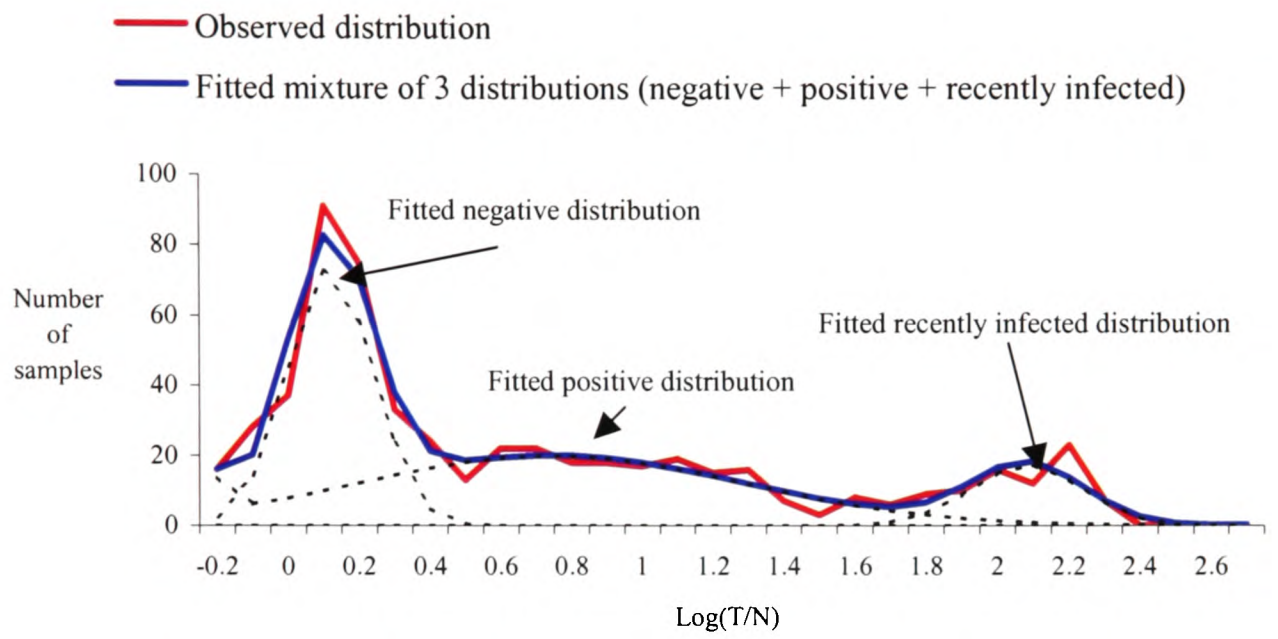
In the older group, the model estimated that results below 0.35 log(T/N) were shared by all negatives (>99% specificity) and about 8% of those with past infection; results between 0.35 log(T/N) and 1.65 log(T/N) were from those with past infection; and results over 1.65 log(T/N) were from those with recent infection and 5% of those with past infection.

Figure 2.14 shows the distribution of the observed GACELISA results for the two age groups and the fitted mixture models. The fitted curves closely followed the distribution of observed results. The sharp curve corresponding to the negative results (Figure 2.14a) and

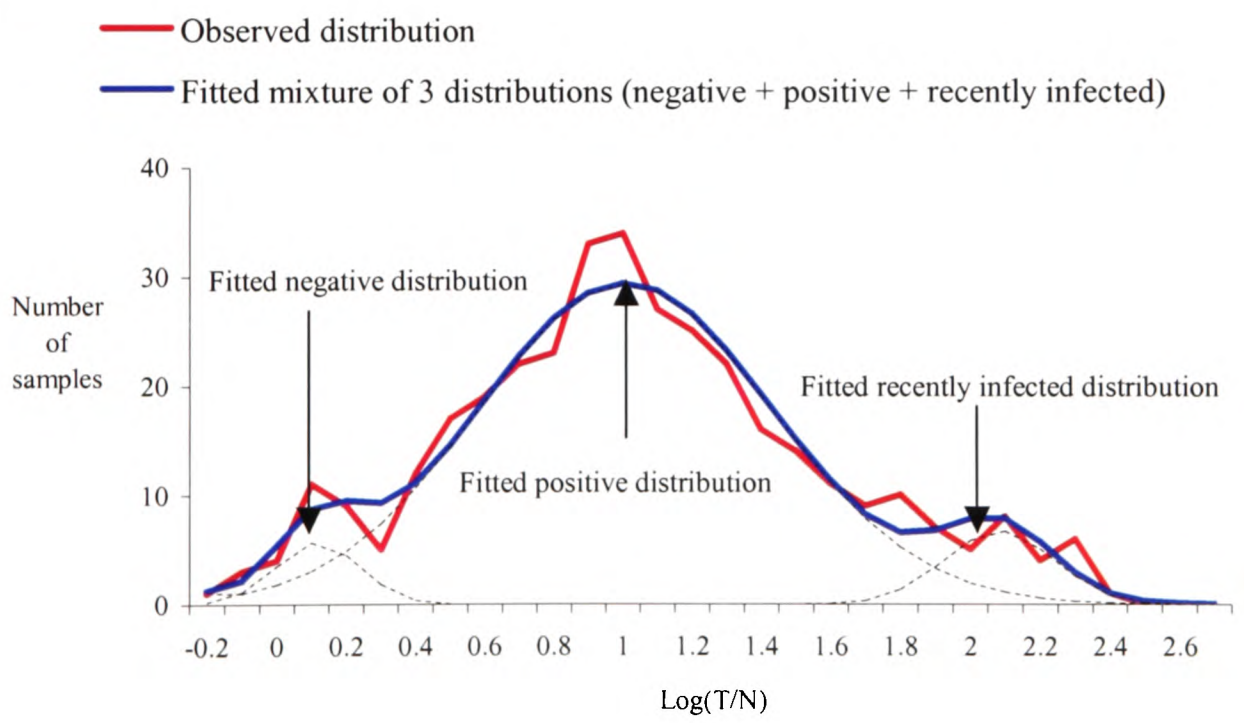
the particularly broad flat curve corresponding to the positive results in the under 20 year old group are particularly noticeable.

Figure 2.14 Distribution of oral fluid GACELISA results for (a) < 20 year olds (n = 565) and (b) > 20 year olds (n = 358).

(a) < 20 years



(b) > 20 years



T/N was calculated as described in Figure 2.13. The red and blue curves represent the observed and fitted data respectively. The dotted lines represent the three separate fitted populations (negative, positive and recently infected).

To independently assess the accuracy of the mixture modelling approach, the mixture model cut-off of 0.35 log T/N was used to categorise the 122 staff oral fluid results which were then compared to the results of the paired serum. Compared to the Biostat assay using serum samples, the oral fluid GACELISA was 100% sensitive and 100% specific (Table 2.12). One sample that was Biostat equivocal and GACELISA negative was not included in the analysis. These results matched exactly the results using the cut-off determined using the serum-oral fluid pairs. Compared to the Behring assay using serum samples, however, the oral fluid GACELISA was 100% sensitive and 53% specific (Table 2.13). Nine samples that were Behring negative were GACELISA positive. Not included in the analysis were eighteen samples that were Behring equivocal and GACELISA positive. These results suggest the Behring is less sensitive than the Biostat assay, rather than it being more specific.

Table 2.12 Comparison of oral fluid GACELISA results using mixture model cut-off (0.35 logT/N) with the Biostat assay

	GACELISA		
BIOSTAT	**Negative**	**Positive**	**Total**
Equivocal	1	-	1
Negative	7	-	7
Positive	-	114	114
Total	8	114	122

Table 2.13 Comparison of oral fluid GACELISA results with the Behring assay

	GACELISA		
BEHRING	**Negative**	**Positive**	**Total**
Equivocal	-	18	18
Negative	8	9	17
Positive	-	87	87
Total	8	114	122

2.4 Discussion

Age-related serological surveys of antibodies to viral and bacterial infections provide valuable information on population immunity and susceptibility. They are particularly useful for informing and refining immunisation strategies. In the UK, the serological surveillance programme has been instrumental in guiding changes to vaccination policy. Examples include the 1994 measles and rubella campaign (Gay *et al.*, 1995) and the introduction of a second dose of MMR at 4 years of age (Gay *et al.*, 1997). The serological surveillance programme relies on the collection of residual serum samples following the completion of biochemical or microbiological investigations so circumvents the need to collect blood samples (Morgan-Capner *et al.*, 1988; Osborne *et al.*, 2000). However, the sera used are not a random sample of the population and lack informative data, such as ethnic group or country of birth. Also, countries with less developed health infrastructures may find it difficult to implement such a system. In other countries, surveys have been conducted using sera specifically donated by people randomly sampled from the population (Esveld, 1994; Fleming *et al.*, 1997). This has the advantage of allowing detailed risk factor information to be obtained but is considerably more expensive.

The potential of serological surveillance will not be fully realised, most especially in the developing world, as long as the collection of blood is required. Blood collection is expensive. Moreover it is invasive and painful so is unaccceptable to many individuals, particulary children, making recruitment difficult.

In contrast, the collection of oral fluid is cheap, easy, painless, and patients evidently prefer to be handed a sponge swab than be subjected to venepuncture. In this study, recruitment to the staff survey would have been better had a matching serum sample not been required. Refusal to enter surveys based on venepuncture has been reported to be up to 13% (de Azevedo Neto *et al.*, 1995).

There is growing evidence that oral fluid can substitute for serum (or plasma) for detecting virus-specific antibodies (Parry *et al.*, 1987; Perry *et al.*, 1993; Rice and Cohen, 1996; Vyse *et al.*, 1997; Ramsay *et al.*, 1998; Nigatu *et al.*, 1999; Vyse *et al.*, 1999; Nokes *et al.*, 2001; Sheppard *et al.*, 2001). The lack of availability of a sensitive oral fluid-based mumps IgG ELISA assay prompted the development and evaluation of the GACELISA described here.

2.4.1 Optimisation of the GACELISA

During the assay development, when BSA was used as a blocking agent in the assay diluent, false positive reactions were identified in wells that did not receive test serum. The false positive reactions occurred because the 'capture' anti-human IgG cross-reacted with the mouse anti-rNP MAb conjugate. Although adding negative serum to the diluent blocked this phenomenon, anti-mouse IgG antibodies were successfully removed from the anti-human IgG preparation by affinity absorption prior to plate coating. Cross-reactions between antibodies of different species have been documented previously (Tijssen, 1985).

Even when the cross-reactions had been eliminated, relatively high background OD values were observed in wells that received negative control serum. This was due to the failure of BSA in the assay diluent to block the non-specific binding of assay components to the solid phase. To overcome this 10% FCS was added to the assay diluent. FCS improved the T/N values by reducing background signals. Previous workers have used FCS as a blocking agent in the assay diluent in antibody capture assays (Perry *et al.*, 1993; Vyse *et al.*, 1997)

Recently, GACELISA's employing the FITC/anti-FITC amplification system have been successfully developed for the detection of rubella and measles-specific IgG in oral fluid (Nigatu *et al.*, 1999; Vyse *et al.*, 1999). Vyse *et al.* found the rubella GACELISA employing the FITC-anti-FITC amplification system was more sensitive than the equivalent radioimmunoassay. Nigatu *et al.* used serum-oral fluid pairs and found the oral fluid measles GACELISA was 97.4% sensitive and 90% specific compared to a serum measles IgG assay. In the present study, an anti-NP-FITC/anti-FITC-HRP system was investigated but was not used due to the poor performance of the anti-NP-FITC conjugate. The amplification step did not compensate for the low reactivity of the anti-NP-FITC conjugate. The reason(s) for the low reactivity of the conjugate was unclear but may have been due to the denaturation of the MAb during storage or perhaps due to inefficient crosslinking. Nevertheless, this highlights the need for good quality reagents to provide sensitive and specific assays.

Most mumps antibody assays employ antigen derived from virus culture. However such antigens may produce false positive results due to reactions with residual non-viral contaminants (Bukrinsky *et al.*, 1989). Also, culturing virus is costly and potentially hazardous. In contrast, highly purified recombinant antigens can be produced relatively quickly, cheaply and in greater abundance compared with antigen extracts. Moreover their use offers the potential for assay improvement. Recombinant antigens expressed in a

variety of expression systems have produced highly sensitive and specific assays. The rNP antigen used in this study was produced in a yeast expression system and has been shown to be highly immunogenic (Samuel *et al.*, 2002). Monoclonal antibodies to rNP were shown to react with native mumps NP virus, as well as purified rNP.

To determine the optimal concentrations of rNP and anti-rNP-HRP, checkerboard titrations were performed which revealed that only very low concentrations of recombinant antigen (50 ng/ml) were required for the GACELISA. A typical indirect assay requires a much greater amount of antigen to be coated on the solid phase (~1 μg/ml).

2.4.2 Oral fluid collection

In this study two different commercial devices, the Oracol and OraSure®, were used to collect oral fluid samples from volunteer laboratory staff. The quality of the oral fluid collected by each device was assessed by measuring the concentrations of total IgG in a sub-sample of 300 oral fluids. All samples contained detectable levels of total IgG, however samples collected by the Oracol contained significantly higher levels, compared to those collected by the OraSure®, when collected under the same conditions (i.e. unsupervised). This difference may have been due to the efficiency of antibody elution from the absorbent material, however the results are more likely to reflect differences in the way the devices are used. Both swabs target the area between the gums and teeth, the part of the oral cavity most likely to be rich in crevicular fluid, however the Oracol is used much more vigourously than the OraSure®. Several earlier studies have compared the quality of samples obtained by different collection devices. Parry *et al.* (1987) compared the OraSure® and Salivette, a device consisting of a cotton wool pad that is chewed to collect oral fluid from all parts of the mouth. Oral fluid samples collected by the OraSure® gave higher mean total IgG concentrations than those collected by the Salivette, perhaps reflecting the targetting by OraSure® of crevicular fluid-rich regions of the mouth. Nokes *et al.* (1998) and Vyse *et al.* (2001) compared the Oracol, OraSure® and the Omni-Sal®, a device consisting of a pad that is placed under the tongue until the stem indicator of the device changes colour. Consistent with the findings presented in this thesis, they found the Oracol device yielded samples with the highest total IgG concentrations. However, the geometric mean levels differed between studies (Table 2.14). The levels detected in this study were similar to those detected by Nokes *et al.*, whereas those detected by Vyse *et al.* were approximately five times lower. This may reflect the different assays used to determine total IgG concentration. Alternatively, it may be due to the age of the study

populations. In this study, samples were collected from adults (>18 year olds). Similarly, a large proportion (57%) of the samples in the Nokes *et al.* study were adults, whereas Vyse *et al.* tested samples collected from 3.5 - 5 year olds. Thieme *et al.* (1994) also found mean total IgG concentrations in oral fluid (collected by OraSure®) were significantly higher in samples collected from over 18 year olds compared to samples from under 18 year olds, suggesting oral fluid total IgG concentrations are age-related.

Table 2.14 Comparison of total IgG concentrations (mg/L) in oral fluids collected by the Oracol and OraSure® in three independent studies

Collection device	Nokes *et al.*	Vyse *et al.*	Present study
Oracol	41.7 (n = 34)	6.08 (n = 42)	36 (n = 50)
OraSure®	22.9 (n = 34)	4.40(n = 45)	26.7 (n = 50)

A wide range of total IgG concentrations was observed (2.4 mg/L to 145.7 mg/L). This may reflect sampling and/or the oral health of subjects. Patients with gingivitis may be expected to exhibit particularly high levels of plasma-derived antibody in their oral fluid, as the disease is characterised by inflammation and bleeding of the gums. Previous studies have determined the minimum total IgG content required for viral specific antibody detection. Parry *et al.* (1993) concluded a minimun total IgG of 0.1 mg/L was required for the measurement of antibody to HIV. While, Vyse *et al.* (2001) found 2 mg/L was required for the detection of oral fluid EBV antibody (Vyse *et al.*, 2001). Although a minimum level of total IgG was not determined here all the oral fluid samples contained over 2 mg/L.

Oral fluid samples collected from different sides of the mouth did not significantly differ in total IgG content, nor did samples taken consecutively. Interestingly, samples collected in the presence of the laboratory personnel supervising the study contained significantly less total IgG than samples collected in their absence. The reason(s) for this are unclear; one would perhaps expect the opposite finding. Subjects may have been more comfortable when collecting the second set of oral fluids in their own time. A previous study found no significant difference between the quality of oral fluids collected by a nurse or self-collected (Sheppard *et al.*, 2001). These findings bode well for future epidemiological studies carried out in community settings.

The OraSure® was more expensive than the Oracol and less acceptable to volunteers as many commented that the taste of the device was unpleasant. However, the Oracol was more difficult to process in the laboratory because it required ackward manual extraction which was time consuming and laborious, as well as presenting a greater risk of aerosol formation. However, recently a prototype container for the Oracol has been developed. This device has been designed to make sample processing much easier and safer (Bernard Cohen, personal communication).

2.4.3 Commercial ELISA

There are a variety of tests on the market for the detection of mumps-specific IgG in serum but no formal comparisons have been published. In this study we assessed the performance of two commercial assays. The results suggested that the Biostat assay was more sensitive than the Behring assay, as more samples were classified as positive.

Sensitivity and specificity of an assay, however, are highly dependent upon the cut-off values provided by the manufacturer. When a scatter graph of the Behring OD values were plotted against the Biostat OD values (Figure 2.10), the distribution of results suggested that the Behring cut-off value was set very high. Manufacturers most likely set cut-off values so as to minimise the number of false-positive results. In determining the immune status of an individual, a false positive result is more harmful than a false negative result as the individual would not be offered vaccine and would remain susceptible. The Behring assay also had a large equivocal range (OD 0.1 - 0.2), which meant 17 samples of 122 were classified as indeterminate. In the clinical setting this is disadvantagous, as the equivocal samples need to be retested which is time-consuming and costly.

2.4.4 Assay reproducibility

It is essential that the reproducibility of an assay is high to provide consistent and accurate results. Here, the GACELISA was shown to be highly reproducible. Both the intra- and inter- assay variations were shown to be low. The assay was also developed such that each run can be validated by testing control sera in parallel with test samples.

2.4.5 Evaluation of GACELISA using serum-oral fluid pairs and mixture modelling

The performance of the oral fluid GACELISA was assessed in two ways: comparison of results from serum/oral fluid pairs collected from staff; and mixture modelling using a large panel of oral fluid samples that had been submitted for MMR testing. The use of serum/oral fluid pairs is the gold standard method for evaluating a new oral fluid IgG assay

and has been used by many investigators (Parry, 1993; Nigatu *et al.*, 1999; Vyse *et al.*, 1999; Nokes *et al.*, 2001). By contrast, mixture modelling has not been widely applied for this purpose (Parker *et al.*, 1990; Sheppard *et al.*, 2001).

Compared to the Biostat serum ELISA, the oral fluid GACELISA had a sensitivity and specificity of 100%. However the specificity estimate was less reliable than the sensitivity estimate due to the small numbers of serum-negative results. To improve the accuracy of the specificity estimate many more paired samples would have to have been collected and tested. This is difficult when many individuals are reluctant to give blood and ethical approval is required.

The OD results (log transformed) of the GACELISA and the Biostat assay correlated very well. This was surprising since the format and antigen preparation of the assays differed considerably. The indirect Biostat assay employed antigen from infected cell cultures whereas the GACELISA employed a recombinant antigen. Most likely NP was well represented in the cell lysate antigen used in the Biostat assay. The structural organisation of the mumps genome results in NP being the first protein expressed (Elango *et al.*, 1988), so it may be produced in greater amounts than the other proteins.

Mixture modelling revealed that subjects with vaccine-induced immunity had relatively lower GACELISA values than those with past infection. Previous studies based on serum samples have obtained similar findings. For example, Weibel *et al.* (1967) showed that mumps neutralising antibody titres were considerably lower after vaccination compared with after natural disease. Also, Christenson and Bottiger (1990) showed that sera from children naturally infected with the mumps virus had significantly higher ELISA values than post-vaccination sera from seroconverting children.

As well as being relatively lower, the GACELISA values were more widely distributed in those with vaccine-induced immunity compared to those with past mumps infection. This larger variation resulted in a larger overlap with the negative distribution, so the estimated sensitivity of the GACELISA was lower (74%) when results from those under 20 years were modelled compared with those over 20 years (92%) (using the optimal cut-off of 0.35 log T/N). Both these estimates were lower than that calculated using the gold standard method of comparison of serum-oral fluid pairs (>99%), suggesting that the model was underestimating sensitivity. It should be noted that the paired serum-oral fluid samples were collected from adults, so the sensitivity estimated should be compared with the older mixture modelling group. The assay specificity calculated by the mixture modelling

(>99%) was the same as that calculated from the paired samples when using the cut-off 0.35 logT/N.

The mixture modelling fitted successfully a mixture of three normal distributions. This was possible since the GACELISA values of those recently infected were much higher and formed a separate distribution to those exposed to natural mumps or vaccination in the past. Thus the assay might be complementary to detecting mumps-specific IgM or IgG avidity testing to diagnose recent infection.

When the mixture modelling-derived cut-off value of 0.35 logT/N was applied to the GACELISA results from volunteer laboratory staff, the sensitivity and specificity estimates were the same as when the cut-off was determined using the serum-negative GACELISA OD values. These findings demonstrate that mixture modelling was a suitable alternative to using paired serum samples (for the identification of a negative population) to determine an appropriate cut-off value. Mixture modelling, however, appeared to underestimate the GACELISA sensitivity, especially in the vaccinated group. Due to the wide spread of results the model estimated a relatively large percentage (26%) of those vaccinated were false-negative (i.e. were GACELISA negative but assumed positive as they had been vaccinated). However, they may have been 'true' negatives (i.e. despite being vaccinated they were negative for mumps-specific IgG). The evidence for this is that the model predicted some vaccinated individuals would have a log(T/N) less than 0, which is unrealistic and secondly, a large percentage (35%) of those recently infected had been vaccinated, suggesting they were not protected (i.e. they were vaccine failures). As mentioned in the general introduction, there have been a number of reports of mumps outbreaks in highly vaccinated populations (Hersh *et al.*, 1991; Briss *et al.*, 1994), which have mostly been attributable to primary and/or secondary vaccine failures. Narita *et al.* (1998) used IgG antibody avidity testing to demonstrate that of 14 patients with vaccine failure, 12 patients, including 7 with a positive IgM response, had high avidity IgG antibodies indicating waning and/or incomplete vaccine-induced immunity. Most countries have now adopted a two-dose regime for vaccination, with the aim of eliminating mumps infection and all its complications. Recently, Pebody *et al.* (2002) investigated the effect of the first and second MMR doses on specific antibody levels 2 - 4 years after receiving a first dose of MMR vaccine at age 12 - 18 months. They found that a large proportion of pre-school children had mumps (23.4%) IgG antibody below the putative level of protection. After a second dose of MMR, however the proportion negative dropped to <

4%. Interestingly, in this study 10 of the 47 (21%) vaccinated subjects with evidence of recent mumps infection had received two doses of the MMR vaccine.

A major advantage of mixture modelling is it does not require uninfected individuals to be identified so eliminates the need for collecting blood samples. However, for cut-off determination, it does rely upon approximately equally numbers of each population (seronegative and seropositives) to be represented. In this study, oral fluids from infants (0.8 – 1.4 years) were used to provide a negative population, as these most likely had not been exposed to mumps and would have lost any maternal antibodies. The drawback of using such a population, however, is that it is not a random sample of the population of seronegatives, and since younger susceptibles are less likely to have experienced other infections, they generally have less contaminating non-specific antibody to raise their OD reading. Thus mixture modelling cannot be used blindly and if known uninfected and infected individuals' samples can be included in the analysis or the accuracy of the components estimated using the mixture modelling approach can be independently assessed then this is best. Here, mixture models were fitted to a mixture of three distributions and the validity of the optimal cut-off for the identification of an individual with past infection was successfully demonstrated.

In summary, the evaluation of the GACELISA demonstrated it to be highly reproducible, sensitive and specific for the detection of mumps-specific IgG in oral fluid. It will allow wider epidemiological studies of mumps infection than are currently possible because of the limitations imposed by the need to collect blood samples. It will also be complementary to mumps-specific IgM assays in the confirmation of recent infection in individuals.

Since oral fluid has relatively low levels of antibodies compared to serum, it has been difficult to distinguish between negative oral fluid samples and those with low levels of antibodies using ELISA technology. It may be that colorimetric detection may not be able to tease apart the very low reactivity oral fluid samples from those with no reactivity. Conventional ELISAs are thought to have a detection sensitivity in the attomole (10^{-18}mol or 10^5 molecules) range, thus specimens containing specific reactive antibodies that fall in this range will not be discriminated from specimens that have no specific antibodies. Thus the use of enzyme labels and colorimetric substrates place theoretical limits on assay sensitivity. The next chapter describes the development of an immuno-PCR assay designed to improve assay sensitivity.

Chapter 3

Immuno-PCR: application to the detection of mumps-specific IgG using DNA-labelled antibodies and real-time PCR

3.1 Introduction

In the previous chapter, it was noted that the sensitive detection of virus-specific antibodies may be limited by the enzyme labels and colorimetric substrates used in enzyme immunoassays. These limitations have been noted previously (Stanley *et al.*, 1985; Ruan *et al.*, 1993) and various amplification systems have been developed to address them. For example, the use of fluorogenic and radioactive substrates or enzyme amplification methods have increased detection limits (Harris *et al.*, 1979; Stanley *et al.*, 1985; Johannsson *et al.*, 1986; Ruan *et al.*, 1993; Zhang *et al.*, 2001). Recently, DNA labels that are detected using PCR have been used in the development of ultra-sensitive I-PCR assays (Sano *et al.*, 1992; Ruzicka *et al.*, 1993; Zhou *et al.*, 1993; Hendrickson *et al.*, 1995; Maia *et al.*, 1995; Case *et al.*, 1997; Wu *et al.*, 2001).

3.1.1 Immuno-PCR

I-PCR is a modification of the ELISA where the enzyme conjugate used to detect an antigen-antibody reaction is replaced by a specific antibody that is either directly or indirectly coupled to a DNA molecule. This DNA can then be used as template for PCR amplification. The technique offers both the specificity of antigen-antibody interactions and the enormous amplification capability of PCR, potentially achieving antibody sensitivity levels far higher than by conventional assays.

The feasibility of I-PCR was first assessed for the detection of an antigen by Sano *et al.* (1992). BSA was immobilised on the surface of microtitre plate wells and subsequently detected using anti-BSA IgG linked to biotinylated DNA via a recombinant streptavidin-protein A chimera; streptavidin binds specifically to biotin residues on modified DNA and Protein A binds to the Fc portion of detecting antibody. The biotinylated DNA was used as template for PCR, the product of which was analysed using gel electrophoresis and ethidium bromide staining. The assay detection limit was a few hundred molecules of antigen, approximately five fold more sensitive than the equivalent ELISA.

Since then several modified I-PCR formats have been described for the detection of a wide range of proteins. One of the main drawbacks of the I-PCR developed by Sano *et al.* was that the chimera was not commercially available. This led others to develop a modified protocol that could be universally adopted. Instead of the chimera, a biotin binding protein, either avidin or streptavidin, was used to link biotinylated DNA with biotinylated detecting antibody. Zhou *et al.* (1993) used this to detect anti-lipoprotein IgG immobilised on microtitre plate wells, and Ruzicka *et al.* (1993) used it to detect

recombinant human proto-oncogene EST1 immobilised on PCR tubes. In both cases, the I-PCR allowed the detection of very low levels of immobilised target. This approach has since been used to detect Bovine Herpesvirus 1 (Mweene *et al.*, 1996), the fish pathogen Pasteurella piscicida (Kakizaki *et al.*, 1996) and various structurally diverse antigens such as oligomeric pyruvate dehydrogenase complex (Case *et al.*, 1997).

In the previous examples either a direct or indirect assay was used. However, using the (strept)avidin/biotin system other assay formats may be adopted. The sandwich-type assay, in which immobilised antibody captures the antigen of interest, has been used to detect α-human atrial natriuretic peptide (Numata and Matsumoto, 1997) and soluble T cell receptors in plasma (Sperl *et al.*, 1995). Successful development of such assays would have been difficult using the chimera protein, as the protein A moiety would bind to the Fc region of immobilised capture antibodies, thus increasing background and compromising assay specificity. Furthermore, protein A is known to have varied affinities for different types of antibodies. Its use would thus be restricted depending on the class or subclass of antibodies being used.

Direct chemical conjugation of antibodies with marker DNA has been the most recent advance in I-PCR. Both single stranded- (ss) and double stranded (ds)-DNA have been shown to function effectively as I-PCR labels when covalently coupled to antigen-specific antibodies (Hendrickson *et al.*, 1995; Joerger *et al.*, 1995; Wu *et al.*, 2001). Although there have only been a few published reports of the use of DNA-antibody chimeras, they may offer advantages over the (strept)avidin/biotin system. Preparation of the conjugates prior to the assay avoids the *in vitro* assembly of components, which has been shown to lack homogeneity and may in turn affect accuracy and reproducibility. In addition, the requirement for fewer incubation and wash steps would simplify and speed up the assay procedure, which has obvious advantages for antibody detection.

To date, investigators have used standard PCR followed by gel electrophoresis and ethidium bromide staining (Sano *et al.*, 1992; Numata and Matsumoto, 1997; Saito *et al.*, 1999), Southern blotting (Maia *et al.*, 1995) or PCR-ELISA (Niemeyer *et al.*, 1997) to detect DNA labels in I-PCR assays. However, these methods are time consuming and pose a risk of contamination due to the opening and closing of tubes. Moreover, accurate quantification of template DNA is not possible; PCR product in the plateau phase of the reaction (i.e. measured by densitometry) does not necessarily correlate with the initial template concentration.

3.1.2 Real-time PCR

Real-time PCR is a powerful technique combining PCR amplification and analysis in a single reaction tube. Several specialised instruments have been developed for performing real-time PCR assays, including the The LightCycler™ and the ABI PRISM® 7700 sequence detection system (ABI PRISM® 7700) manufactured by Roche Diagnostics (Mannheim, Germany) and Perkin-Elmer (Foster City, CA) respectively. These instruments have important differences and have been designed to run different real-time PCR assays, including the SYBR Green I DNA binding assay (Morrison *et al.*, 1998), the ResonSense® assay (Lee *et al.*, 2002), the hybridisation assay (Wittwer *et al.*, 1997a) and the hydrolysis (or 5'-exonuclease) assay (Heid *et al.*, 1996). All four formats can be run on the LightCycler™, whereas the ABI PRISM® 7700 is currently configured to run the SYBR Green I and hydrolysis assays.

Both instruments consist of a combined fluorimeter and thermal cycler. PCR products are detected as they accumulate during cycling by fluorescent probes. Fluorescence increases within the exponential and log-linear phases of PCR correlate directly with increases in product. Absolute quantification is possible if external standards are run in parallel with test samples. The standards are used to create a standard curve of crossing threshold versus cycles, which can be used to determine the initial template concentration. Data analysis, including standard curve generation and copy number calculation, are performed automatically.

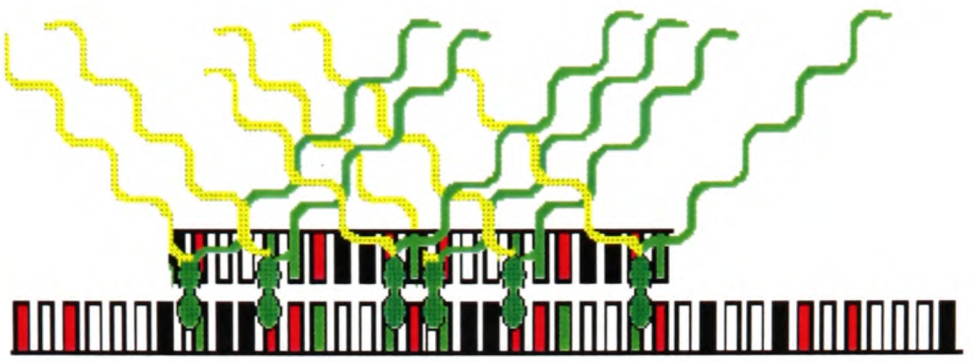
One of the unique features of the LightCycler™ is the very short PCR cycle times. Reactions are carried out in very small-volume glass capillaries whose surface area to volume ratio is large (Wittwer *et al.*, 1997b). This factor, combined with an air-based thermal system, enables rapid heating and cooling of the capillaries so amplification reactions can be completed in as little as 20 to 30 minutes. Light from a blue light-emitting diode is used to excite the samples and fluorescence is usually read once per cycle by photodetection diodes that have different wavelength filters so allow the use of spectrally distinct fluorescent probes [channels F1 (530 nm), F2 (640 nm) and F3 (710 nm) are optimised to detect emissions from SYBR Green I or fluorescein, Cy5 and Cy5.5 respectively]. Different PCR products can be discriminated through the use of melting-point analysis. Melting-point analysis is performed after the completion of the amplification reaction by the continuous measurement of fluorescence as the temperature of the reaction is gradually increased. When the dissociation temperature (melting-point) of the ds DNA reaction product is reached, SYBR green I and/or fluorescent probe is

released and the fluorescence decreases. Since the melting-point is dependent on guanine-cytosine (GC) content, length and sequence of the PCR product, non-specific products such as primer artifacts can be easily differentiated from specific product. This feature of the LightCycler™ makes it ideal for SYBR Green I and hydridisation probe-based assays, however it can also detect the fluorescence generated in the hydrolysis assay.

The ABI PRISM® 7700 (often referred to as the TaqMan) is also a combined fluorimeter and thermal cycler but reactions are performed in thin-walled 96-well microtitire plate wells or tubes, which are irradiated with a laser light via a multiplexed array of optical fibres. Fluorescence emissions return via the fibres and are directed to a spectograph with a charged coupled device array. The machine is currently configured to run the SYBR Green I assay and the hydrolysis assay.

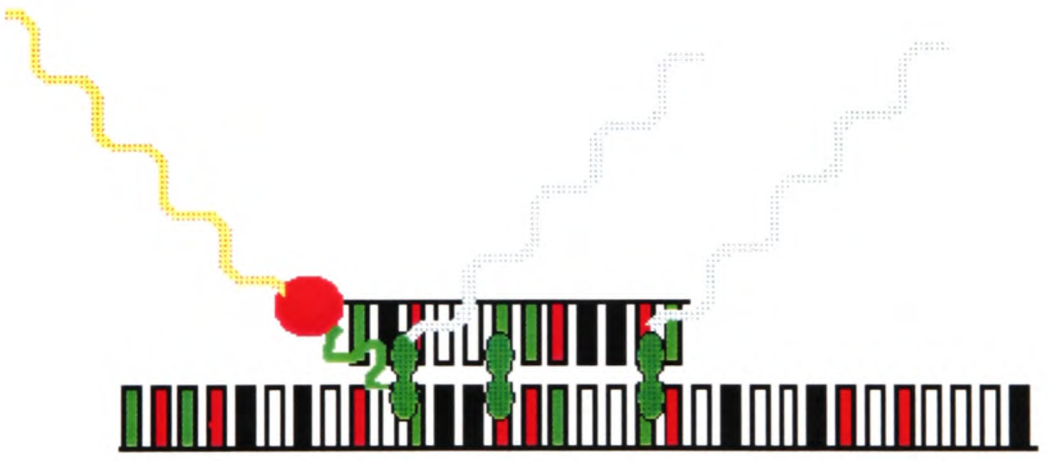
The simplest format for the detection of real-time PCR product uses SYBR Green I, which fluoresces when it binds to any ds DNA (Figure 3.1a). ResonSence® probes (Figure 3.1b) are sequence specific oligonucelotides that are labelled with a fluorescent molecule (fluor), usually Cy5 (Lee *et al.*, 2002). When the probe binds to its complementary sequence on the PCR template, SYBR Green I present in the reaction mix binds to the ds DNA portion created. When irradiated, the excited dye transfers energy to the nearby Cy5 which subsequently fluoresces. This process is called fluorescent resonance energy transfer (FRET). Hybridisation probes differ from ResonSense® probes in that two oligonucleotides are used, one of which is labelled with a donor fluor (e.g. fluorescein) and the other with an acceptor fluor (e.g. Cy5). The two oligonucelotides are designed to bind to the template in a head to tail arrangement, so the fluors are brought into close proximity and FRET can occur (Figure 3.1c). Hydrolysis (or TaqMan) probes are oligonucelotides that contain a reporter (e.g. FAM), typically on the 5' base, and a quencher (e.g. TAMRA), typically on the 3' base. When irradiated, the excited reporter transfers its energy to the nearby quencher rather than fluorescing, resulting in a nonfluorescent substrate. The probe is designed to bind to an internal region of the PCR product. Once PCR amplification begins, T*aq* DNA polymerase (using its 5' - 3' exonuclease activity) cleaves the probe, and the reporter is released. Once the reporter dye is separated from the quencher dye during every amplification cycle, it generates a sequence-specific fluorescent signal (Figure 3.1d).

Figure 3.1a Binding of SYBR Green I to DNA



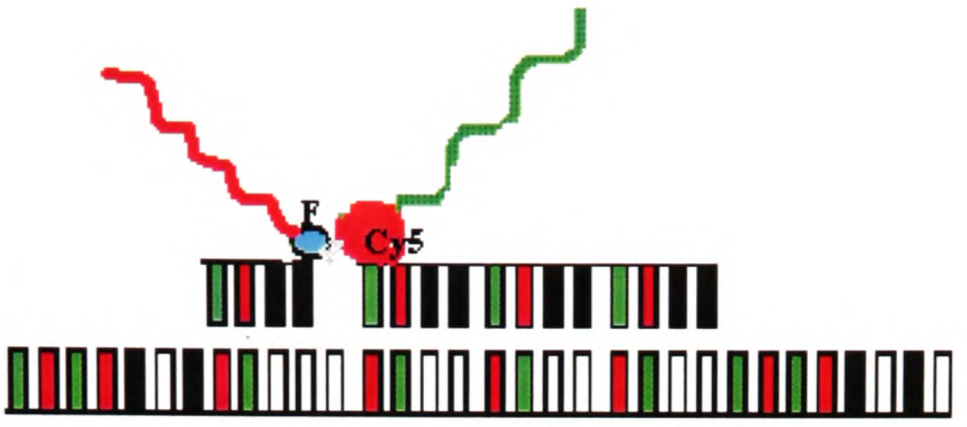
SYBR Green I (green symbol) binds to ds DNA and fluorescence increases due to a conformation change. The green wavy lines represent input blue light and the yellow lines represent measured green light.

Figure 3.1b ResonSense® probe system



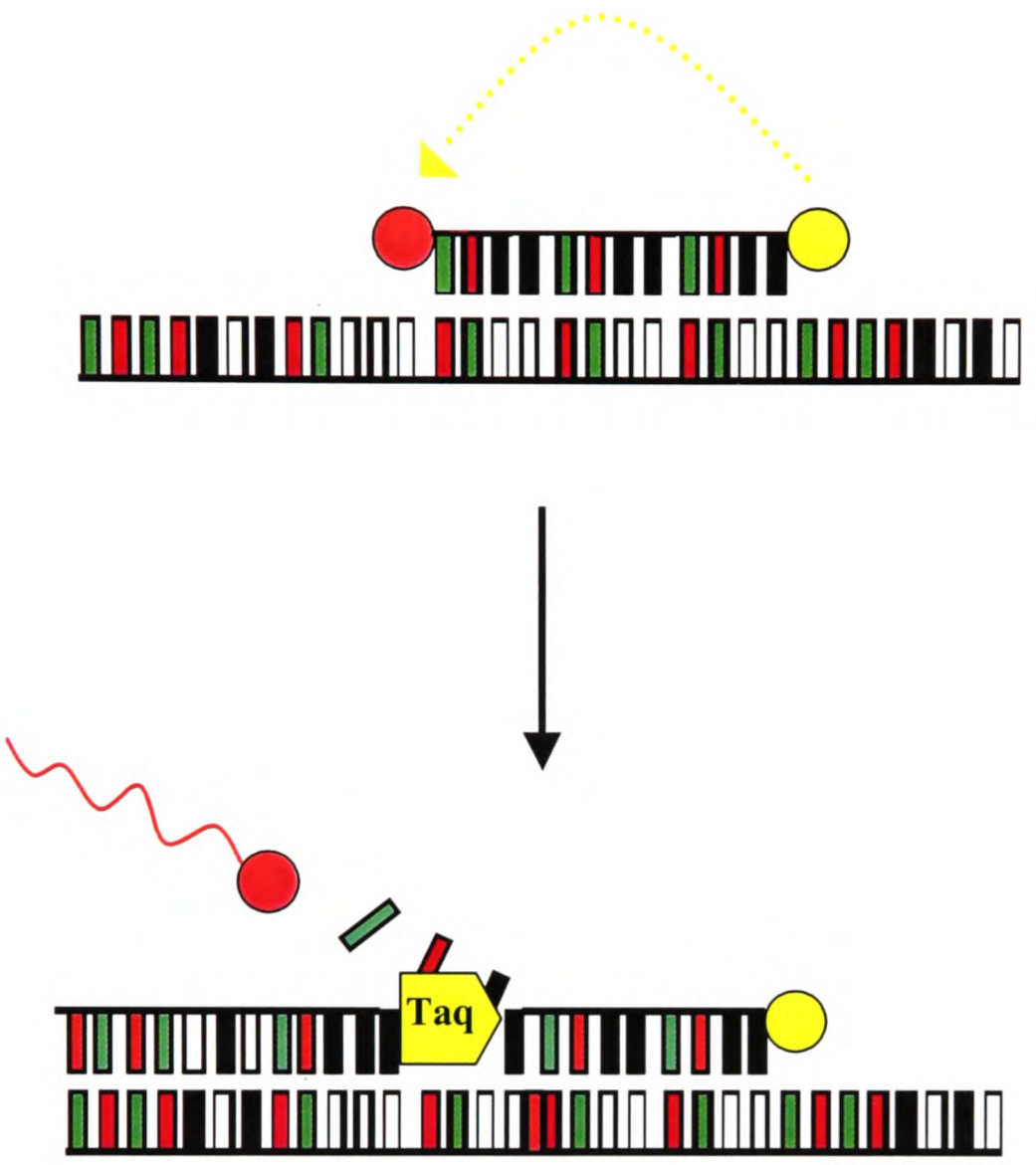
A signal is produced by FRET between Cy5 (red circle) and SYBR Green I (green symbol). Blue lines represent light directed into the system. Orange line represents light emited by the Cy5 molecule.

Figure 3.1c Hybridisation probes



FRET takes place between Fluorescein (F) and Cy5. The red and green wavy lines represent excitation and emitted photons respectively.

Figure 3.1d Hydrolysis probe system



When the probe binds to its complementary sequence, the fluorescence produced by FAM (red circle) is quenched by TAMRA (yellow circle). During amplification the probe is degraded by the T*aq* DNA polymerase, thus liberating FAM which fluoresces.

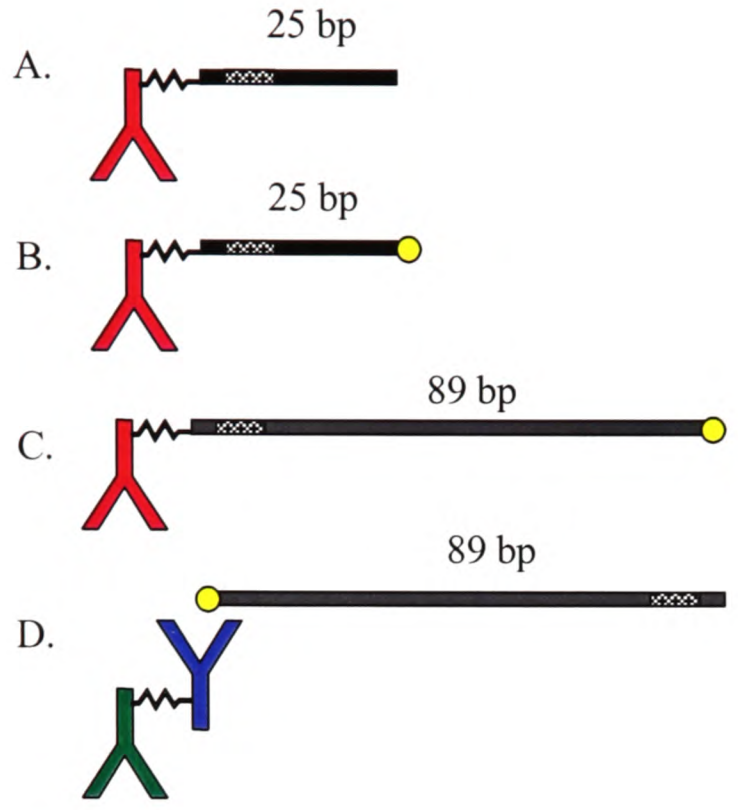
To date, I-PCR has not been applied to the detection of antibodies to human pathogens. The aim of this study was to develop a quantitative I-PCR assay, using DNA-antibody conjugates and real-time PCR, and assess its use to detect antibodies to mumps.

During the course of the study, four I-PCR conjugates, designated A to D, were synthesised and these are shown in Figure 3.2. Conjugates A to C were designed for use in an indirect assay (Figure 3.3a), where the basic immunoreaction was kept constant i.e. microtitre plate wells were coated with rNP and then incubated with human serum (positive or negative for mumps-specific IgG antibodies). Bound antibodies were detected using anti-human IgG conjugates that were variously labelled with oligonucleotides. Conjugates A and B were labelled with a short capture oligonucelotide (25 bp) that was able to hybridise with a longer synthesised target oligonucleotide (tDNA of 89 bp). After washing, hybridised tDNA was released into solution by restriction enzyme digestion and subsequently quantified by real-time PCR using the LightCycler™ (or ABI PRISM® 7700). The amount of tDNA released from the conjugate was proportional to the level of mumps-specific IgG antibody in the test sample.

Conjugate C consisted of anti-human IgG directly linked to the 5' end of tDNA. tDNA was released for real-time PCR by incubation with capture oligonucleotide, followed by restriction enzyme digestion. Conjugates B and C were labelled with FITC so that the conjugates could be used not only for I-PCR but also in an indirect ELISA, where the FITC was detected with anti-FITC-HRP (Figure 3.3b).

In contrast to conjugates A to C, conjugate D was designed for use in an antibody capture or competitive assay. It consisted of two covalently crosslinked MAbs so had the dual function of binding to rNP and FITC-labelled tDNA (Figure 3.3c). This allowed the immunoreaction to be carried out prior to adding the tDNA, used for PCR amplification.

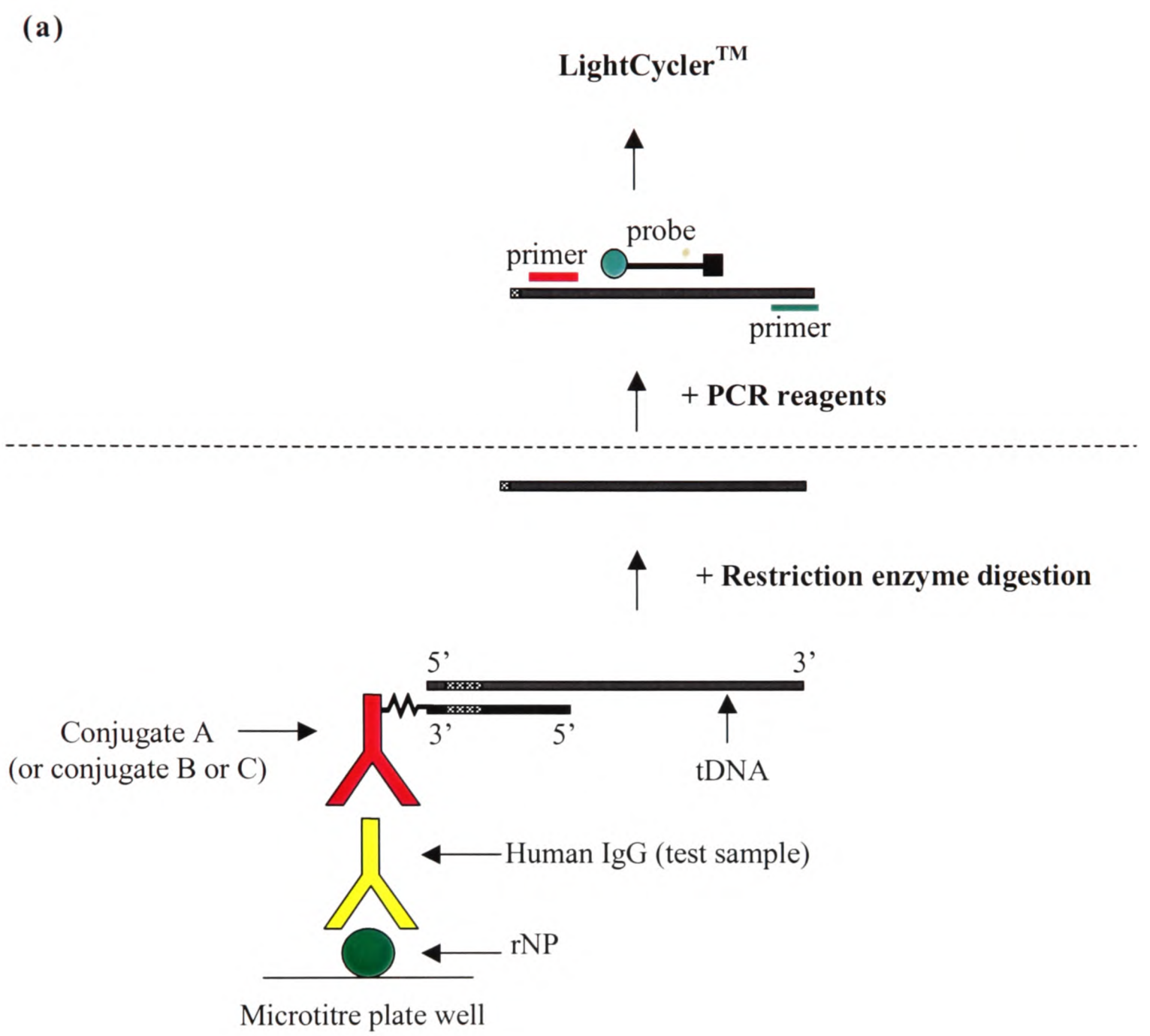
Figure 3.2 Design of DNA-antibody conjugates for I-PCR



Key:

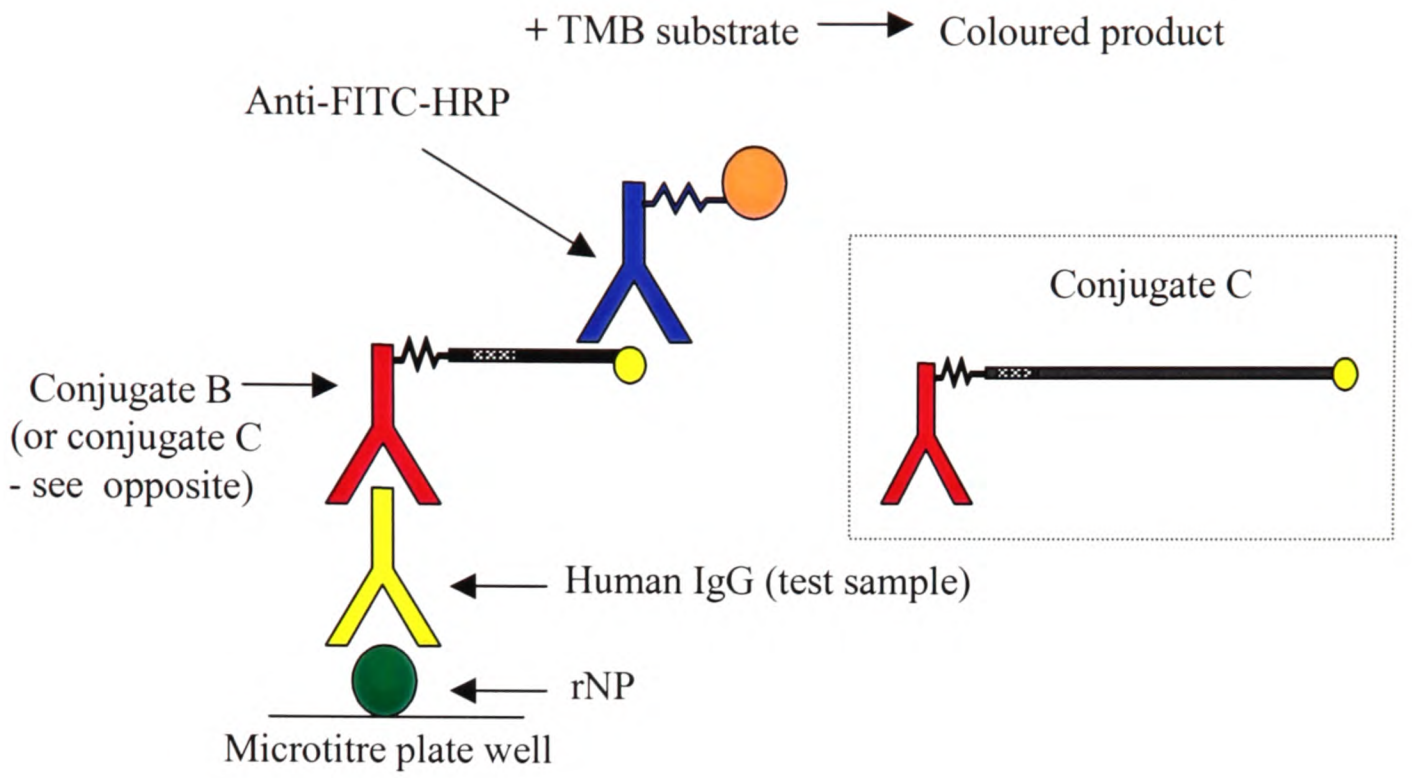
Four conjugates were synthesised and evaluated for use in I-PCR assays. Conjugates A to C consisted of anti-human IgG covalently coupled with ss DNA (either capture oligonucelotide or target DNA) and were designed for use in an indirect assay to detect human IgG. In contrast, conjugate D was designed for use in an antibody capture or competitive assay and was composed of anti-rNP-MAb covalently coupled with anti-FITC-MAb, where the latter binds to FITC-labelled tDNA.

Figure 3.3 Schematic diagrams of assays described in this study.



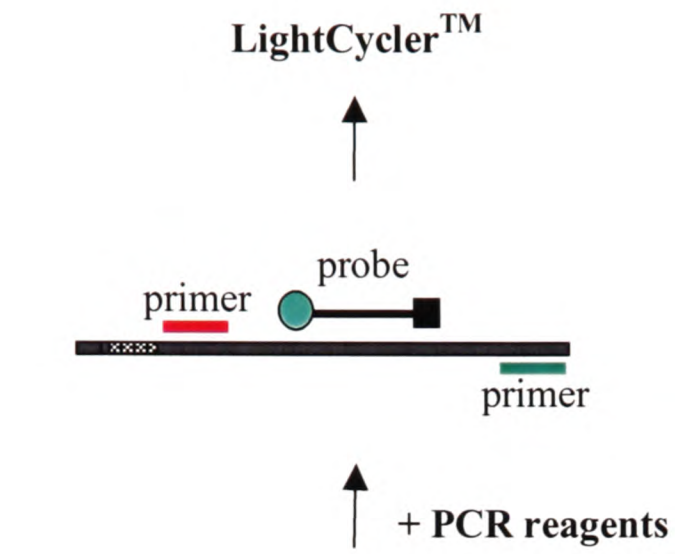
Indirect I-PCR: serum samples were incubated in wells coated with rNP. After washing, wells were incubated with a mixture of conjugate (A, B or C) and either tDNA (if using conjugate A or B) or capture oligonucleotide (if using conjugate C); the capture oligonucleotide was complementary to the 5'of tDNA so that they could hybridise. After washing, hybridised tDNA was released into solution by restriction enzyme digestion and an aliquot was subsequently quantified by real-time PCR using the LightCycler™. The amount of tDNA released from the conjugate was proportional to the level of specific-mumps IgG antibody in the test sample. The steps below the dotted line were performed in the microtitre plate well.

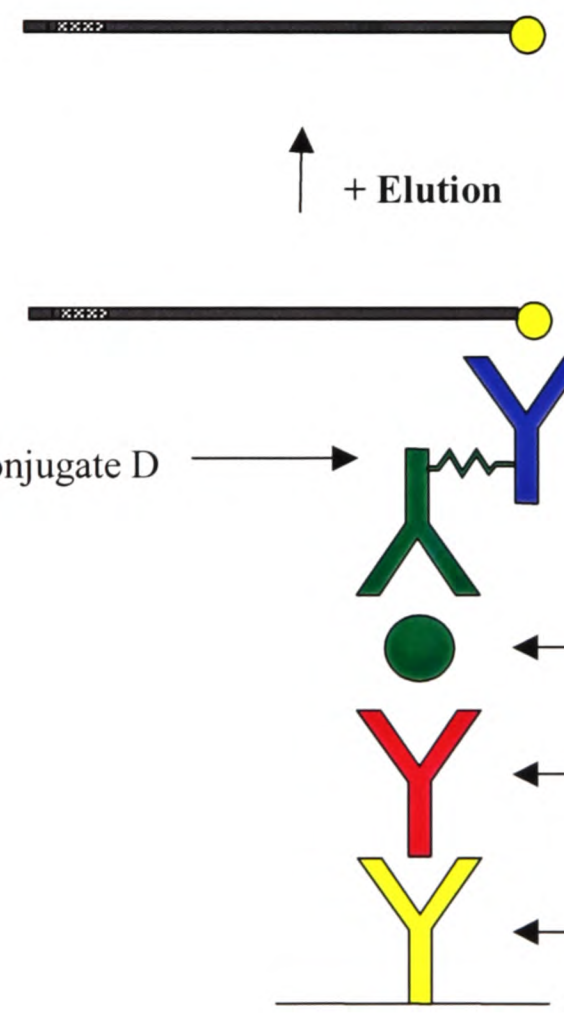
(b)



Indirect ELISA: as (a) except the conjugate (B or C) was reacted with anti-FITC-HRP, followed by standard ELISA detection.

(c)





Antibody capture I-PCR: serum samples were incubated in wells coated with anti-human IgG. After washing, wells were sequentially incubated with test sample, rNP, conjugate D and tDNA. After washing, tDNA was released into solution and subsequently quantified by real-time PCR using the LightCycler™. The steps below the dotted line were performed in the microtitre plate well.

3.2 Material and Methods

3.2.1 Design of tDNA, primers and probes

Oligonucleotides were synthesised by MWG-Biotech, UK (Milton Keynes, England) and are shown in Table 3.1. For the Lightcycler™ ResonSense® probe assay, tDNA (ss amplification target oligonucleotide) was designed with six defined sequence regions: complementary sequence to capture oligonucleotide; *Hin*d III and *Bam*H I restriction sites; 5' primer sequence; complement sequence to the 3' primer; and a ResonSense® probe sequence. Two primers were used to amplify a 67 bp region of the tDNA. They had a G+C content of 45 - 50%, similar melting temperatures and no complementary sequences. Two additional oligonucleotides were used. The ResonSense® probe was designed to hybridise to a sequence between the primer sites and was used to detect specific PCR product during amplification. It was labelled with Cy5 at the 5' terminal and blocked with biotin at the 3' terminal to prevent it acting as a primer during PCR. The capture oligonucleotide was complementary to the 5' end of the tDNA and contained *Hin*d III and *Bam*H I restriction sites to enable the release (by either enzyme) of hybridised tDNA into solution prior to DNA quantification.

The TaqMan assay was designed using the Primer Express software package (Perkin-Elmer). Two primers were designed to amplify a 78 bp region of the tDNA. A hydrolysis probe of 30 bp was selected: to have a T_m 8°C greater than the T_m of the primers so as to ensure the probe was fully hybridised during primer extension; to bind very close to the reverse primer as probes with more C's produce higher fluorescent signals; and so as not to have a G at the 5' end, as this is thought to cause quenching of the fluorescent signal generated after cleavage. The probe was labelled with the reporter dye FAM at the 5' terminal and the quencher dye TAMRA at the 3' terminal.

Table 3.1 Oligonucleotides

Region[a]	Length (bp)	GC%[c]	T_m (°C)[d]
(a) The ResonSense® probe assay[a] **Target DNA** 5'-TGCTC**AAGCTT**A*GGATCC*ATAGCATGACTACGTGACTCGGTTGGAAGCTAT ACGTTCGATGGCTGCTGCATCAGTACGCAATCGGTATG	89	49.4	> 75
Capture oligonucleotide[b] 5'-CTAT*GGATCC*T**AAGCTT**GAGCATTT –biotin/amine	25	40	59.7
Primer forward 5'-CATGACTACGTGACTCGGTT	20	50	57.3
Primer reverse 5'-CATACCGATTGCGTACTGAT	20	45	55.3
ResonSense® probe 5'-Cy5-ATACGTTCGATGGCTGCTGC- biotin	20	55	59.4
(b) The TaqMan assay **Target DNA** As above	78	49.4	>75
Primer Forward 5'-GCTCAAGCTTAGGATCCATAGCAT	24	45.8	61.0
Primer Reverse 5'-GCGTACTGATGCAGCAGCCA	20	60	61.4
Hydolysis Probe 5'-6FAM -CGAACGTATAGCTTCCAACCGAGTCACGTAG - TAMRA	31	51.6	69.5

[a] *Hin*d III and *Bam*H I restriction sites are in bold and italics respectively.
[b] Two forms of the capture oligonucleotide were synthesised, with either a biotin or amino link at the 3' end.
[c] GC% denotes percentage of G and C bases in oligonucleotide.
[d] T_m denotes the melting temperature.

3.2.2 Amplification of tDNA with the ResonSense® probe assay on the LightCycler™

Five external DNA standards, containing 10^4 copies to 10^8 copies of tDNA, were run in parallel with test samples to allow accurate quantification. The standards were prepared by making serial tenfold dilutions of tDNA of known concentration in nuclease-free water (Promega, Southampton, UK) containing 5 μg/ml of herring sperm DNA (Sigma, Poole, Dorset). In addition at least one negative control containing no tDNA was included in each batch of PCR tests. PCR reactions were carried out using the LightCycler™ (Bio/Gene, Kimbolton, England) in a final volume of 10 μl containing 50 mM Tris-HCl, pH 8.3; 0.5 μg/μl BSA; 3 mM $MgCl_2$; 200 μM deoxynucleoside triphosphates (dNTPs; Life Technologies); 0.4 units Platinum® *Taq* DNA polymerase (Life Technologies); 1:10,000 SYBR Green I (Bio/Gene, Kimbolton, England); 0.5 μM each primer; 0.25 μM of ResonSense® probe internal probe; and 1μl of tDNA or H_2O. Reaction mixtures were loaded into glass capillaries and centrifuged for 5 s at 1000 g. The capillaries were sealed with a plastic plug and placed in the LightCycler™ for fluorescence monitoring. The thermal cycling conditions were an initial denaturation at 93°C for 15 s, followed by 50 amplification cycles of four steps: 93°C for 0 s; 55°C for 0 s; 58°C for 1 s; and 74°C for 2 s. Fluorescence was measured once per cycle after the 58°C step. Maximum ramp rates (20°C/s) were used except for the transition from 55 - 58°C when the ramp rate was 3°C/s. Immediately after amplification a two-step melting programme followed: 95°C for 0s; and 45°C to 95°C at a ramp rate of 0.2°C/s with continuous fluorescent monitoring. The whole cycling process took under 30 min. Data analysis was performed using the LightCycler™ software. A standard curve was constructed using the cycle number obtained from the external standards. The cycle number values of the test samples were plotted on the standard curve and the copy number was determined. Runs were acceptable if the negative control gave the expected result, the standards were within 0.5 log of their target value and the standard curve gave a mean squared error of 10^{-2}. PCR product identity was confirmed using melting curve analysis.

3.2.3 Amplification of tDNA with the TaqMan assay on the ABI PRISM® 7700

The external standards were prepared as described above. PCR reactions were carried out using the ABI PRISM® 7700 sequence detection system in a final volume of 50 μl containing 0.12 units of AmpliTaq® Gold (Applied Biosystems, Foster City, California, USA), 3.5 mM $MgCl_2$, 250 μM of each of dATP, dCTP and dGTP, 400 mM of dUTP, 0.25

units of uracyl-*N*-glycosylase, 300 μM of each of the two primers and 200 μM of FAM-labelled probe. Reaction mixtures were added to wells of a 96 well plate, which was loaded onto the ABI PRISM® 7700 and DNA amplification was carried out by heating at 50°C for 5 min, 95°C for 10 min followed by 40 cycles of a two stage temperature profile at 95°C for 15 s and 60°C for 1 min. Data points collected following primer extension were analsyed at the end of thermal cycling. A threshold value was determined as 10 standard deviations above the mean of the background fluorescence emission for all wells between cycles 1 and 15. The cycle number at which the fluorescence signal from an unknown sample crossed the threshold was recorded. A standard curve was plotted using the threshold cycles determined for the external standards, which was used to determine the number of copies tDNA in the test sample.

3.2.4 Capture and release of tDNA

tDNA (3×10^{12} tDNA copies/μl) was incubated for 10 min at 50°C, in the presence and absence of biotinylated capture oligonucleotide. Each mixture was diluted 1:100 (final concentration of tDNA approximately 1.5×10^{10} copies/μl) with PBS and 40 μl were added to streptavidin-coated (Thermo Labsystems, Helsinki, Finland) or negative control wells. After removal of unbound material by washing with PBST, wells were incubated with 40 μl of buffer containing 1U *Hin*d III (New England Biolabs (UK) Inc., Hitchin, U.K.), or mock digested, for 1 h at 37°C. Eluted material was collected and 1μl was used as template for quantitative PCR using the LightCycler™.

3.2.5 Preparation of microtitre plate wells for immunoassays

Microtitre plate wells (MaxiSorb®, Nunc, Life Technologies) were coated with either 2μg/ml of affinity purified human IgG (Chemicon International Inc.), 2 μg/ml of BSA, 10 μg/ml of absorbed anti-human IgG in 0.05 M sodium carbonate buffer, pH 9.6 (Appendix A), or 1 μg/ml of rNP (Microimmune Ltd.) in PBS azide overnight at 4°C. The wells were aspirated and non-specific protein binding sites were blocked with 250 μl of 5% SoluPro (Dynagel Inc.) in distilled water for 2 h at 37°C. The wells were aspirated, left to dry ON at 37°C and stored in a sealed container until use.

3.2.6 Protein A affinity chromatography

Protein A affinity chromatography is a simple and reliable method for purifying total IgG from crude protein mixtures, such as serum and ascitic fluid. Protein A binds to the Fc portion of IgG.

(i) Purification of IgG from human serum

Two millilitres of protein A Sepharose^{TM} 4 Fast Flow (Amersham Pharmacia Biotech, High Wycombe, UK) was added to an empty chromatography column containing PBS azide. The column was packed by allowing it to flow by gravity and then equilibrated by washing with PBS azide. Two millilitres of human serum was filtered through a 0.2 μm membrane (Millipore) and slowly applied to the column. After incubating for 1 h at RT, the column was washed with PBS azide until the OD (280 nm) of the flow-through was below 0.05. Bound proteins were eluted with 0.1 M glycine-HCl, pH 2.5 (Appendix A). One millilitre fractions were collected and monitored for protein by measuring the OD at 280 nm. Peak fractions were pooled and dialysed against PBS azide or 0.1 M sodium bicarbonate buffer overnight at 4°C. Regeneration of the column was accomplished by washing with 0.1 M glycine-HCl, pH 2.5 and re-equilibrating with PBS azide.

(ii) Purification of anti-FITC MAb from ascitic fluid

Anti-FITC MAb was purified as for human IgG except a freshly packed protein A Sepharose^{TM} Fast Flow column was equilibrated with ImmunoPure IgG Binding Buffer (Pierce, Rockford, Illinois, USA) and a 1:1 suspension of ascitic fluid and ImmunoPure IgG Binding Buffer (Pierce) was applied to the column. In addition, the column was eluted with ImmunoPure IgG Elution buffer (Pierce) instead of glycine buffer.

3.2.7 Preparation of affinity columns

Columns were prepared by coupling purified antibody (human IgG, mouse Ig, anti-FITC MAb) or coumarin active ester to CNBr-S4B. All columns were stored at 4°C.

(i) Human IgG column

0.6 g of CNBr-S4B (Amersham Pharmacia Biotech) was washed for 15 min with 1mM HCl as recommended by the manufacturer. The swollen mix was poured into an empty PD-10 column, which was centrifuged at 2500 rpm for 2 min. The contents of the column were added to 20 mg of affinity purified human IgG in 0.1 M sodium bicarbonate buffer, pH 8.3 and allowed to mix for 2 h at 37°C on a circular rotator. Afterwards the mix was added to an empty PD-10 column. The column was centrifuged at 2500 rpm for 5 min and the OD (280 nm) of the eluate was measured to estimate the percentage of bound antibody (~96%). The column contents were added to 20 ml of 0.1 M Tris-HCl, pH 8.0 and allowed to mix for 1 h at 37°C on a circular rotator and then overnight at 4°C to block any remaining active groups. The column was packed by centrifugation at 2500 rpm for 5 min.

(ii) Anti-FITC MAb column

The anti-FITC MAb column was prepared as for the "Human IgG column" except 0.15g of CNBr-S4B was washed and added to 4.3 mg of affinity purified anti-FITC MAb.

(iii) Mouse Ig column

See section 2.2.4.

(iv) Coumarin column

Coumarin-S4B columns were a gift from Dr R. Abuknesha (Department of Biochemistry, Kings College, London).

3.2.8 Purification of anti-human IgG

Three millilitres (2 mg/ml) of rabbit anti-human IgG (Chemicon International Inc.) was purified by a two-step procedure. First, the preparation was applied to a mouse Ig column equilibrated with PBS azide to remove antibodies that cross-reacted with mouse Ig. The column was washed and 1 ml fractions were collected and monitored for protein by measuring the OD at 280 nm. Peak fractions were pooled and reapplied to the column after column regeneration to remove any remaining cross-reacting antibodies. Pooled flow-through fractions were then applied to a human IgG column. After incubating for 1 h at RT, the column was washed until the OD (280 nm) of the flow-through was below 0.05. Bound antibodies were eluted with 0.1 M glycine-HCl, pH 2.5 and 1 ml fractions were

collected and monitored for protein by measuring the OD at 280 nm. A second elution with 0.1 M glycine-HCl, pH 2.5 containing 20% acetonitrile was then performed to improve the recovery of protein. Antibody-containing fractions were pooled, dialysed against PBS azide and concentrated using an Ultrafree centrifugal filtering device and tube (Millipore).

3.2.9 Conjugations using heterobifunctional cross-linking reagents

(i) Conjugate A (Capture oligonucleotide-anti-human IgG conjugate)

Amino-modified capture oligonucleotide was chemically conjugated to rabbit anti-human IgG (Chemicon International Inc.) using the heterobifunctional cross linkers N-succinimidyl-3- (2-pyridyldithio) propionate (SPDP) and succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) by Microimmune Ltd. The conjugate was isolated by gel filtration chromatography on a PD-10 column containing Sephadex® G-25 equilibrated with PBS azide. One millilitre fractions were collected and monitored for protein and DNA by measuring the OD at 280 nm and 260 nm respectively. Peak fractions were pooled and analysed by I-PCR (see 3.2.14i).

(ii) Conjugate B (Fluorescein-labelled capture oligonucelotide-anti-human IgG conjugate)

Affinity-purified rabbit anti-human IgG (Chemicon International Inc.) was activated with SPDP and the mix was reacted with thiol-modified FITC-labelled capture oligonucleotide as follows:

Two microlitres of 10 mg/ml SPDP in N,N-Dimethylformamide (DMF) was added to 1.2 mg (8 nmol) of purified anti-human IgG with gentle stirring on a magnetic stirrer and left to react for 1 h at RT and then overnight at 4°C. The mix was applied to a Nick® column containing Sephadex® G-50 (Amersham Pharmacia Biotech) equilibrated with PBS and incubated for 1 h at RT. The column was washed (to remove excess reagent and by-products) and 250 µl fractions were collected and monitored for protein and SPDP by measuring the OD at 280 nm and 414 nm respectively. Peak fractions containing the SPDP-derivatised IgG were pooled and concentrated using an Ultrafree centrifugal filtering device and tube (Millipore). The IgG concentrate was added to 106.5 nmol (903 µg) of 5' thiol-modified FITC-labelled capture oligonucleotide with gentle stirring on a magnetic stirrer and left to react for 2 h at RT and then overnight at 4°C. The mix was applied to a Nick® column containing Sephadex® G-50 column (Amersham Pharmacia Biotech)

equilibrated with PBS azide and incubated for 1 h at RT to remove free reagents. The column was washed and 250 µl fractions were collected. An aliquot of each fraction was diluted 1/50 in diluent B (Appendix A) and analysed for the presence of conjugate by ELISA (see section 3.2.15i).

(iii) Conjugate C (Fluorescein-labelled tDNA-anti-human IgG conjugate)

Thiol-modified fluorescein-labelled tDNA was conjugated to rabbit anti-human IgG (Chemicon International Inc.) using the heterobifunctional cross linker SPDP by Microimmune Ltd.. The conjugate was isolated by gel filtration chromatography on a column containing Sephacryl S300 (Amersham Pharmacia Biotech) equilibrated with PBS azide. One millilitre fractions were collected and analysed for the presence of conjugate and tDNA (conjugated and any remaining free label) by ELISA and real-time PCR respectively. For the ELISA, an aliquot of each fraction was diluted 1/1000 in diluent B and analysed for the presence of conjugate (see section 3.2.15i). For real-time PCR, an aliquot of each fraction was diluted 1/10,000 in nuclease-free water (Promega) and 1 μl was used as template for quantitative PCR using the LightCycler^{TM}.

(iv) Conjugate D (Anti-FITC MAb-anti-rNP MAb conjugate)

Separate heterobifunctional crosslinking reagents were used to independently activate anti-rNP MAb and anti-FITC MAb, which were then mixed to facilitate spontaneous coupling of the antibodies:

Activation of anti-rNP MAb with SPDP: 20.8 μl of 10 mg/ml SPDP in dry ethanol was added to 10 mg of anti-rNP MAb in 490 μl of 0.1 M sodium bicarbonate buffer with gentle stirring on a magnetic stirrer and left to react for 45 min at RT. The mix was applied to a PD10 column containing Sephadex^{TM} G-25 equilibrated with PBS (to remove unconjugated crosslinker). The column was washed and 1 ml fractions were collected and monitored for protein by measuring the OD at 280 nm. Peak fractions were pooled and allowed to react with 7.64 μl of 100 mg/ml Tris (2-carboxyethyl)phosphine hydrchloride (TCEP-HCl) for 15 min at RT to reduce disulphide bonds. The reaction was monitored by measuring the OD at 343 nm to determine the concentration of the pyridine-2-thione released (Table 3.2). The total amount of pyridine-2-thione released was 0.46 μmoles (extinction co-efficient of thiopyridine = 8.08 x 10^3 M^{-1} cm^{-1}). The conjugate was concentrated, using an Ultrafree filter and tube, by centrifugation at 3000 rpm for 30 min to a final volume of 500 μl. The concentrate was applied to a PD10 column equilibrated with

PBS containing 1 mM EDTA to remove impurities and ions. The column was washed and 200 μl fractions were collected and protein was monitored by measuring the OD at 280 nm. The conjugate was concentrated to a final volume of 500 μl.

Table 3.2 Absorbance at 280nm and 343nm before and after reduction of SPDP-derivatised anti-human IgG

	Optical Density	
	280 nm	**343 nm**
Before reduction	2.78	0.11
Immediately after reduction	3.00	1.54
15 min after reduction	2.39	1.96

Activation of anti-FITC MAb with SMCC: 22 μl of 10 mg/ml SMCC in DMF was added to 10 mg of anti-FITC MAb in 833 μl of PBS with gentle mixing and allowed to react for 1 h at RT. The mix was applied to a PD10 column containing Sephadex^{TM} G-25 equilibrated with PBS containing 1 mM EDTA (to remove excess crosslinker and impurities). The column was washed with PBS containing 1 mM EDTA and 1 ml fractions were collected and monitored for protein by measuring the OD at 280 nm. Peak fractions were collected and pooled.

Conjugation: SMCC-activated anti-FITC MAb was added to SPDP activated, reduced anti-rNP MAb with gentle mixing and allowed to react for 1 h at RT and then overnight at 4°C. The conjugate was concentrated to a final volume of 1.5 ml, analysed by SDS-PAGE and tested for activity by ELISA (see 3.2.15ii).

3.2.10 Purification of anti-rNP MAb-anti-FITC MAb conjugate using a coumarin affinity column

A coumarin-S4B column was washed with 10 ml of 0.1 M glycine-HCl, pH 2.5 and equilibrated by washing with PBS azide. Anti-rNP MAb-anti-FITC MAb conjugate was slowly applied to the column and incubated for 30 min at RT. The column was washed with equilibration buffer until the OD (280 nm) of the flow-through was below 0.05. Bound proteins were eluted with 0.1 M glycine-HCl, pH 2.5 and 1 ml fractions were

collected and monitored for protein by measuring the OD at 280 nm. Peak fractions were pooled and analysed by I-PCR (3.2.14iii).

3.2.11 SDS-PAGE

(i) Sample Preparation

Samples were diluted to 1.1 x the desired concentration by diluting with NuPage 4 x sample buffer and deionised H_20. Immediately prior to electrophoresis, 0.5 M dithiothreitol (DTT; Sigma) was added to the sample solution (for reducing conditions) or deionised H_2O was added to bring the sample to the final volume. The samples were heated for 10 min at 70°C, vortexed and 10 μl was loaded into the appropriate wells.

(ii) Gel electrophoresis

Gel electrophoresis was performed using the XCELL II™ Mini-Cell apparatus (Invitrogen, Paisley, Scotland). NOVEX Tris-Glycine polyacrylamide gels (Invitrogen) were run according to the manufacturers instructions. Briefly, gels were rinsed with deionised water and the tape was peeled off the bottom of the cassettes. The comb was removed and the wells were thoroughly rinsed with NOVEX Tris-Glycine running buffer (Invitrogen). The gels were placed into the Mini-Cell so that the notched well side of the cassette faced inwards towards the buffer core. The gels were locked into place with the gel tension wedge. If one gel was run, the plastic buffer dam replaced the second gel cassettes. The upper buffer chamber (inner) was filled with running buffer so that the buffer level was greater than the level of the wells. Ten microlitres of each sample was loaded onto the gel and then the lower (outer) buffer chamber was filled with 600 ml of 1 x running buffer. The gels were run for approximately 50 min at 200 V. After the run was complete the power was shut off, the electrodes were disconnected and the gels were removed from the Mini-Cell. The three bonded sides of the cassette were separated using the gel knife and the gel was placed into transfer buffer (Appendex A) for western blotting or Coomassie blue for protein staining.

(iii) SDS-PAGE Gel staining with Coomassie Blue

Gels were stained with Coomassie blue (Appendex A) for 1 – 2 h and then destained (Appendex A) overnight with gentle agitation. Gels were scanned using an AGFA SNAPSCAN 600 scanner.

3.2.12 Western Blotting

Western transfer was performed according to the manufacturers instructions (Invitrogen). Briefly, a sheet of Hybond™ ECL nitrocellulose membrane (Amersham Life Science, Little Chalfont, Buckinghamshire, UK) was cut to approximately the same size as the gel (8 x 8 cm) and soaked briefly in transfer buffer. Six pieces of Whatmann paper (8 x 8 cm) and the blotting pads were soaked in transfer buffer until they were saturated. Two blotting pads were added to the cathode core of the blot module (Invitrogen), followed by three pieces of Whatman paper, the gel, the Hybond™ ECL membrane, another three pieces of Whatman paper and another three blotting pads. The anode core was placed on top of the pads and the module was placed into the Mini-Cell. The blot module was filled with transfer buffer until the gel/membrane sandwich was covered. The outer buffer chamber was filled with 600 ml of deionised water. The lid was placed on top of the unit and transfer was performed for 2 h at 30 V. After the run was complete the membrane was removed and immunostaining was performed as follows: the membrane was incubated with blocking buffer (Appendix A) for 2 h to block reactive binding sites. The membrane was washed 3 times for 5 min with PBST and then incubated with 1/2000 dilution of anti-FITC-HRP (Microimmune Ltd.) for 30 min. After washing, the membrane was incubated with blocking solution (Appendix A) for 30 min at 37°C (to localise the blue product at the site of the HRP catalysed reaction). The solution was aspirated and TMB was added for 15 - 30 min until the blue/purple bands appeared. All steps were performed at RT with gentle agitation. The membrane was scanned using a AGFA SNAPSCAN 600 scanner.

3.2.13 Serum Samples

Mumps-specific IgG negative, weak positive and strong positive sera (as determined with a commercial ELISA Kit; Behring Enzygnost; Behringwerke AG, Marburg, Germany) were used to optimise the I-PCR assays, and subsequently used as controls in further experiments. A panel of 88 additional sera, which had been submitted for diagnostic testing by the same kit, was used to evaluate the indirect I-PCR assay with conjugate A.

3.2.14 Immuno-PCRs

Wells were washed four times with PBST using an automated plate washer (Labsystems Wellwash 4Mk 2) unless indicated otherwise.

(i) Binding of DNA-anti-human IgG conjugates to human IgG

Microtitre plate wells coated with 2 μg/ml of human IgG, or BSA as a control, were incubated with 40 μl of conjugate diluted as indicated in diluent B (Appendix A) for 1 h at 37°C. Blank wells contained diluent only. After washing, 40 μl of tDNA or capture oligonucleotide diluted as indicated in diluent B was added to wells and incubated for 1 h at 37°C. After washing, wells were digested with 1 U of *Hin*d III or *Bam*H I (New England Biolabs (UK) Inc.) for 1 h at 37°C. Released tDNA was collected and 1 µl was used as template for quantitative PCR using the LightCycler™. Results were quantified in terms of copies of amplified tDNA per µl eluted from each well.

(ii) Use of capture oligonucelotide-anti-human IgG conjugate to detect mumps-specific IgG in serum

Microtitre plate wells coated with 1 μg/ml of rNP were incubated with 20 μl of sera diluted 1/200 in diluent C (Appendix A). After washing 12 times with PBST, conjugate/tDNA mixture was prepared by adding 1 µl of conjugate and 50 µl of tDNA (10^{10}copies/µl) to 1 ml of diluent C. Twenty microlitres were added to the wells and incubated at 37°C for 1 h. Wells were washed 12 times with PBST and digested with 1U of *Hin*d III (New England Biolabs (UK) Inc.) for 1 h at 37°C. Eluted material was collected and quantified as described in 3.2.14i.

(iii) Binding of anti-rNP MAb -anti-FITC MAb conjugate to rNP

Conjugate/FITC-tDNA mixture was prepared by adding 10 μl of conjugate diluted as indicated and 1 μl of FITC-tDNA (10^{10} copies/μl) to 1 ml of diluent B. Forty microlitres were added to wells coated with 1 μg/ml of rNP and incubated for 1 h at 37°C. Blank wells received diluent only. After washing, wells were heated for 5 min at ~90°C to release FITC-DNA. Eluted material was collected and quantified as described in 3.2.14i.

(iv) Use of anti-rNP MAb-anti-FITC MAb conjugate to detect mumps-specific IgG in serum

Microtitre plate wells coated with 10 μg/ml of anti-human IgG (Chemicon International Inc.) were incubated with 40 μl of sera diluted 1/200 in diluent C for 30 min at 37°C. After washing 12 times with PBST, wells were incubated with 40 μl of 0.1 μg/ml rNP in diluent C for 1 h at 37°C, followed by 40 μl of conjugate/FITC-tDNA mix (see 3.2.14iii) for 1 h at 37°C. The wells were washed between steps. FITC-DNA was then released and quantified as described in section 3.2.14iii.

3.2.15 ELISAs

(i) Binding of DNA-anti-human IgG conjugates to human IgG

Conjugate was diluted as indicated in diluent B and 40 μl was incubated in wells coated with 2 μg/ml of human IgG, and BSA as a control for 1 h at 37°C. After washing, 40 μl of anti-FITC-HRP (Microimmune Ltd.) diluted 1/2000 in diluent B was added to the wells and incubated for 30 min at 37°C. After washing, wells were incubated with 40 μl of TMB for 15 min and the reaction was stopped with 40 μl of 0.5 M HCl. The amount of bound conjugate was determined by measuring the OD at 450 nm (620 nm reference).

(ii) Binding of anti-rNP MAb-anti-FITC MAb conjugate to rNP

Microtitre plate wells coated with 1 μg/ml of rNP were incubated with 100 μl of conjugate diluted as indicated in diluent B for 1 h at 37°C. Blank wells received diluent only. After washing, wells were incubated with 100 μl of 1 μg/ml BSA-FITC (Microimmune Ltd.) in diluent B for 30 min at 37°C, followed by 100 μl of anti-FITC-HRP diluted 1/2000 in diluent B for 30 min at 37°C. The wells were washed between steps and the amount of bound conjugate was determined as described in 3.2.15i.

(iii) Use of FITC-labelled tDNA-anti-human IgG conjugate to detect mumps-specific IgG in serum.

The method described in 3.2.14ii was followed except after the sera incubation, wells were washed 12 times with PBST and incubated with 40 μl of conjugate diluted as indicated in diluent C. Bound conjugate was detected as described in 3.2.15i.

3.2.16 Statistical Methods

(i) Optimisation experiments

Paired t-tests were used to determine if there was a significant difference between mean tDNA values in optimisation experiments.

(ii) Cut-off of indirect I-PCR assay with conjugate A

A positive I-PCR result was defined as any value greater than two SDs above the mean value of the negative control, which was tested in parallel with samples. The SD was determined by the analysis of a panel of 17 sera negative for mumps specific IgG by the Behring ELISA.

(iii) Reproducibility of indirect I-PCR assay with conjugate A

The reproducibility data were analysed in the BMDP program 8V, which performs general mixed model analysis of variance for equal cell sizes (Dixon, 1992). The data were analysed for each serum sample separately to obtain serum specific estimates. In the analysis, wells and PCRs were declared to be random effects.

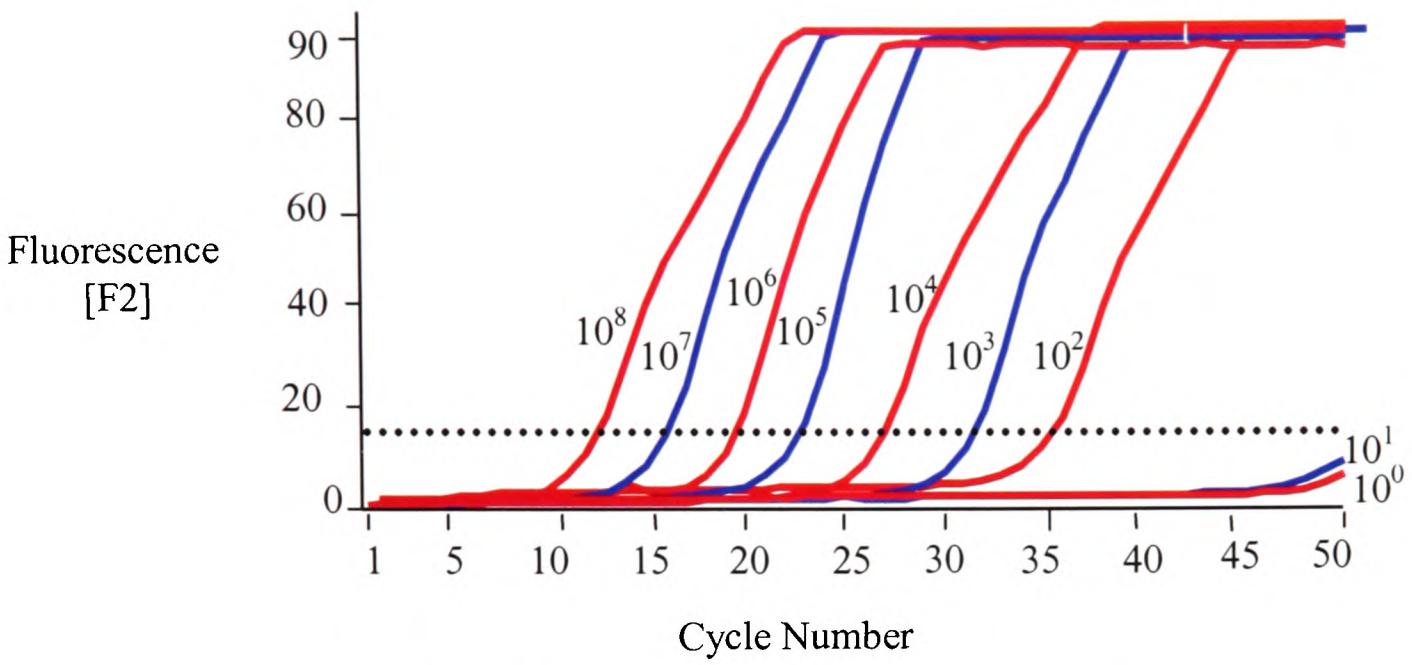
3.3 Results

3.3.1 Real-time PCR on the LightCycler™

As shown in Figure 3.3a, the I-PCR approach required the detection of hybridised tDNA. The tDNA selected was an 89 bp ss oligonucelotide and a ResonSense® probe assay was designed to amplify it on the LightCycler™. The PCR conditions for the amplification and quantification of the tDNA were investigated.

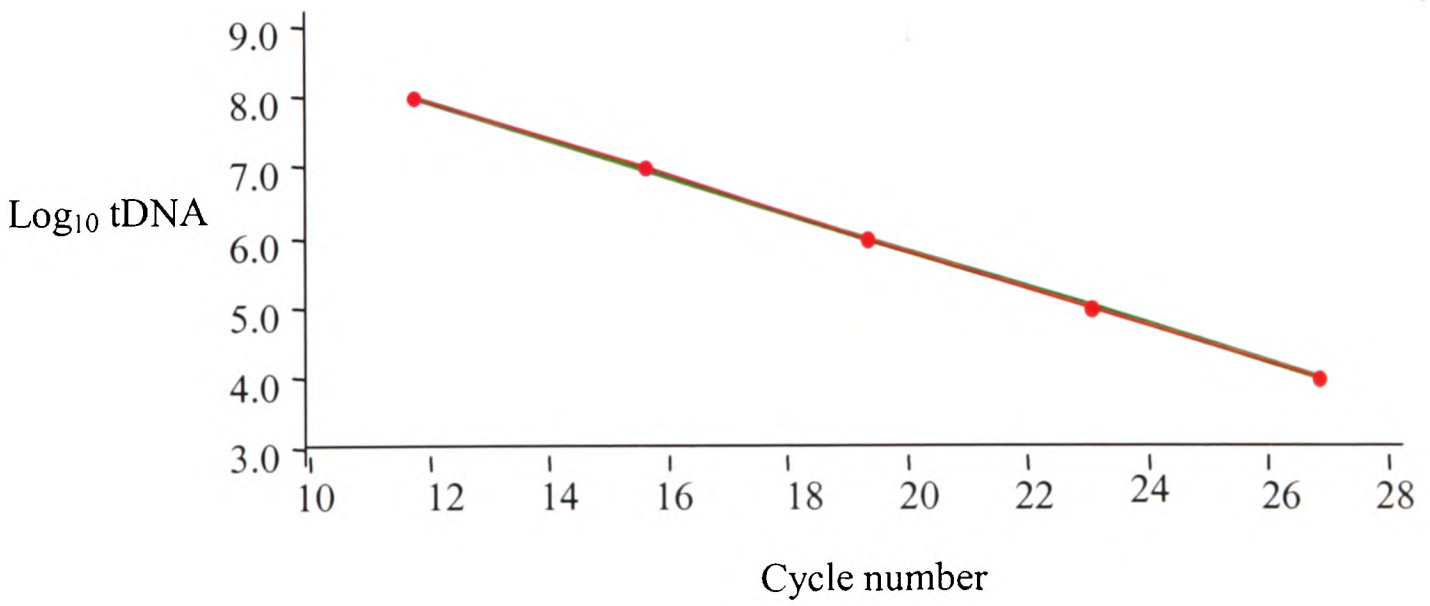
Under optimised amplification conditions, the PCR was able to detect 10^2 copies of tDNA, with a wide dynamic range of > 7 logs (Figure 3.4). For quantification, the log input copy numbers of the external standards (10^4 - 10^8 copies of tDNA) were plotted against the cycle number at the crossing point to produce a standard curve (Figure 3.5). Melting curve analysis revealed peaks at 63°C and 84°C, corresponding to the T_m of the ResonSense® probe/tDNA duplex and the full-length product respectively (Figure 3.6).

Figure 3.4 Sensitivity of LightCycler™ assay.



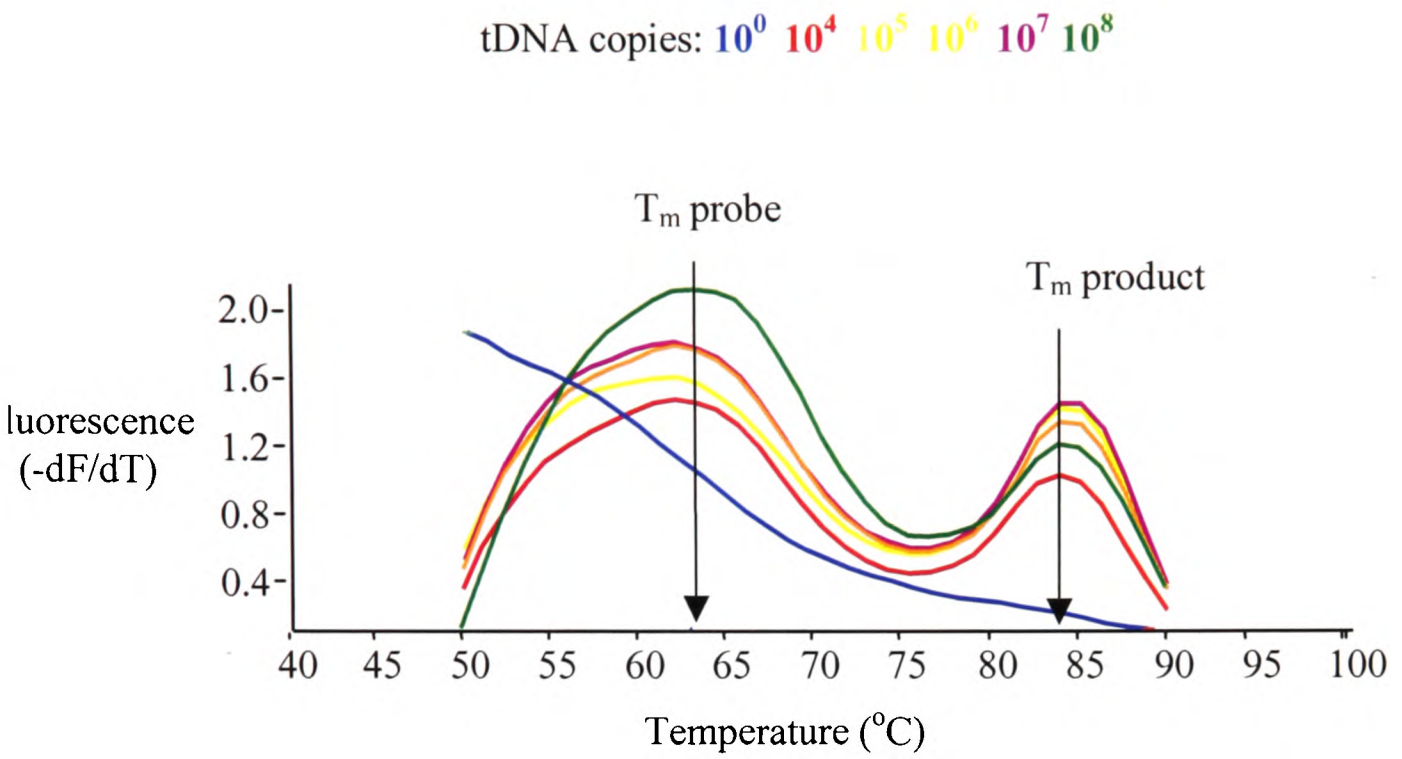
A ten-fold serial dilution of tDNA (10^8 - 10^0 copies) was amplified on the LightCycler™. The fluorecence [F2] was plotted against the cycle number at the crossing point.

Figure 3.5 Representative standard curve.



The concentration of tDNA in the external standards, 10^4 to 10^8 copies, was plotted against the crossing point cycle number (log_{10} scale).

Figure 3.6 Melting curve analysis of external standards.



Melting peaks were derived by plotting the negative derivative of fluorescence (-dF/dT) with respect to temperature.

3.3.2 Capture and release of tDNA

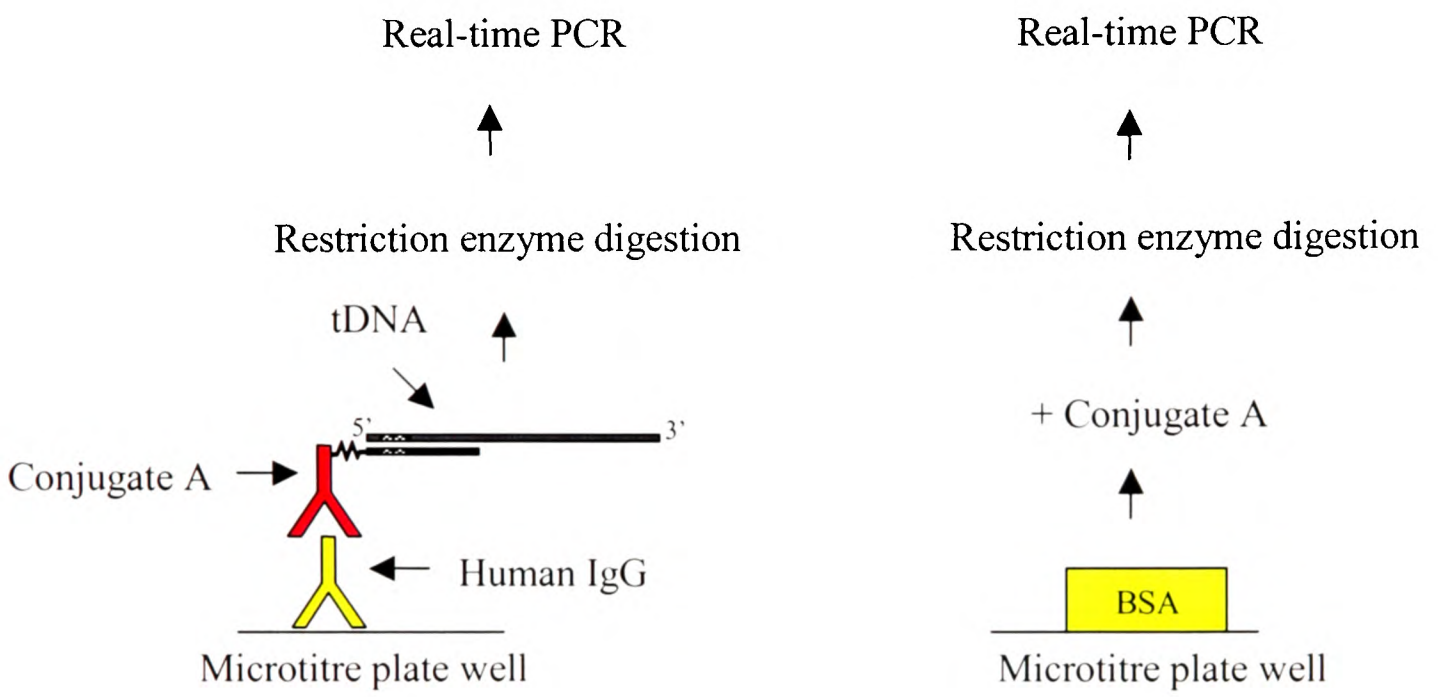
For the indirect I-PCR (Figure 3.3a), tDNA and capture oligonucleotide need to hybridise to one another. Subsequently tDNA needs to be released prior to its detection by real-time PCR. Experiments were performed to determine whether the tDNA would hybridise to the capture oligonucleotide, and to determine the optimal conditions for the release of hybridised tDNA prior to PCR. Binding of tDNA to streptavidin-coated (Strep+) and uncoated (Strep-) wells, in the presence or absence of biotinylated capture oligonucleotide, was investigated. tDNA was found to bind at very low concentrations to Strep– wells, both in the presence and absence of capture oligonucleotide (1×10^4 copies tDNA/µl out of 1.5×10^{10} copies tDNA/µl added bound to the wells). A similar level of binding was observed when tDNA was added to Strep+ wells in the absence of capture oligonucleotide. In contrast, in the presence of streptavidin and capture oligonucleotide, high levels of tDNA (1×10^9 copies tDNA/µl) were detected. This suggested that the tDNA was binding to the streptavidin-coated wells via the biotinylated capture oligonucleotide. The optimal release of hybridised tDNA from Strep+ wells was achieved by restriction digestion (1×10^9 copies tDNA/µl detected), however the tDNA could also be eluted simply by incubating wells with buffer for a similar time period (3×10^8 copies tDNA/µl detected). The reason(s) for this was unclear.

3.3.3 Conjugate A - Capture oligonucleotide-anti-human IgG conjugate

(i) Binding of conjugate A to human IgG

Conjugate A was initially evaluated with respect to its capacity to bind to immobilised human IgG and BSA as a control (Figure 3.7).

Figure 3.7 Binding of conjugate A to human IgG and BSA.



As shown in Table 3.3, when a constant amount of tDNA was incubated with ten-fold dilutions of conjugate, very low levels of tDNA were detected in the BSA-coated wells compared with the human IgG wells. The dilution of conjugate which resulted in the highest T/N value was $1/10^3$, hence this was used in subsequent experiments. At a conjugate dilution of $1/10^4$, the signal obtained was approximately ten-fold lower, but the level of non-specific binding remained the same. In wells that received tDNA but no conjugate, only background levels of tDNA were detected. There was evidence of cross-contamination between wells, as traces of tDNA were detected in wells to which none had originally been added.

A separate experiment was performed to determine the optimal amount of tDNA to use in the I-PCR assay, where the conjugate was fixed at a dilution of $1/10^3$ and the tDNA was varied. The optimal amount was found to be 10^{10} copies/well (Table 3.4).

Table 3.3 Binding of conjugate A to human IgG to determine the optimal conjugate dilution to use in I-PCR

Conjugate Dilution Factor	tDNA (copies/well)	BSA-coated wells (tDNA/μl)	Human IgG-coated wells (tDNA/μl)	T/N[a]
10^1	10^{11}	1.9×10^5	1.0×10^8	526
10^2	10^{11}	4.9×10^4	1.0×10^8	2041
10^3	10^{11}	1.3×10^4	4.6×10^7	3538
10^4	10^{11}	1.4×10^4	5.1×10^6	364
10^5	10^{11}	2.1×10^3	1.2×10^6	571
-	10^{11}	1.4×10^3	6.1×10^3	4
10^1	-	1.8×10^0	3.4×10^1	19
-	-	3.2×10^{-1}	2.9×10^1	91

[a] T/N denotes test to negative ratio (amount of tDNA eluted from single human IgG-coated wells divided by the amount eluted from single BSA-coated wells).
- denotes no conjugate and/or tDNA was added to the wells.

Table 3.4 Binding of conjugate A to human IgG to determine the optimal concentration of tDNA to use in I-PCR

Conjugate Dilution Factor	tDNA copies/well	BSA-coated wells (tDNA/μl)	Human IgG-coated wells (tDNA/μl)	T/N[a]
10^3	10^6	8.4×10^2	8.5×10^4	101
10^3	10^7	1.1×10^3	6.1×10^5	555
10^3	10^8	7.7×10^4	4.0×10^6	52
10^3	10^9	2.8×10^4	7.6×10^7	2714
10^3	10^{10}	2.8×10^5	9.7×10^8	3464
10^3	10^{11}	2.9×10^6	2.5×10^9	862
10^3	-	1.1×10^3	8.1×10^1	0.07
-	10^{11}	3.1×10^5	1.0×10^5	0.33

[a] T/N is the amount of tDNA eluted from single human IgG-coated wells divided by the amount eluted from single BSA-coated wells.
- denotes no conjugate and/or tDNA was added to the wells.

(ii) Use of conjugate A to detect mumps-specific IgG in serum

Initially representative negative, weak positive and strong positive mumps sera (which had been defined using the Behring Enzygnost ELISA) were used to evaluate the I-PCR assay to detect mumps-specific IgG. In wells that did not receive sera, background levels of tDNA were detected (6.6×10^3 copies/μl). In wells that received negative mumps sera, 5.2×10^4 copies/μl tDNA were detected, approximately 1 log greater than in the absence of sera suggesting that human IgG was binding non-specifically to the wells. In the presence of weak and strong positive sera good signals were obtained (6.8×10^5 and 6.0×10^6 copies/μl tDNA respectively).

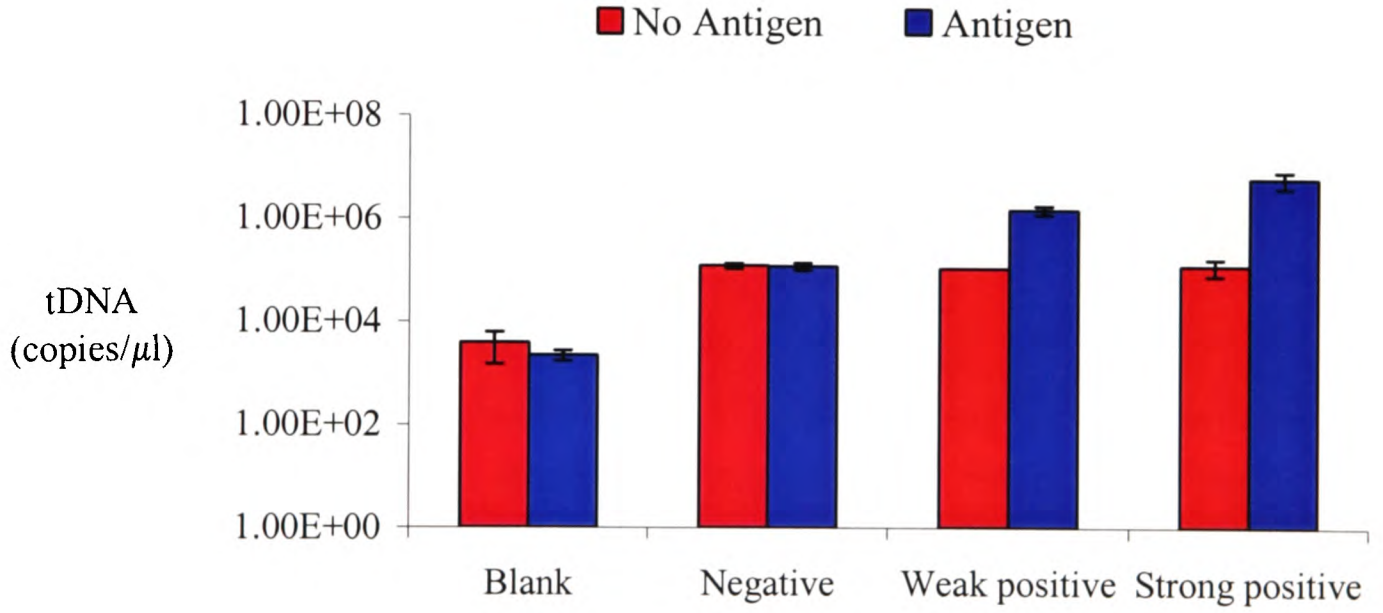
(iii) Non-specific binding

To address the problem of non-specific binding several experiments were performed and these are described below.

(a) Recombinant nucleoprotein-associated mumps antigen

The recombinant mumps antigen employed was expressed in yeast (Samuel *et al.*, 2002). It was possible that the non-specific binding observed was due to reactions of serum antibodies against residual yeast proteins that may have been left after purification of the antigen. To investigate this, I-PCR was performed in the presence and absence of antigen coated on microplate wells. As shown in Figure 3.8, the amount of tDNA detected in wells that received negative serum was independent of antigen, suggesting that serum antibodies were not reacting non-specifically with the recombinant antigen. Further, when negative sera were incubated with yeast extract prior to I-PCR, the amount of tDNA detected did not decrease compared to when incubated in the absence of yeast extract (data not shown).

Figure 3.8 I-PCR in presence and absence of immobilised antigen

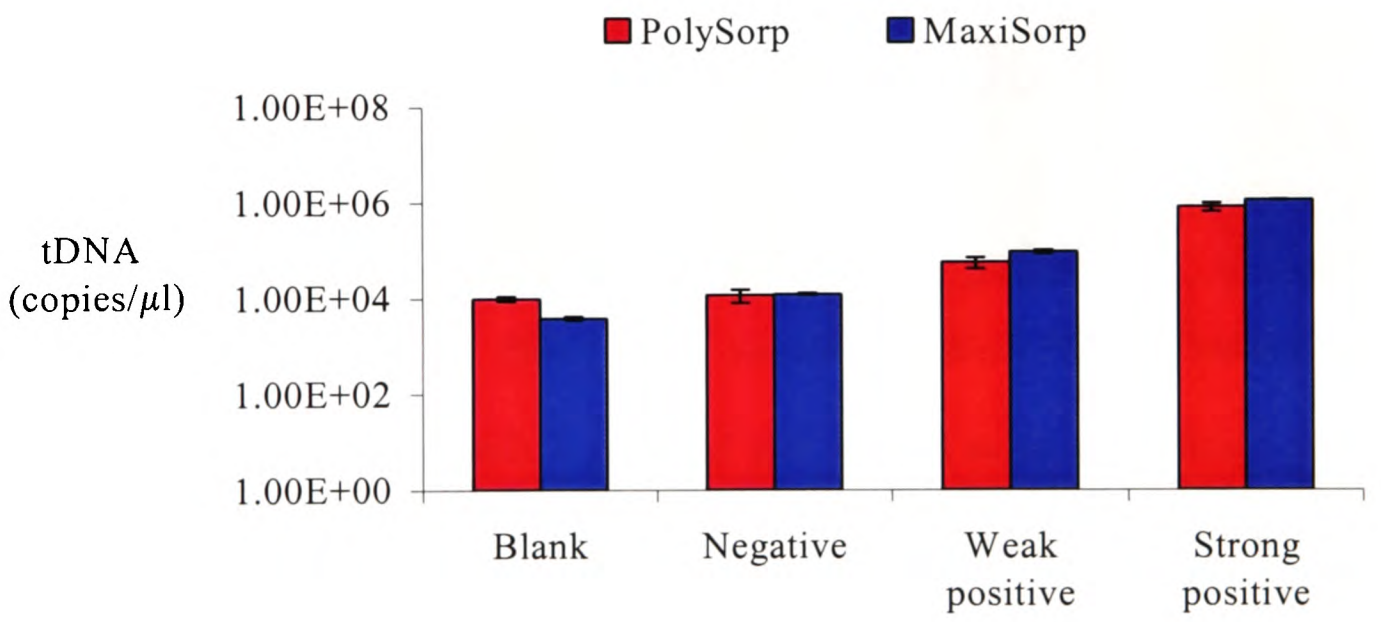


Microtitre plate wells coated with 1 μg/ml of rNP in PBS azide, or PBS azide only, were blocked with 5% Solupro and incubated with 40 μl of the following at 37°C: sera diluted 1/200 in diluent B (Appendix A); conjugate diluted 1/1000 in diluent B for 1 h; tDNA (10^{10} copies/well) in diluent B for 30 min. Wells were washed between incubations with PBST. tDNA was eluted and quantified on the LightCycler™. Samples were tested in duplicate and the mean values were plotted in a histogram. Error bars represent one SD.

(b) Comparison of MaxiSorp™ and PolySorp™ microtitre plate wells

MaxiSorp™ microtitre plate wells (as used in previous experiments) were compared with PolySorp™ microtitre plate wells to determine whether the well surface affected the assay performance. Both well types are made of polystyrene. They differ in that the MaxiSorp™ surface is more polar so has a higher binding capacity for proteins with polar or hydrophilic groups, including antibodies. I-PCR was performed on both well types and the results were compared. As shown in Figure 3.9, the amount of tDNA detected in the PolySorp™ wells was the same as the amount detected in MaxiSorp™ wells in the presence of negative serum. As a result of these findings MaxiSorp™ wells were used in further experiments.

Figure 3.9 MaxiSorp™ versus PolySorp™ wells

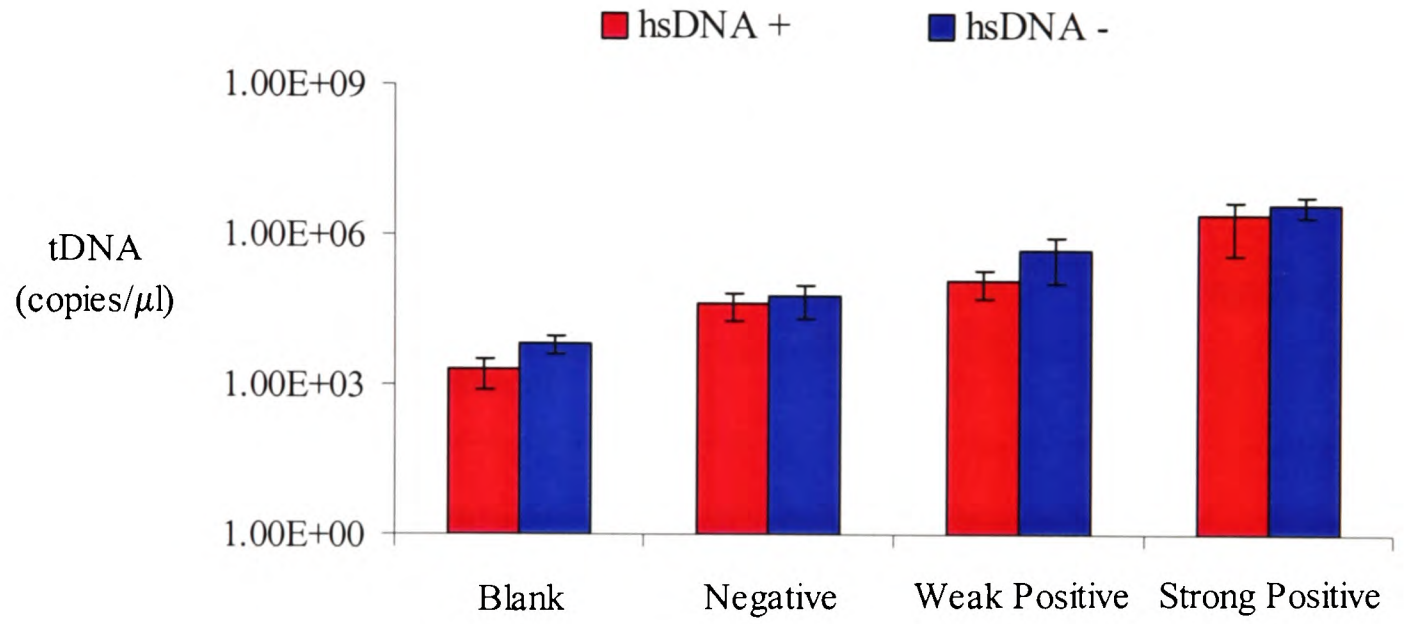


MaxiSorp™ and PolySorp™ microtitre plate wells were coated with 1 μg/ml of rNP and then blocked with 5% SoluPro. Other conditions were as those described in Figure 3.8. Samples were tested in duplicate and the mean values were plotted in a histogram. Error bars represent one SD.

(c) Herring sperm DNA

Fish sperm DNAs have previously been used to limit the non-specific binding of DNA to microtitre wells or PCR tubes (Sano *et al.*, 1992; Kakizaki *et al.*, 1996). Thus an experiment was performed to determine whether adding herring sperm DNA to the assay diluent would reduce the amount of tDNA binding non-specifically to the solid phase and thus improve the performance of the assay. As shown in Figure 3.10, slightly higher mean tDNA values were detected when hsDNA was not added to the assay diluent, however this was not significant ($p = 0.28$). In further experiments hsDNA was not added to the diluent.

Figure 3.10 Effect of herring sperm DNA on assay performance



Microtitre plate wells coated with 1 μg/ml of rNP in PBS azide and blocked with 5% Solupro were incubated with 40 μl of the following at 37°C: sera diluted 1/200 in diluent B (Appendix A) +/- 0.5% hsDNA; conjugate diluted 1/1000 in diluent B +/- 0.5% hsDNA for 1 h; tDNA (10^{10} copies/well) in diluent B +/- 0.5% hsDNA for 30 min. Wells were washed between incubations with PBST. tDNA was eluted and quantified on the LightCycler™. Shown are the mean tDNAs of four wells tested in two separate PCR runs. Error bars represent one SD.

(iv) Optimisation experiments

A series of experiments were performed to optimise the assay conditions that were aimed at improving the discrimination between negative and positive I-PCR results. These are summarised in Table 3.5. The optimal conditions were used in subsequent assays.

Table 3.5 Summary of optimisation experiments

Experiment	Parameter(s) investigated	Optimal result[c]
Blocking wells after antigen coating[a]	Well blocking agent	Milk or SoluPro
Blocking agent added to assay diluent[b]	Diluent blocking agent	Milk
Serum incubation time	30 min or 1 h	30 min
Serum incubation temperature	37°C or RT	37°C
Number of washes between incubations	4 or 12	12
Incubation of conjugate and target DNA	Together or sequentially	Together
% Tween 20 in assay diluent	0 - 5%	0.1%

[a] Wells were blocked with skimmed milk (Marvel), Solupro (Dynagel), gelatin (Boehringer-Mannheim, Germany), normal goat serum (Sigma), or bovine serum albumin at 5% for 2 h at 37°C prior to I-PCR.
[b] Blocking agents mentioned in [a] were added to assay diluent (PBST) at 2%.

(vi) Reproducibility of the I-PCR assay

The reproducibility of the optimised I-PCR assay was investigated by adding control sera to different wells on the same microtitre plate in quadruplicate. tDNA from each well was then quantified in four separate PCR assays. Table 3.6 shows the mean values (log_{10} scale) and SDs obtained. Both the inter-PCR and inter-well SDs were low indicating the assay to be highly reproducible.

Table 3.6 Reproducibility of the I-PCR assay

Control Sera	Mean (log_{10})	SD (log_{10}) Wells[a]	SD (log_{10}) PCR[b]	SD (log_{10}) All[c]
Negative	4.86	0.08	0.09	0.13
Weak Positive	6.09	0.04	0.08	0.1
Strong Positive	6.69	0.07	0.10	0.13

[a] SD (log_{10}) for the averaged measurements from different wells
[b] SD (log_{10}) for the averaged measurements of different PCRs from the same well
[c] SD (log_{10}) obtained for all the measurements.

(vii) Evaluation of the I-PCR assay

The I-PCR assay for mumps-specific IgG was evaluated in its optimised format, using 88 serum samples previously tested in the commercial ELISA (Behring). Concordant results were obtained for 84 sera. Of the remaining four, one specimen was ELISA positive and I-PCR negative, one was ELISA negative and I-PCR positive, and two ELISA equivocal were I-PCR negative. For three of these discrepancies the ELISA result was very close to the cut-off (Table 3.7). Compared to the ELISA, the I-PCR was 98.6% (71/72) sensitive and 92.9% (13/14) specific. When the ELISA absorbance readings were plotted against the I-PCR results for the 88 sera, there was good correlation (r = 0.88) between the values (Figure 3.11).

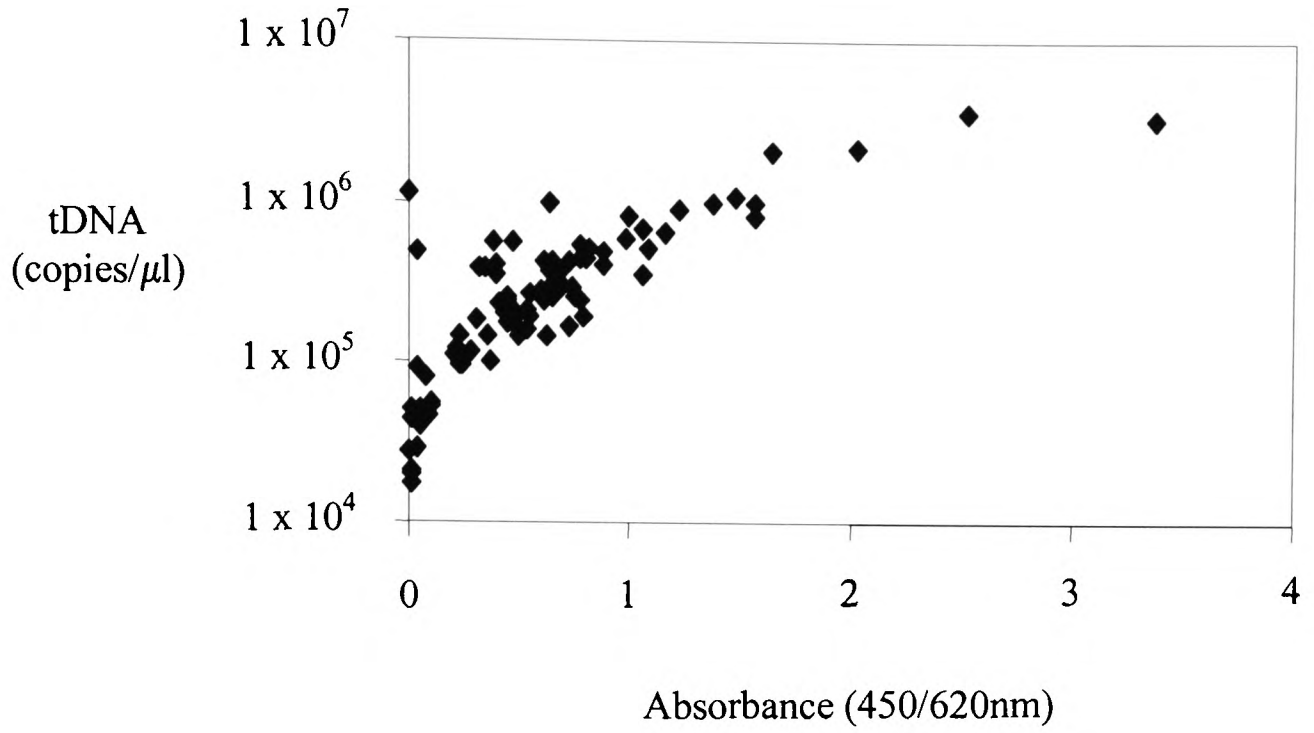
When the I-PCR assay was used to detect mumps-specific IgG in oral fluid no discrimination was observed between negative and positive samples (data not shown).

Table 3.7 Data on discrepant samples

SampleNo.	Age (Years)	Clinical Details	Mumps-specific IgM[a]	Mumps-specific IgG (OD)[b]	I-PCR
6	6	No information	Negative	Equivocal (0.106)	Negative
14	25	Hospital staff immunity check	Not Tested	Positive (0.213)	Negative
31	32	Acute parotid swelling	Negative	Negative (0.041)	Positive
73	37	? Recent mumps	Negative	Equivocal (0.102)	Negative

[a] Mumps-specific IgM Antibody Capture Radioimmunoassay (Perry *et al.* (1993)
[b] Behring Enzygnost ELISA (Negative = < 0.1; Equivocal = 0.1 - 0.2; and Positive = >0.2).

Figure 3.11 Scatter Plot comparing Behring ELISA and I-PCR test results.



Number of copies of tDNA/μl (log_{10} scale) plotted against the ELISA absorbance readings at 450/620nm for the 88 sera tested.

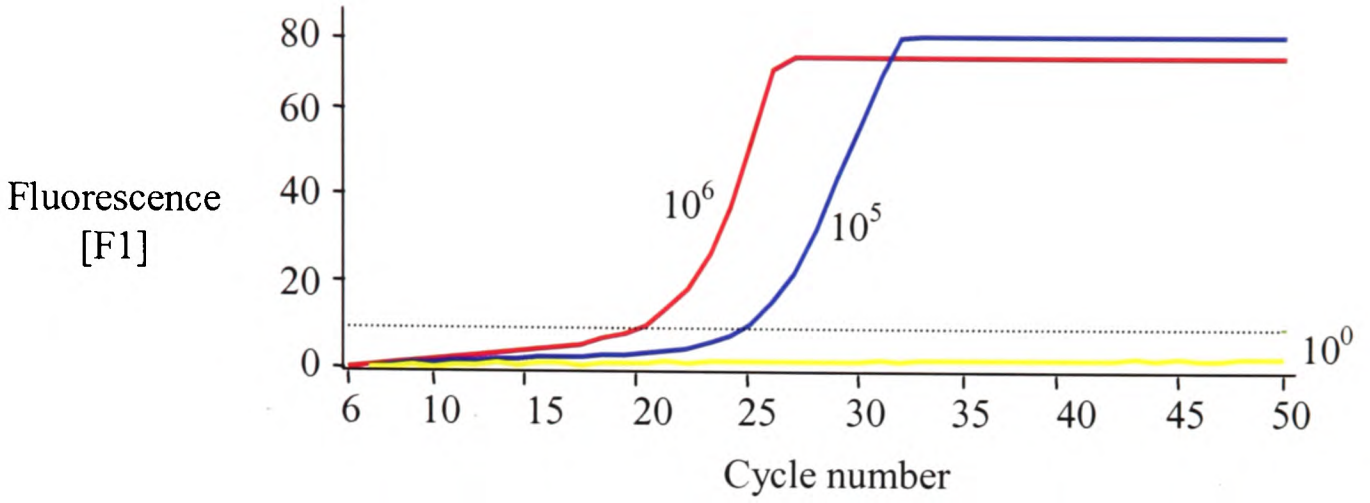
3.3.4 Real-time PCR on the ABI PRISM® 7700

During the course of this study, a real-time PCR assay (TaqMan assay) was designed to amplify tDNA on the ABI PRISM® 7700. The ABI PRISM® 7700 offers the advantage of being able to simultaneously analyse 96 samples in one run compared with 32 on the LightCycler™. Moreover, PCR reactions are performed in microtitre plate wells so the same wells could be used for antibody detection and real-time PCR. This would greatly simplify the I-PCR assay procedure by removing the need to elute the tDNA prior to amplification.

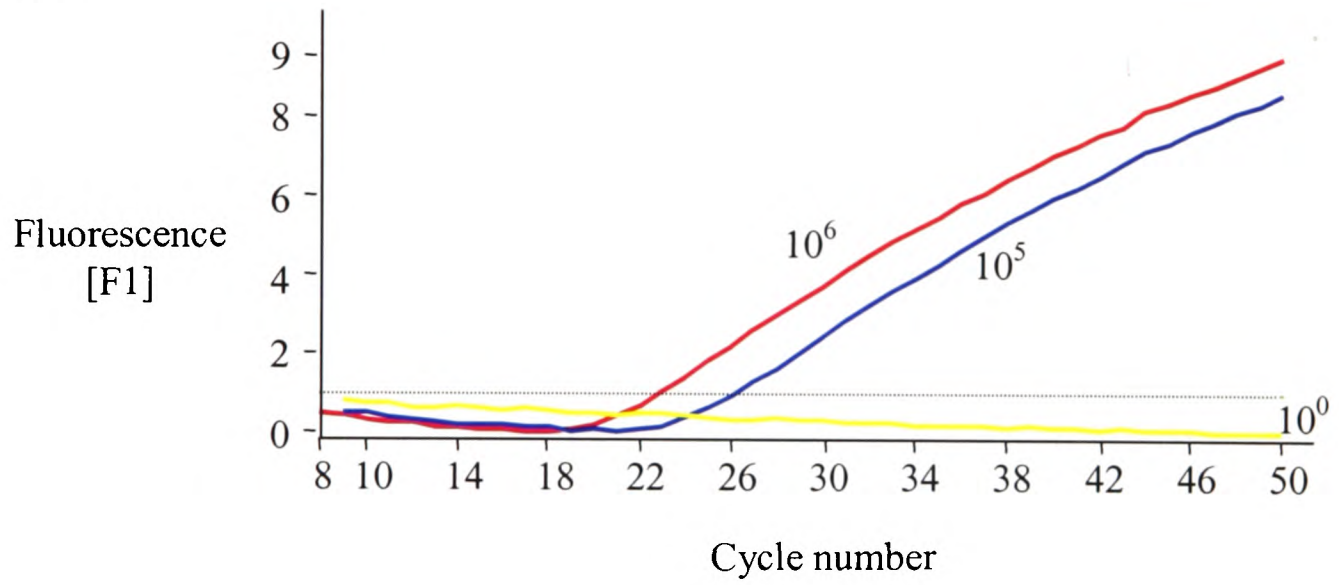
Initially, the TaqMan assay was analysed on the LightCycler™ and the results are shown in Figure 3.12. The TaqMan primers successfully amplified tDNA (10^5 and 10^6 copies) in the presence of SYBR Green I and hydrolysis probe. The fluorescent signals were greater and detected earlier for the amplification of tDNA in the presence of SYBR Green I compared with hydrolysis probe. This was expected since many SYBR Green I molecules bind to dsDNA and fluoresce whereas only one fluorescent signal is generated upon binding and cleavage of hydrolysis probe.

Figure 3.12 Amplification of tDNA using TaqMan primers in presence of (a) SYBR green I and (b) hydolysis probe on LightCycler™.

(a)



(b)



tDNA (10^5 and 10^6 copies) were amplified on the LightCycler™. The thermal cycling conditions for (a) were: an initial denaturation at 93°C for 15 s, followed by 50 amplification cycles of four steps: 93°C for 0 s; 58°C for 0 s; 60°C for 0s; and 74°C for 2 s. Fluorescence was measured once per cycle after the 74°C step. The conditions for (b) were: an initial denaturation at 93°C for 15 s, followed by 50 amplification cycles of three steps: 93°C for 0 s; 58°C for 0 s; 60°C for 2 s. Fluorescence was measured once per cycle after the 60°C step. Maximum ramp rates (20°C/s) were used except for the transition from 58 - 60°C when the ramp rate was 3°C/s.

The TaqMan primers and hydrolysis probe were then used to amplify the external standards (10^4 – 10^8 copies tDNA) on the ABI PRISM® 7700. A standard curve was constructed by plotting the $\log_{10}$ input copy number of each standard against the crossing point and this was compared with the standard curve obtained using the ResonSense® probe assay on the LightCycler™ (Figure 3.13). The efficiency of amplification (slope) and sensitivity (y-intercept) was marginally better when tDNA was amplified using the TaqMan assay on the ABI PRISM® 7700. However considering the chemistries of the probe systems were different and different real-time instruments were used the standard curves were very similar.

Figure 3.13 Comparison of standard curves obtained using the TaqMan assay and ResonSense® assay.

The $\log_{10}$ input copy number (tDNA copies) of each standard was plotted against the crossing point cycle number.

The TaqMan assay (performed on the ABI PRISM® 7700) was subsequently used to quantify tDNA eluted from I-PCR wells and the results were compared with those obtained using the ResonSense® probe assay on the LightCycler™ (Table 3.8). As shown in Table 3.8, slightly higher tDNA values for negative, weak positive and strong positive samples were observed by using the TaqMan assay on the ABI Prism 7700, however this was not significant ($p = 0.3$). In addition, the T/N ratios were very similar.

Table 3.8 Quantification of tDNA eluted from I-PCR wells using the LightCycler™ and ABI PRISM® 7700.

Sample[a]	ResonSense assay Mean tDNA copies (T/N)	TaqMan assay Mean tDNA copies (T/N)
Blank	1.4×10^4	1.5×10^4
Negative	6.3×10^4	1.6×10^5
Weak positive	3.5×10^5 (6)	1.5×10^6 (9)
Strong positive	4.5×10^6 (71)	1.2×10^7 (75)

[a] Shown are the mean tDNA values of four replicate samples.

3.3.5 I-PCR using the ABI PRISM® 7700

Initially, an experiment was performed to determine whether the thin-walled ABI PRISM® 7700 microtitre plate wells were compatible with antigen coating. The ABI PRISM® 7700 and MaxiSorp™ microtire plate wells were coated with 1 μg/ml of rNP and then ELISA and I-PCR were used to compare coating efficiency. The results are shown in Table 3.9. In wells that did not receive sera (blank), low amounts of tDNA and low OD values were obtained. In wells that received negative sera, approximately 0.5 logs more tDNA bound to the MaxiSorp™ wells compared with the ABI PRISM® 7700 wells, suggesting less human IgG was binding non-specifically to the ABI PRISM® 7700 wells. However, in the presence of weak and strong positive sera relatively lower signals were obtained in ABI PRISM® 7700 wells compared with the MaxiSorp™ wells resulting in similar I-PCR T/Ns for both well types. The findings suggested the ABI PRISM® 7700 microtire plate wells could be used for both antibody detection and real-time PCR. However, when the wells were used in this way, high amounts of tDNA were detected in the negative control wells relative to the amount of tDNA detected in the positive control wells resulting in low T/N values. The most likely explanation for this was inconsistent

washing of the wells. The ABI PRISM® 7700 plates were incompatible with the automated plate washer so they were manually washed. Since washing was vital to reduce non-specific binding, further experiments were not conducted due to the lack of an appropriate automated plate washer.

Table 3.9 Coating of MaxiSorp™ and ABI PRISM® 7700 microtitre well plates with rNP[a]

Sample	I-PCR tDNA copies/μl (T/N)		ELISA[b] OD (T/N)	
	MaxiSorp™ wells	ABI PRISM® 7700 wells[c]	MaxiSorp™ wells	ABI PRISM® 7700 wells[c]
Blank	4.1×10^4	1.8×10^4	0.016	0.014
Negative	1.8×10^5	5.4×10^4	0.059	0.021
Weak Positive	1.5×10^6 (8)	4.8×10^5 (9)	0.817 (14)	0.46 (22)
Strong Positive	1.2×10^7 (67)	3.6×10^6 (67)	2.605 (44)	1.762 (84)

[a] The coating conditions were the same for both plates (see section 3.2.5). Samples were tested in duplicate. T/N is the mean tDNA or OD of positive samples divided by the mean tDNA or OD of negative samples.
[b] Wells were incubated with sera followed by anti-human IgG-HRP (Dako) diluted 1/3000 in diluent C (Apendix A).
[c] The ABI PRISM® 7700 wells were not compatible with the ELISA plate reader so the well contents were transferred to fresh MaxiSorp™ wells prior to spectrophotometric analysis.

3.3.5 Conjugate B – FITC-labelled-capture oligonucleotide-anti-human IgG conjugate

In earlier experiments when investigating the capture and release of tDNA, the optimal release of tDNA was achieved by restriction enzyme digestion, however tDNA could also be released by simply washing the wells with buffer. Although the reason for this was unclear, to improve the specificity of the assay the first 12 bases of the tDNA and the complementary bases on the 3' end of the capture oligonucleotide were altered to incorporate more G/C bp's as these form stronger bonds than A/T bp's (Table 3.10). In addition, the capture oligonucleotide was synthesised with a 3' thiol and a 5' FITC. The thiol enabled the capture oligonucleotide to be reacted directly with SPDP-activated anti-human IgG: the thiol group reacts with the 2-pyridyl disulphide residue of SPDP. The capture oligonucleotide was labelled with FITC so the conjugate could be used for ELISA (using an anti-FITC –HRP conjugate) as well as for I-PCR.

Table 3.10 Modified tDNA and capture oligonucleotide

Oligonucelotide	**Sequence**[a]
tDNA	5'-GGCTCCGACTCC**GGATCC**ATAGCATGACTACGTGA CTCGGTTGGAAGCTATACGTTCGATGGCTGCTGCATCAGTA CGCAATCGGTATG-3'
FITC-capture oligonuclotide	5'-FITC-CTAT**GGATCC**GGAGTCGGAGCCTTT-thiol-3'

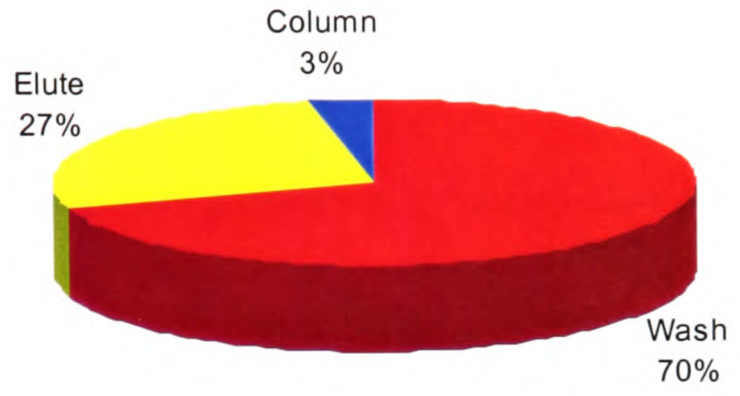
[a] *Bam*H I digestion site in bold and the base changes compared to the original tDNA are underlined.

(i) Purification of anti-human IgG

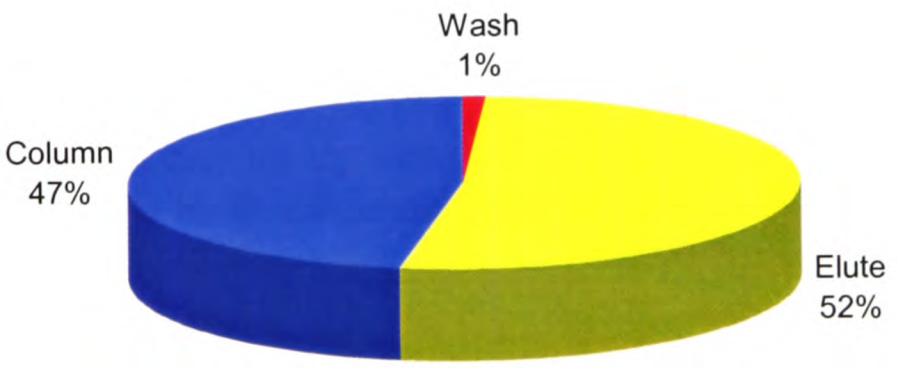
Prior to the conjugation of anti-human IgG and FITC-capture oligonucleotide, the anti-human IgG was purified by absorbing out mouse Ig reactivity and then affinity purifying on a human IgG immunosorbent column. Figure 3.14 summarises the results of the purification. Of the 6 mg of anti-human IgG applied to the mouse Ig column, 70% (4.2 mg) was washed from the immunosorbent (= flow-through), 27% (1.6 mg) was eluted, and 3% (0.2 mg) remained bound to the column. When the wash fractions were reapplied to the column, no further proteins were retained indicating that all the anti-mouse Ig reactivity had been removed from the preparation during the first application. The flow-through from the mouse-Ig column was then applied to the human IgG immunosorbent, where 1% (0.05 mg) was washed from the column, 52% (2.2 mg) was eluted, and 47% (1.95 mg) remained on the column (high affinity antibodies that could not be removed). A second elution was performed by applying glycine buffer containing 20% aceonitrile to the human IgG column however only a negligible amount of protein was eluted. The % recovery of protein was 37% (2.2 mg).

Figure 3.14 Purification of anti-human IgG on (a) mouse Ig; and (b) human IgG chromatography columns.

(a) Mouse Ig column



(b) Human IgG column

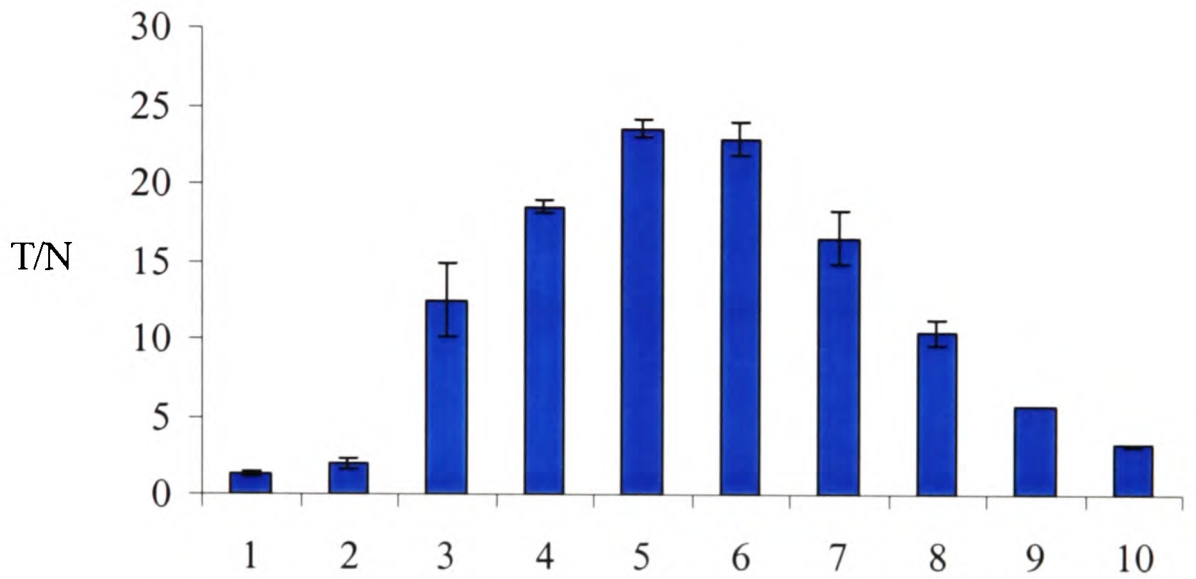


Anti-human IgG (6mg) was applied to a mouse Ig column and then the wash fractions (or flow-through) were collected and applied to a human IgG column (as described in section 3.2.8). The amount of protein (expressed as a percentage) eluted, washed or remained on the column, determined by measuring the absorbance at 280 nm, is represented in yellow, red and blue respectively. The eluate from the mouse Ig column contained the cross-reacting antibodies.

(ii) Isolation and analysis of conjugate B

After the coupling reaction, conjugate B was isolated using gel filtration chromatography. The fractions collected from the column were analysed for the presence of conjugate by ELISA. Figure 3.15 shows the ELISA T/N values of the fractions collected from the column. The highest T/N values were detected in fractions 3 to 8 so these were pooled.

Figure 3.15 Analysis of conjugate B fractions by ELISA after gel filtration chromatography.



An aliquot of each fraction was diluted 1/50 in diluent B (Appendix A) and analysed for the presence of conjugate B by ELISA as described in 3.2.15i. T/N is the OD of human IgG wells divided by the OD of BSA wells. Error bars represent one SD.

Analysis of the pooled conjugate by ELISA (Table 3.11) showed the conjugate was poorly immunoreactive as relatively low OD values (< 1) were obtained in human IgG wells. The most likely explanation for this was that the high affinity anti-human IgG antibodies were removed during the affinity purification.

Since in conjugate B the capture oligonucelotide was linked to the antibody via a disulphide (S-S) bond, the reducing agent, DTT, was investigated as an alternative to restriction enzyme digestion to release the modified capture oligonucleotide. Wells were incubated in the presence and absence of 0.1 M DTT for 15 min prior to incubation with anti-FITC-HRP. DTT resulted in a 3.8 fold drop in OD in human IgG wells suggesting that it may be useful as an alternative to restriction enzyme digestion to release tDNA (Table 3.11)

Table 3.11 Binding of conjugate B to human IgG[a]

Conjugate Dilution Factor	BSA wells OD	Human IgG wells OD	T/N
-	0.035	0.023	
10	0.034 (0.039)	0.83 (0.219)	24
20	0.029	0.825	28
50	0.031	0.517	17
100	0.030	0.380	13

[a] Conjugate dilutions were tested in duplicate. T/N is the mean OD of human IgG wells divided by the mean OD of BSA wells. The results given in brackets were those obtained after DTT treatment of the wells.

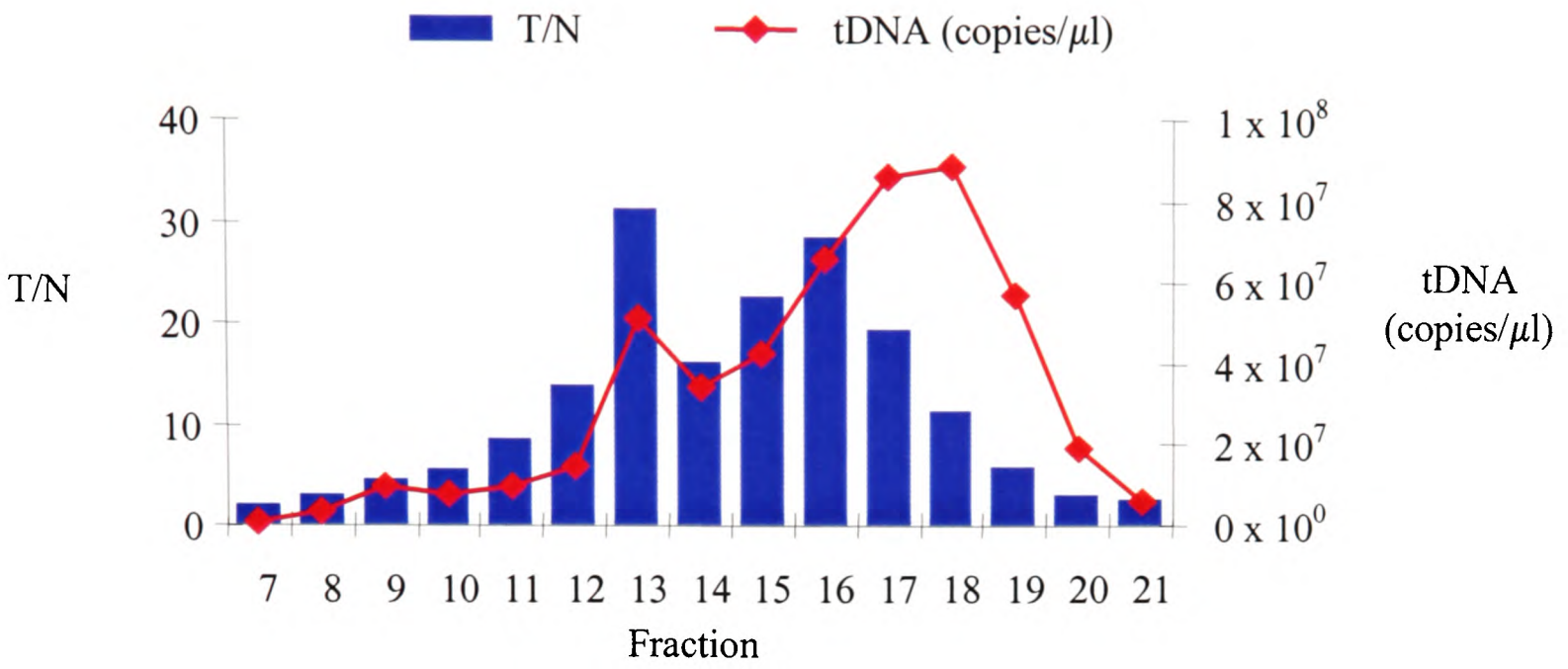
3.3.6 Conjugate C - FITC-tDNA-anti-human IgG conjugate

Instead of coupling capture oligonucleotide to anti-human IgG, tDNA was covalently linked to anti-human IgG. The tDNA was labelled with FITC so that it could be used in an ELISA and the anti-human IgG preparation was not affinity purified prior to the conjugation.

(i) Isolation and analysis of conjugate C

Conjugate C was isolated using gel filtration chromatography. The fractions collected from the column were analysed for the presence of conjugate by ELISA using anti-FITC HRP. Also, the amount of FITC-tDNA (conjugated and free) in each fraction was determined using real-time PCR. Figure 3.16 shows the ELISA T/N values and the amounts of FITC-tDNA detected in fractions 7 - 21. Two T/N peaks were observed at fractions 13 and 16. Since larger molecules are eluted from the column earlier, the conjugate represented by the first peak was most likely antibody conjugated to more than one DNA molecule, whereas conjugate represented by the second peak was antibody conjugated to one DNA molecule. The PCR results followed the ELISA results in they both contained 2 distinct peaks, although the second tDNA peak was identified in a later fraction. Most likely fractions 17 and 18 contained a large amount of free, unconjugated tDNA. As a result of these findings fractions 12 - 16, containing heterogenous conjugate, were pooled.

Figure 3.16 Isolation of conjugate C by gel filtration chromatography.



An aliquot of each fraction collected after gel filtration chromatography was diluted 1/1000 in diluent B and 1/10,000 in nuclease-free water and analysed for the presence of conjugate by ELISA (blue columns) and tDNA (red line) by real-time PCR respectively. T/N is the OD of single samples from human IgG wells divided by the OD of single samples from BSA wells.

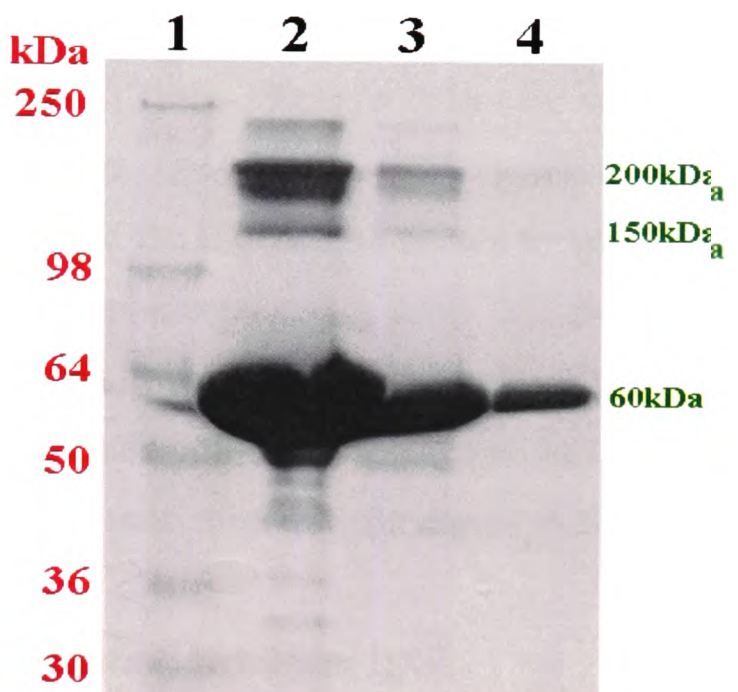
(ii) Characterisation of conjugate C

The size and purity of conjugate C was analysed by SDS-PAGE and Western blotting (Figures 3.17a and b). Coomassie blue-staining revealed a intense band at ~ 60 kDa, which represented the BSA added to the conjugate preparation as carrier protein. Several bands were observed between 150 and 250 kDa. The band at 150 kDA most likely represented unconjugated anti-human IgG that was not removed by gel filtration chromatography, whereas the higher molecular weight bands (~180 - 240kDa) represented conjugate, where varying numbers of FITC-tDNA molecules were coupled on the anti-human IgG molecule. The expected size of the conjugate containing one FITC-tDNA was 180 kDa (150 kDa (antibody) + 30 kDa (F-tDNA; 89 mer x 330 Da). Western blot analysis using anti-FITC-HRP revealed similar results: a diffuse band representing conjugate was observed between 180 - 225kDa. However, Western blot analysis also identifed a smaller 70 kDa band not present by SDS-PAGE. This band most likely represented FITC-labelled BSA. The absence of a band corresponding to free ~30 KDa FITC-tDNA in the Western blot (Figure

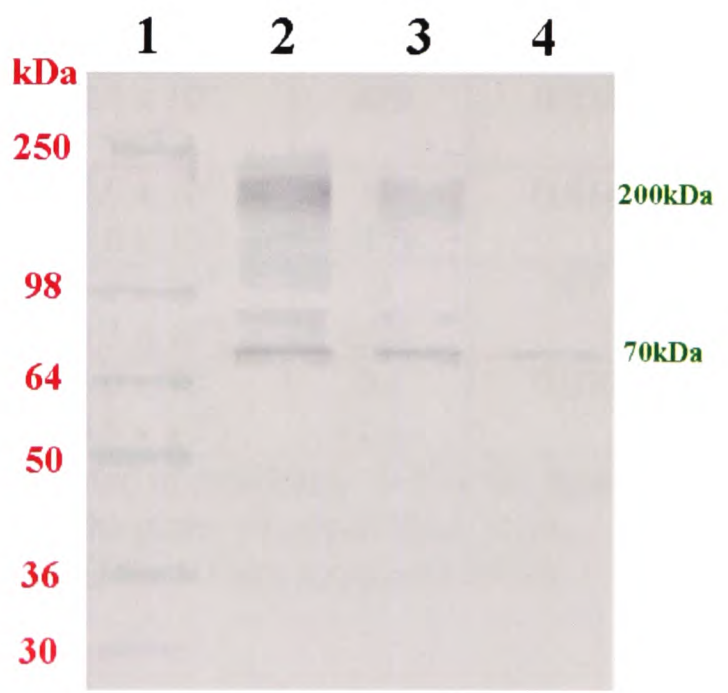
3.17b) suggested that all free DNA was removed from the conjugate preparation; alternatively free FITC-tDNA did not bind to the nitrocellulose. These findings support the observations made in the previous experiment that the conjugate was heterogeneous.

Figure 3.17 Characterisation of conjugate C by (a) SDS-Page and Coomassie blue-staining and (b) Western blotting with anti-FITC-HRP.

(a)



(b)



Lane 1 contained molecular weight marker and lanes 2, 3 and 4 contained conjugate B diluted 1/10, 1/100 and 1/1000 respectively.

(iii) Binding of conjugate C to human IgG

The effect of conjugation on the ability of conjugate C to bind to human IgG and BSA as a control was investigated using I-PCR and ELISA. As shown in Table 3.12, very low OD values and amounts of tDNA were detected in the BSA-coated wells compared to the human IgG-coated wells. The dilution of conjugate that resulted in the highest T/N value was $1/10^2$. At this dilution the I-PCR T/N values were approximately 4 fold greater than the ELISA T/Ns. At the conjugate dilution of $1/10^3$ the T/N values obtained were approximately ten-fold lower. Under similar I-PCR conditions, conjugate A gave significantly higher T/N values (Table 3.3). To determine whether the relatively lower signals were due to the inefficient release of the tDNA by restriction enyme digestion, heat denaturation was used to release the tDNA. As shown in table 3.12, however, the T/N values did not improve when heat treatment was used. Interestingly, when wells that had undergone I-PCR and/or ELISA were washed and reanalysed by ELISA, relatively high amounts of bound conjugate could still be detected (see Table 3.13 for a representative experiment). This suggests the restriction enzyme digestion did not release all the FITC-labelled tDNA into solution.

Table 3.12 Binding of conjugate C to human IgG[a]

Conjugate Dilution Factor	I-PCR tDNA copies/μl detected after restriction digest (heat denaturation[b])			ELISA OD		
	BSA wells	Human IgG wells	T/N[b]	BSA wells	Human IgG wells	T/N[b]
10^2	5.6×10^3 (NT)	2.4×10^6 (NT)	429	0.014	1.516	108
10^3	6.5×10^3 (2.1×10^4)	2.6×10^5 (3.6×10^5)	40 (17)	0.014	0.173	12
10^4	4.9×10^3 (4.3×10^3)	4.5×10^4 (4.3×10^4)	9 (10)	NT	NT	-
-	4.9×10^1 (5.6×10^0)	6.0×10^0 (8.8×10^0)	0.1 (1.6)	0.016	0.021	1.3

[a] Conjugate dilutions were tested in duplicate. T/N is the mean tDNA copies/μl or OD of human IgG wells divided by the mean values of BSA wells.
[b] Wells were incubated at 94°C for 10 min to release tDNA.

Table 3.13 Analysis of microtitre plate wells by ELISA after I-PCR and ELISA[a]

Conjugate Dilution Factor	I-PCR tDNA copies/μl (OD)			ELISA OD		
	BSA wells	Human IgG wells	T/N	BSA wells	Human IgG wells	T/N
-	0.01 (7.0×10^{-1})	0.03 (7.5×10^{0})	3 (11)	0.007	0.022	31
10^2	0.008 (2.8×10^{4})	1.4 (1.1×10^{6})	175 (39)	0.008 (0.008)	0.693 (1.554)	87 (194)
10^3	0.008 (1.4×10^{3})	1.555 (1.8×10^{5})	194 (129)	0.008	0.189	24

[a] Conjugate dilutions were tested in duplicate. T/N is the mean tDNA copies/μl or OD of human IgG wells divided by the mean values of BSA wells. The results in brackets are the initial I-PCR and ELISA results. - denotes no conjugate was added to the wells

(iv) Comparison of conjugate C with a commercial conjugate for the detection of mumps-specific IgG

The immunoreactivity of conjugate C was compared with a commercial conjugate for the detection of mumps-specific IgG in serum by ELISA and the results are shown in Table 3.14. In the presence of weak and strong positive sera, higher OD and T/Ns values were obtained when the commercial conjugate was used to detect specific antibody. As a result of its relatively low performance characteristics compared with conjugate A and the commercial conjugate, conjugate C was not tested further.

Table 3.14 Comparison of a commercial conjugate with conjugate C for the detection of mumps-specific IgG in serum[a]

Sample	Anti-human IgG-HRP[b] OD	T/N	Conjugate C OD	T/N
Blank	0.018	-	0.034	-
Negative	0.087	-	0.037	-
Weak Positive	0.84	10	0.093	3
Strong Positive	2.32	27	0.65	18

[a] Samples were tested in duplicate. T/N is the mean OD of positive wells divided by the mean OD of negative wells.
[b] Obtained from Dako.

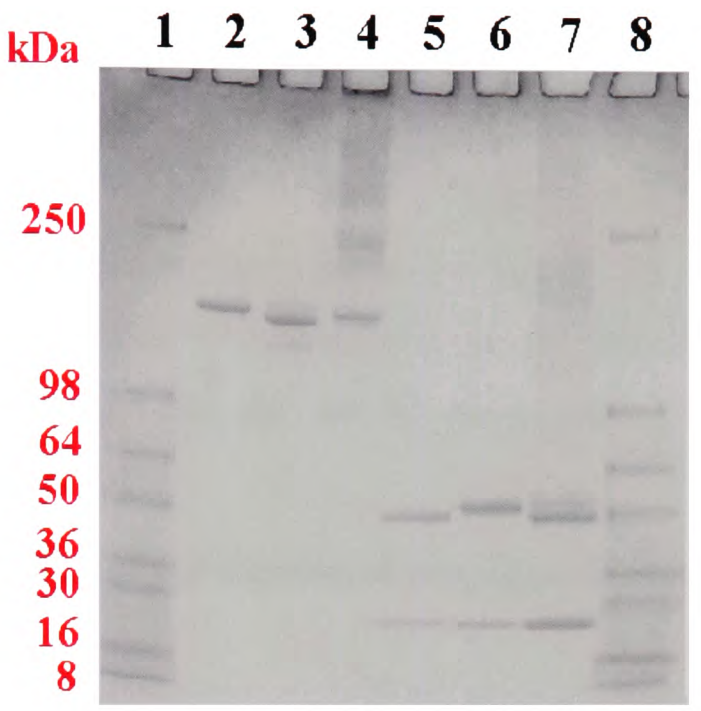
3.3.7 Conjugate D - Anti-rNP MAb-anti-FITC MAb conjugate

A chimera consisting of two MAbs that bind to mumps antibodies and FITC-tDNA was synthesised (Figure 3.3d) as an alternative to the DNA-antibody chimeras described above to: assess the feasibility of coupling two MAbs; to enable the immunoreaction to occur before the addition of tDNA in I-PCR; and to provide a conjugate for use in an antibody capture or competitive assay configuration.

(i) Characterisation of conjugate D

The size and purity of conjugate D was analysed by SDS-PAGE (Figure 3.18). In lanes 2 and 3, the bands observed at 150 kDa represented anti-FITC MAb and anti-rNP MAb respectively. In lane 4, several diffuse bands of > 240 kDa were observed, which represented anti-rNP MAb coupled to different epitopes on the anti-FITC MAb and vice versa. The presence of a band at 150 kDa in lane 4 suggested unconjugated antibody was present in the conjugate preparation. The heavy (55 kDa) and light chain (25 kDa) immunoglobulin bands were clearly visible in the reduced lanes (lanes 5 - 7). In lane 7, as well as the heavy and light chain bands, a smear was observed which represented reduced conjugate: reduction would not effect the covalent bond between the anti-rNP MAb and anti-FITC MAbs.

Figure 3.18 SDS-PAGE analysis of conjugate D.



Coomassie blue stained SDS-PAGE gel: lanes 1 and 8, molecular weight marker; lane 2 anti-FITC MAb; lane 3: anti-rNP MAb; lane 4; conjugate D; lane 5: reduced anti-FITC MAb; lane 6 reduced anti-rNP MAb; lane 7: reduced conjugate.

The effect of conjugation on the ability of conjugate D to bind to rNP was analysed by ELISA. As shown in Table 3.15, high OD values were detected in rNP-coated wells compared with BSA-coated wells. Very similar T/N values were obtained at conjugate dilutions of $1/10^2$ and $1/10^3$.

Table 3.15 Binding of conjugate D to immobilised rNP[a]

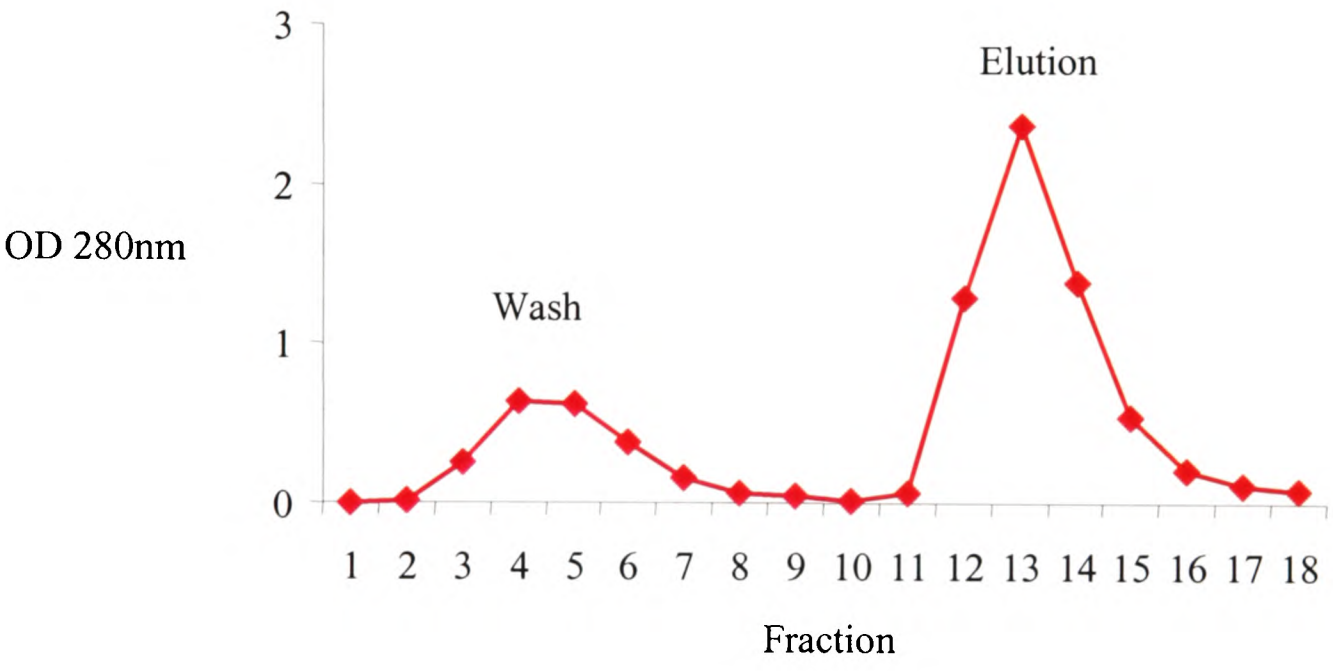
Conjugate Dilution Factor	BSA-coated wells OD	rNP-coated wells OD	T/N[a]
Blank	0.010	0.011	1.1
10^2	0.011	1.326	120
10^3	0.011	1.444	131
10^4	0.011	0.971	88

[a] Conjugate dilutions were tested in duplicate. T/N is the mean OD of rNP wells divided by the mean OD of BSA wells.

(ii) Purification of conjugate D

Since SDS-PAGE revealed the presence of unconjugated MAb in the conjugate preparation and free anti-rNP MAb has the potential to interfere with the binding of the conjugate to rNP in the antibody capture assay, conjugate D was purified by affinity chromatography on a coumarin-column. Derivatives of 7–hydroxy coumarin have structural similarities to FITC and can be used to purify anti-FITC antibodies (Samuel and Abuknesha, 1990). The elution profile is shown in Figure 3.19. Two peaks were observed at fractions 4/5 and 13. The first peak represented the wash fractions containing unconjugated anti-rNP MAb and the second peak represented conjugate (and any unconjugated anti-FITC MAb).

Figure 3.19 Elution profile of purification of conjugate D on coumarin column



Conjugate D was purified on a coumarin column as described in 3.2.10. Wash and elution fractions were collected and analysed for the presence of protein by measuring the absorbance at 280 nm.

(iii) Evaluation of conjugate D using I-PCR

Coumarin-purified conjugate was initially evaluated with respect to its capacity to bind to immobilised rNP by I-PCR. Instead of using restriction enzyme digestion, DTT was used to release FITC-tDNA from the solid phase. In the absence of conjugate, low levels of tDNA were detected (8.9×10^3 copies/μl) whereas good signals were observed in the presence of conjugate (1.3×10^6copies/μl). Subsequently, the conjugate was used to detect

mumps-specific IgG in serum using an antibody capture assay format (see Figure 3.3c). However, very little discrimination between positive and negative serum samples was observed (data not shown) due to low signals in the presence of positive serum.

3.4 Discussion

The use of labelled antibodies in immunoassays offers the potential for developing highly sensitive and specific tests provided that labels of high specific activity are employed and non-specific binding of labelled antibody is minimised (Ekins, 1981). A wide range of labels with various specific activities have been employed in immunoassays. Radioisotopic labels such as I^{125} have relatively low specific activities, in the order of five disintegrations per minute per attomole (Ishikawa *et al.*, 1989). Non-isotopic labels such as chemiluminescent and fluorescent substances are capable of yielding higher specific activities. For example, the specific activity of MAbs labelled with a chemiluminescent acridium ester was shown to be 50 photon counts per attomole of label (Weeks *et al.*, 1984).

Enzymes have the potential for signal amplification since one molecule of enzyme may catalyse the conversion of many substrate molecules to detectable product. In practice, however, the colorimetric substrates used to monitor enzyme activity limit the sensitivity with which enzyme labels can be detected since relatively large concentrations of chromogens need to be generated for spectrophotometric detection. Enzyme assays have therefore not usually demonstrated much greater sensitivity than radioisotopic methods.

Recently DNA labels have been employed for the development of sensitive I-PCR assays (Sano *et al.*, 1992; Ruzicka *et al.*, 1993; Zhou *et al.*, 1993; Hendrickson *et al.*, 1995; Maia *et al.*, 1995; Case *et al.*, 1997; Wu *et al.*, 2001). Sano *et al.* (1992) reasoned that since single target nucleic acid molecules can potentially be detected by PCR, the use of DNA labels would permit highly sensitive assays. Using this approach they were able to detect as few as 580 molecules of immobilised BSA. Until now, I-PCR has been used for the sensitive detection of a variety of soluble protein molecules such as cytokines (Sanna *et al.*, 1995; Saito *et al.*, 1999), tumour markers (Suzuki *et al.*, 1995) and hormones (Hendrickson *et al.*, 1995). In this study, the method was adapted for the detection of antibodies to mumps virus. The distinctive features of the I-PCR assays were (a) the conjugates; (b) the use of restriction enzyme digestion to release specifically hybridised tDNA; and (c) the quantification of tDNA by real-time PCR.

Of the four conjugates evaluated, the best results were obtained using conjugate A, which was designed for use in an indirect assay format. It consisted of anti-human IgG covalently conjugated to a short capture oligonucleotide and had the dual function of binding to human IgG and capturing tDNA; the 5'end of the tDNA was complementary to the capture oligonucleotide. The conjugate was designed to be generic so could potentially be used to detect any human IgG.

In previous I-PCR assays heat treatment was used to release antigen-antibody-marker DNA complexes from the solid phase (Ruzicka *et al.*, 1993; Sanna *et al.*, 1995). By contrast, in the present study restriction enzyme digestion was used to release hybridised tDNA from the solid phase. The reasoning for using restriction enzyme digestion was to improve the specificity of the test, however the experiments indicated that the release of tDNA could be achieved simply by incubating wells with restriction enzyme digestion buffer at 37°C. The nature of this release was unclear; the buffer would not be expected to denature the tDNA from the capture oligonucleotide, but it may be that the antigen or antibodies desorb from the solid phase over time.

tDNA released into solution was amplified by real-time PCR using the LightCycler™, which provided rapid and sensitive quantification of tDNA. Once tDNA samples were ready for PCR, amplification and analysis were completed within 30 min, considerably faster than by conventional PCR (2 – 3 h). The PCR could detect accurately 100 copies of tDNA. This level of sensitivity is consistent with other single round PCR reactions (Brechtbuehl *et al.*, 2001). The assay had a wide dynamic range; the upper limit was 2 $\log_{10}$ higher than the highest standard (10^{10}copies/μl). In previous I-PCR assays conventional PCR was used so accurate quantification was not possible.

Melting curve analysis was used to determine whether the fluorescence detected by the LightCycler™ was due to the specific amplification of tDNA. Two peaks were generated, corresponding to the melting temperature of the ResonSense® probe/tDNA duplex and the full-length product. The full-length product peak was generated due to spectral overlap. Although channel F2 is optimal for the detection of emissions from Cy5, emissions from SYBR Green I are also detected.

As well as being fast, sensitive and specific, another advantage of real-time PCR is the low risk of contamination since post PCR product analysis is not required. Amplicon is contained within sealed capillaries that are discarded immediately after each run.

Quantitative PCR was essential to allow the relative levels of non-specific binding of tDNA, conjugate and human IgG to be determined accurately. In previous studies using

conventional PCR based on the detection of amplicons after gel electrophoresis, it was not possible to determine accurately the contribution of the specific signal compared to the non-specific signal. In the present study, non-specific binding of human IgG was the main factor limiting the sensitivity of the assay, compromising the advantages gained by PCR amplification. It was necessary therefore to define the experimental results on the basis of T/N values rather than the presence or absence of specific product. The non-specific binding observed was not due to reactions of serum antibodies against the recombinant antigen, suggesting the antigen was highly purified.

To improve the specificity of the assay extensive optimisation experiments were performed. Most critical were the working concentrations of conjugate and tDNA; decreasing the conjugate concentration by ten-fold resulted in an approximate ten-fold reduction in T/N.

The type of well surface used for antigen coating did not affect the amount of background signal. In the presence of negative serum, similar amounts of tDNA were detected in PolySorp™ and MaxiSorp™ wells. This was surprising since the PolySorp™ surface is more hydrophobic than the MaxiSorp™ surface so is less suited to binding glycoproteins such as antibodies but is recommended for binding viral antigens. Thus one might expect better signals and lower background with PolySorp™ wells.

An improvement in assay specificity may have been achieved by the use of wells designed for covalent attachment of coating antigen. Several commercial companies supply wells with amino groups attached that serve as bridges for covalent binding to the functional groups of proteins and other molecules; Covalink™ NH Modules (Nunc), Xenobind (Corning) and TopYield™ Strips (Xenobind) are examples. The advantages of covalent binding are that thorough washing of wells is possible and it allows for the orientation of the immobilised molecules and better recognition by the detection molecule. Sperl *et al.* (1995) compared Xenobind covalent binding plates with passive binding plates and found the results differed depending on the assay format. There was little difference in assay performance between the wells when used for direct and direct sandwich ELISAs however considerably lower background was detected with an indirect ELISA.

The use of an appropriate blocking agent was essential to limit non-specific binding. Of the reagents tested, skimmed milk was the most effective. This is consistent with the results of other studies (Kenna *et al.*, 1985; Bjercke *et al.*, 1986; Vogt *et al.*, 1987; Pratt and Roser, 1989; Case *et al.*, 1997). Sperl *et al.* (1995) tested 3% solutions of BSA, low fat dry milk, gelatin and ovalbumin and found that low fat dry milk gave the lowest background.

Similarly, Pratt and Roser (1989) found casein (which is the main protein present in milk) was more effective than BSA or newborn calf serum for blocking the binding of rat serum to MaxiSorp^{TM} microtitre plate wells. They further showed the high blocking efficiency of casein was due to its content of small MW species. High-pressure liquid chromatography revealed casein contained a heterogeneous mixture of molecular species from > 60 KDa to < 10 KDa, whereas newborn calf serum NBCS consisted of proteins > 60 KDa; BSA has a MW of ~ 66 KDa. Fractionation of casein by ultrafiltration into three MW grades, MW < 10 KDa, MW > 30 KDa and 10 KDa < MW < 30 KDa enabled them to show that blocking potency was inversely proportional to the MW of the component proteins.

Nonetheless the use of milk as a blocking agent in immunoassays must be approached with care because of several potential problems: individuals with antibodies to milk constituents may cause false-positive reactions; milk consists of a complex mixture of substances which may interfere with some assays; and it may mask other solid phase proteins causing the loss of immunoreactivity of antigens bound to surfaces (Spinola and Cannon, 1985; Spierto *et al.*, 1987). In the present study, milk did not adversely affect the binding of the assay components.

As reported by others (Sano *et al.*, 1992; Kakizaki *et al.*, 1996; Chang and Huang, 1997) and confirmed in the present study, it was essential to extensively wash the wells with detergent-containing buffer to remove unbound reagents and thereby minimise background noise. Sano *et al.* (1992) washed wells 15 times for 10 min to remove unbound reagents. Chang *et al.* (1997) recommended at least ten washes after incubations.

Other interesting findings of the optimisation experiments were that high concentrations of Tween 20 (3%) in the assay diluent did not adversely affect the binding of assay components, and decreasing the temperature and time that the serum was incubated in the wells did not improve the assay performance; incubation at room temperature reduced the background signal but also the test signal.

To minimise the level of non-specific binding of tDNA, sheared sperm DNA has been used by several groups (Sano *et al.*, 1992; Maia *et al.*, 1995; Sperl *et al.*, 1995; Kakizaki *et al.*, 1996). However, addition of herring sperm DNA to the assay diluent did not improve the performance characteristics of the assay developed in the present study.

The reproducibility of the assay was assessed after the assay was optimised. The reproducibility between wells on the same plate (tDNA amplified in the same PCR run) was marginally better than the reproducibility of the real-time PCR as the SDs for the analysis of different wells (same PCR run) were lower than the SDs for different PCR runs.

Improvements in the accuracy of the I-PCR for a single sample could therefore be achieved most effectively by performing multiple PCR runs on the same well.

When the optimised I-PCR assay was compared with the ELISA, concordance was good. Discrepant findings were obtained in four cases, two of which were attributable to equivocal values in the commercial ELISA. The use of different antigen preparations for capturing the anti-mumps antibody in the two assays is a possible explanation for differences in the results.

During the course of the present study access to an ABI PRISM® 7700 prompted the development of a real-time hydrolysis assay. The reason for this was two fold. Firstly, the ABI PRISM® 7700 is capable of analysing 96 samples in one run, compared to 32 on the LightCycler™. The ABI PRISM® 7700 is therefore more appropriate for testing large numbers of samples as less hands on time is required. Secondly, as reactions are performed in 96-well microtitre plates, the immunoreaction and real-time PCR could be performed in the same well. In addition to reducing the number of steps, this avoids losses of sample in transfers from the conventional microtitre plates to the LightCycler™ capillaries.

A hydrolysis assay was successfully developed to amplify tDNA. The assay was initially analysed on the LightCycler™. This was possible because the LightCycler™ can detect (in channel F1) the fluorescence emitted from the reporter FAM, released from the 5'end of the hydrolysis probe by *Taq* polymerase during amplification. The hydrolysis assay was subsequently optimised for use on the ABI PRISM® 7700 and the assay was found to be comparable in sensitivity to the LightCycler™ ResonSense® probe assay.

Preliminary experiments to determine whether the ABI PRISM® 7700 plate wells were compatible with antigen coating were promising. Compared with the rigid polystyrene MaxiSorp™ wells, the thin-walled polycarbonate ABI PRISM® 7700 plates produced relatively lower test signals in ELISA and I-PCR (where tDNA was released from the solid phase and then quantified on the LightCycler™) but background signals were also lower, so T/N values were comparable. This was not surprising as the polycarbonate thermal cycling wells are often advertised as being ideal for PCR because they have good heat transfer characteristics and have low protein binding properties to minimise loss of material by adsorption. Case *et al.* (1999) have reported previously that polycarbonate plates may be used for antigen coating. They found that polycarbonate wells bound similar amounts of antigen to conventional ELISA wells. Moreover they were able to use the coated wells to produce sensitive I-PCR assays for the detection of a variety of antigens. In the present study, however, when the ABI PRISM® 7700 plates were used for antibody detection and

real-time PCR very little discrimination was obtained between negative and positive samples due to high background. The most likely reason for this was insufficient washing. The ABI PRISM® 7700 plates were not compatible with the automated plate washer so were manually washed which may have been inadequate.

A rational approach to enhance the sensitivity and specificity of the I-PCR assay was to improve the DNA-antibody conjugate. A repeat conjugation of anti-human IgG to capture oligonucelotide was performed with several modifications. First, to minimise 'leaching' of tDNA the nucleotide sequence of the capture oligonucleotide was changed to incorporate more G and C bases. However, this did not reduce the level of leaching (data not shown). Second, the capture oligonucelotide was synthesised with a thiol group on the 3' end rather than an amino group so that it could be reacted with SPDP-activated antibody in a more simple crosslinking procedure. Third, to obtain a highly purified anti-human IgG preparation for conjugation, the commercial preparation was further purified. The anti-human IgG had been affinity purified using immobilised Protein A by the manufacturers. Protein A is widely used for purifying antibodies but it is not specific as it binds to all IgG. Thus, the conjugate was further purified by adsorption on a mouse Ig column to remove cross-reacting antibodies, followed by affinity purification on a human IgG absorbent. However, a large proportion (~50%) of the anti-human IgG antibodies could not be retrieved from the affinity column. These were likely the highest affinity antibodies and may explain the poor immunoreactivity of conjugate B.

In another attempt to improve the conjugate, instead of capture oligonucleotide, antibody was covalently coupled with tDNA to produce conjugate C. The tDNA was tagged with FITC to enable the conjugate to be easily detected using anti-FITC-HRP in Western blotting and ELISA. SDS-PAGE and Western blotting showed the conjugate was heterogeneous in size, perhaps reflecting the different positions and numbers of coupled tDNA. The conjugate was evaluated initially using human IgG-coated microtitre plate wells. Unfortunately, T/N values were not as high as those obtained using conjugate A. The reason(s) for this are unknown. Perhaps the cross-linking procedure was not optimal, or contaminating unconjugated antibody interferred with the assay. Alternatively the FITC-DNA may have been attached near or close to the antigen-binding site thus causing steric hindrance or affecting the conformation of the antibody. Joerger *et al.* (1995) synthesised DNA-antibody conjugates containing 85bp, 95bp ss DNA labels and a 401bp ds DNA label and found the length of label did not effect the sensitivity of the I-PCR assay. However, the cross-linking procedure produced conjugates consisting of an antibody to

DNA ratio of 1:1. In this study, more than one DNA molecule was coupled to some of the antibodies, which may have affected their activity.

As well as improved reagents, the type of assay format chosen can impact on assay sensitivity and specificity. Most of the background signal detected in the indirect assay was due to the conjugate detecting non-specifically bound human IgG antibodies. An alternative assay format may have been better suited to the task of quantifying antibodies in serum, such as an antibody capture or competitive assay configuration.

Conjugate D was designed with this in mind and consisted of anti-rNP MAb covalently coupled with anti-FITC MAb. The main reason for conjugating the two MAbs was that it would allow the immunoreaction to be performed before adding the DNA.

SDS-PAGE revealed the presence of unconjugated antibody in the conjugate preparation so the eluate was further purified on a coumarin immunosorbent. This was performed to remove free anti-rNP MAb as these antibodies would potentially interfere with the binding of conjugate to rNP. Analysis of the conjugate by indirect ELISA and I-PCR revealed the immunoreactivity of the MAbs were retained following conjugation and purification. However, when the conjugate was used in an antibody capture assay format to detect mumps-specific IgG in serum, very low signals were observed in the presence of positive serum. The reason(s) for this was unclear but perhaps the binding of this relatively large conjugate to rNP in the capture assay format was sterically hindered by the mumps-specific IgG antibodies already bound to rNP.

In summary, the present study demonstrates the feasibility of using I-PCR for the detection of virus-specific antibodies in human serum. In contrast to previously published reports an enhanced sensitivity over the ELISA was not demonstrated mainly due to non-specific binding of assay components to the solid phase. Improvements in specificity might be obtained through the use of wells designed for covalent attachment of coating antigens. This would allow more stringent washing conditions to remove the weakly bound complexes without disturbing the specific antigen-antibody reactions. The development of solid supports that minimise background and have sufficient thermal stability to allow thermal cycling would be preferable. Further improvements should also be obtained through the use of highly purified and specific antibodies, and standardised protocols for crosslinking DNA and antibody.

Chapter 4

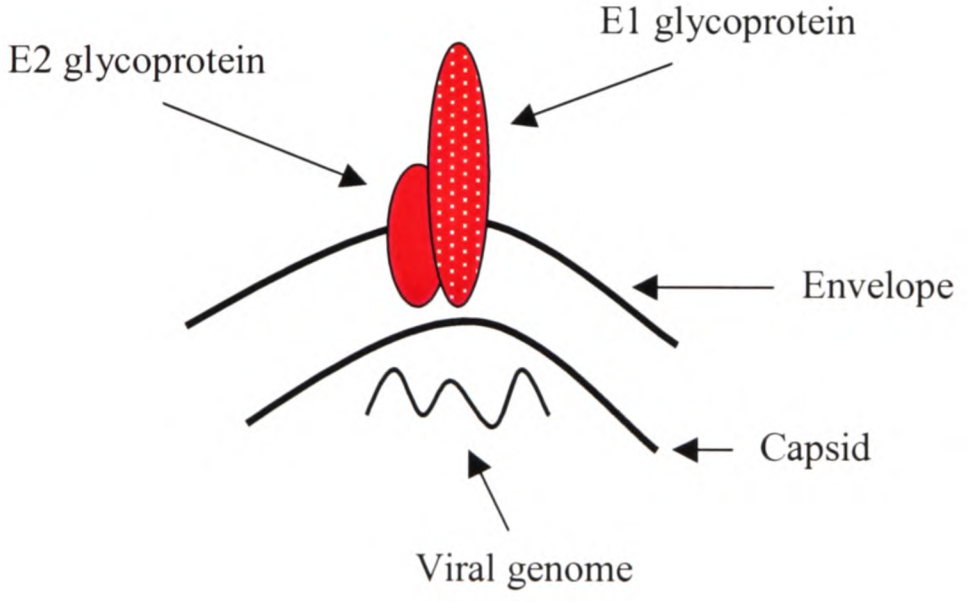
Production of recombinant rubella virus E1 fusion proteins using the PinPoint^{TM} Xa-1 T-vector system

4.1 Introduction

Recombinant antigens have been increasingly used in immunoassays to detect antiviral and antibacterial antibodies (Eroglu *et al.* 2000; Liu *et al.* 2001; Lahdenne *et al.* 2003). The use of recombinant antigens circumvents the time-consuming, costly and inefficient production of antigens in cell culture. Moreover, they can be highly characterised and purified so offer the potential for improved assays.

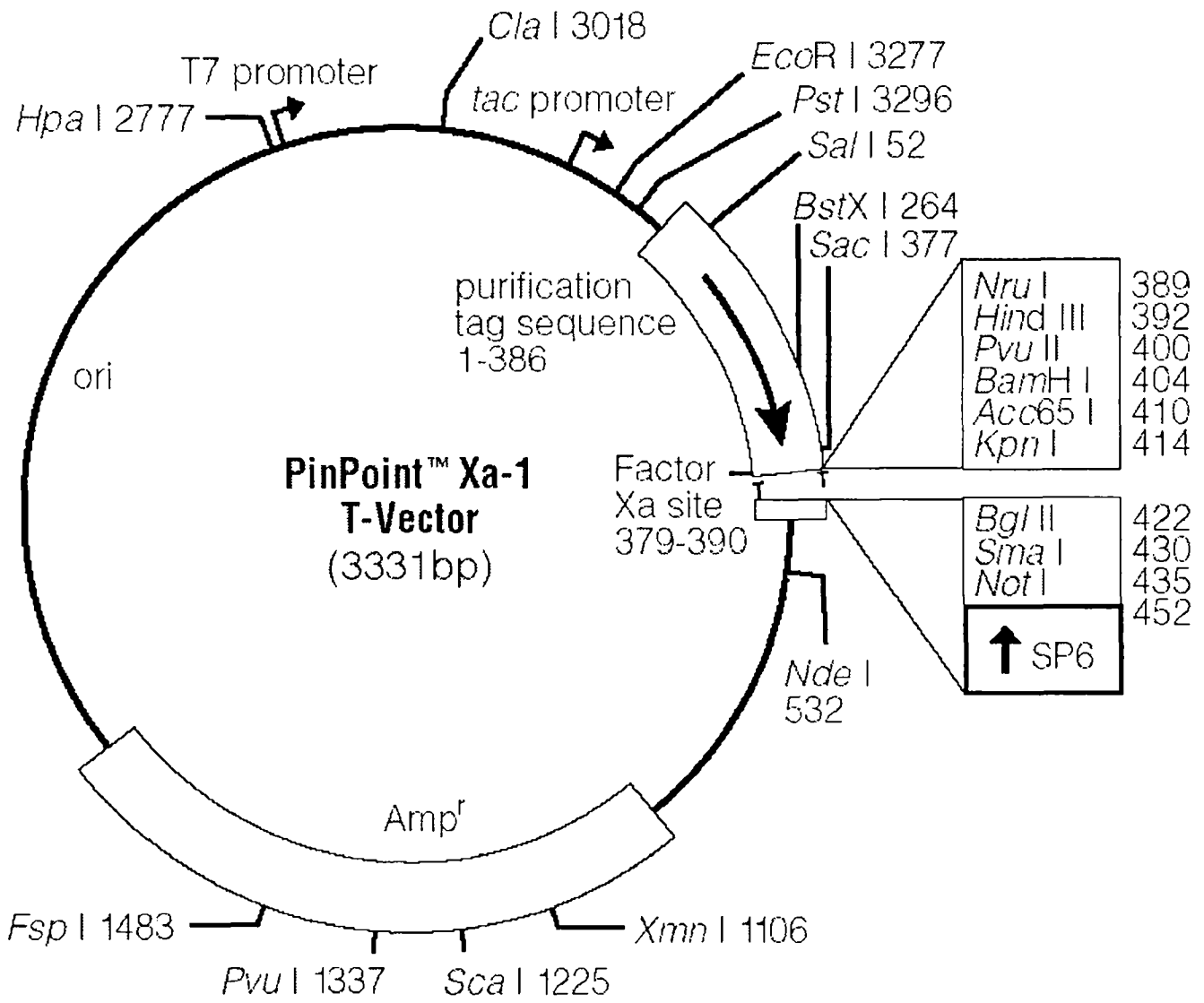
As mentioned in the general introduction (section 1.2.2), RV contains two glycoproteins, namely E1 and E2. E1 (481bp) is larger than E2 (282bp) and is the immunodominant antigen of the RV. It is thought that the E1 is more projected from the surface of the virion than the E2 glycoprotein (Figure 4.1); E2 is relatively inaccessible to the action of enzymes and the immune response (Waxham and Wolinsky, 1985). Thus, E1 has good potential as an immunogenic reagent.

Figure 4.1 Schematic diagram of RV envelope glycoproteins.



Various expression systems are available for the production of recombinant antigens. The PinPoint™ Xa-1 T-Vector (PV) system is a prokaryotic expression system that can be used to express protein antigens encoded by PCR products. It has previously been used to express a portion of the feline foamy virus *gag* protein for use in ELISA (Winkler *et al.*, 1997). A map of the PV is shown in Figure 4.2. It is 3331 bp in length and has 3'-deoxythymidine (T) overhangs to enable the efficient ligation of PCR products that have a single deoxyadenosine (A) on their 3' ends; some thermostable polymerases add a single deoxyadenosine (A) on 3' ends of PCR products in a nontemplate-dependent manner. The first 386 bp of the vector encodes a peptide that is naturally biotinylated by biotin ligase in *E. coli* (Cronan, 1990). Biotinylated fusion proteins are created by selecting primers such that the amplified genes are expressed in-frame with the biotinylation domain. Alternatively the multiple cloning region may be used. The vector encodes the cleavage sequence for Endoproteinase Factor Xa at the carboxyl terminus of the biotinylated region, providing a way to separate the purification tag from the native protein. The biotinylated proteins produced can be affinity purified using Softlink™ Soft Release Avidin Resin or directly on streptavidin-coated ELISA wells.

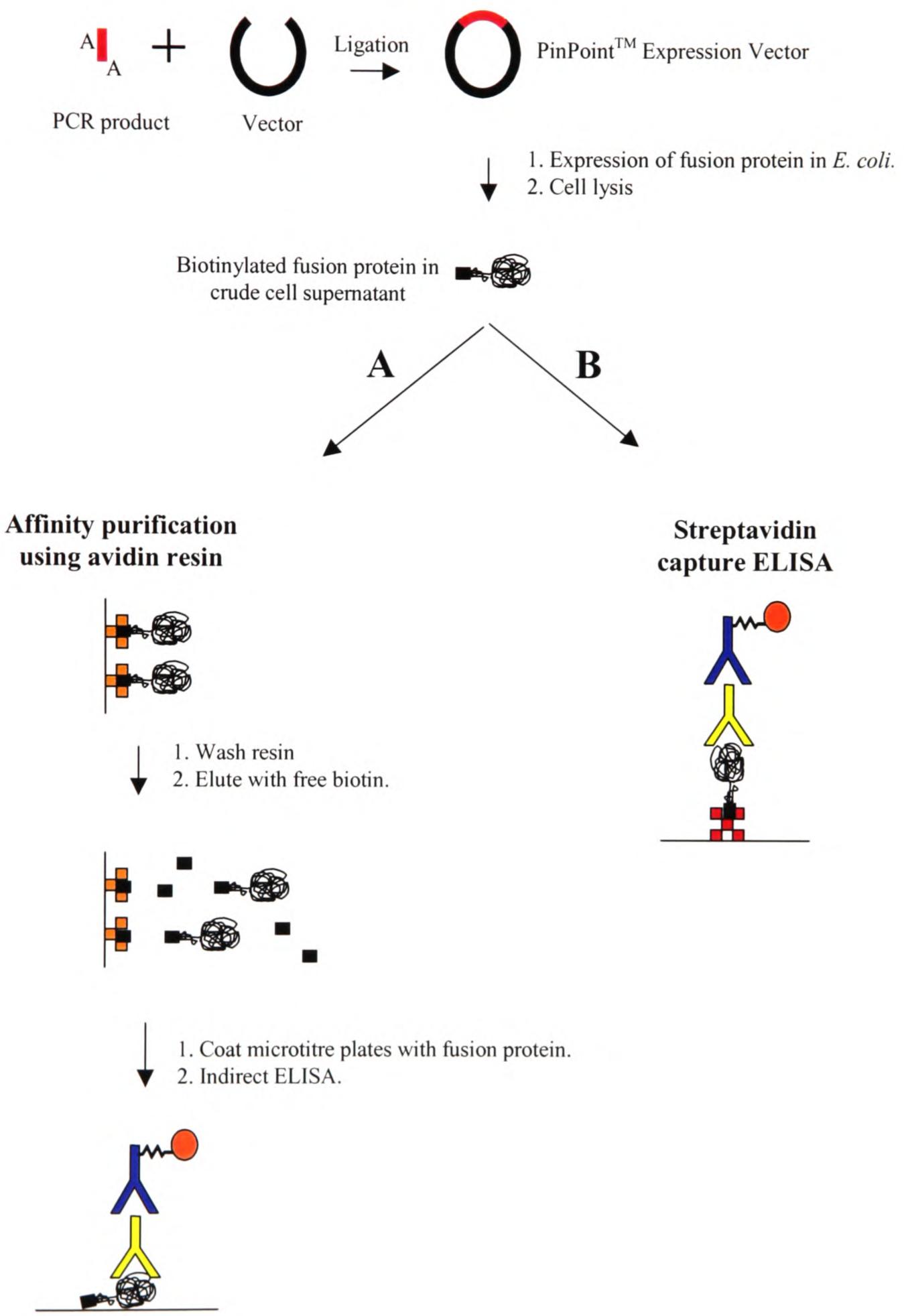
Figure 4.2 PinPoint Xa-1 T-Vector circle map.



Source: Anon, 1990.

In this study, the PV system was used to produce 10 overlapping recombinant biotinylated fusion proteins that span the RV E1 glycoprotein. The suitablility of the recombinant proteins as antigens to detect rubella-specific lgG in ELISA for assessment of immune status was then investigated. A schematic diagram of the procedure is shown in Figure 4.3.

Figure 4.3 Schematic diagram of procedure using the PinPoint™ Xa-1 T-Vector system to produce biotinylated fusion proteins for ELISA.



4.2 Material and Methods

4.2.1 RV strain

The RA27/3 vaccine strain of RV.

4.2.2 Monoclonal antibodies

RV haemagglutinin-specific murine MAb labelled with FITC (MAb-FITC) was kindly supplied by Bernard Cohen (ERNVL). A panel of 6 unlabelled and HRP-labelled MAbs to wild type RV specific for E1 (12F3, 8F9, and 2G10) and E2 (15C3, 14G1 and 11H67) were a gift from D. Dembrow (Chemicon International Inc.).

4.2.3 Sera

A panel of 92 serum samples submitted to ERNVL for routine RV antibody screening were analysed. Thirty-three samples had been previously tested by latex agglutination (Rubagen, Launch Diagnostics, Kent, UK) and 59 had been tested by a commercial ELISA (Behring Enzygnost) for the detection of RV-specific IgG.

4.2.4 Generation of RV E1 sequences for cloning and expression

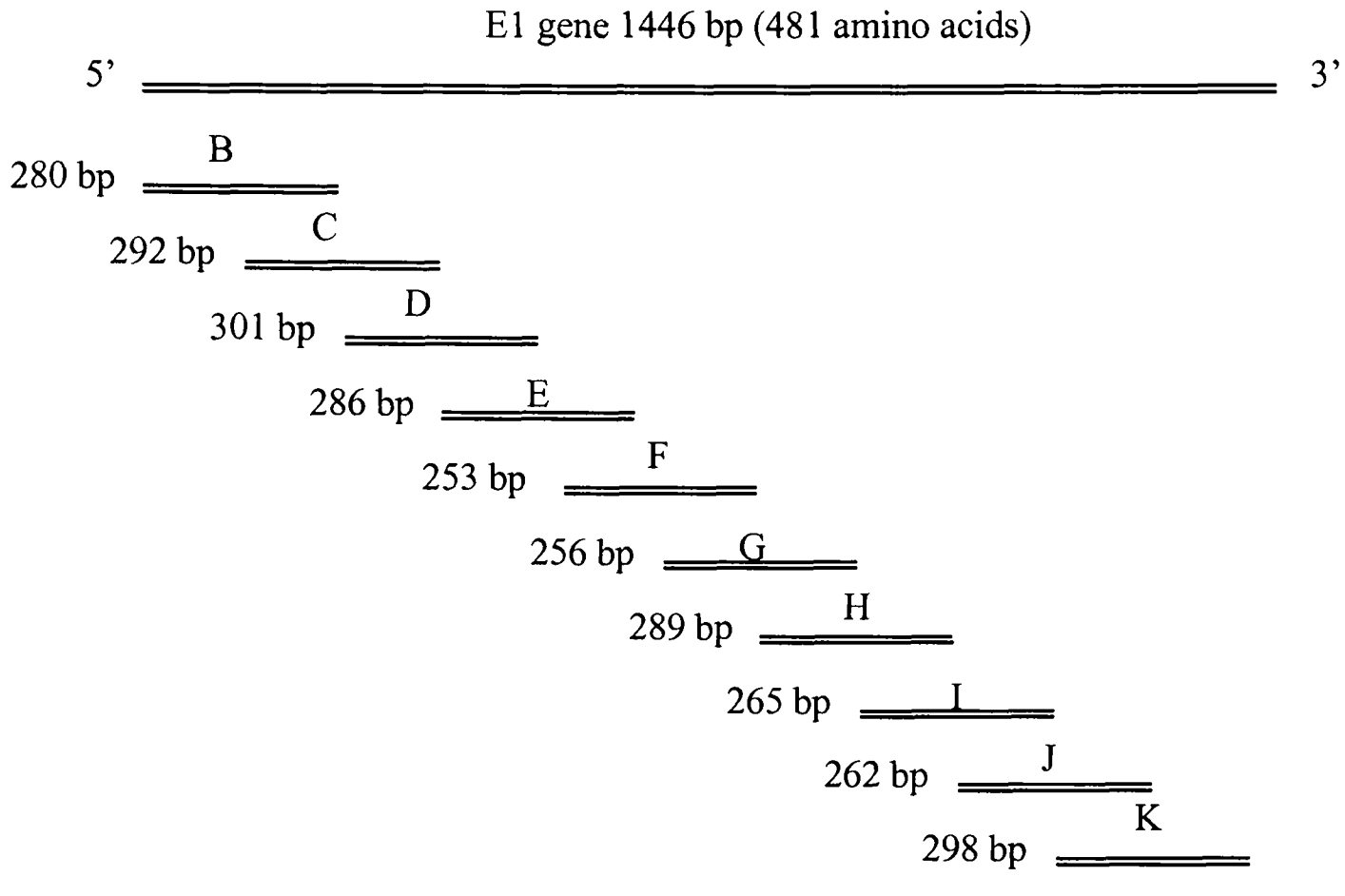
(i) Primers

Twenty primers (Table 4.1) were designed to amplify 10 overlapping cDNA fragments, designated B to K, spanning the whole of the RV E1 gene (Figure 4.4). The forward primers were designed so the E1 fragments were in the same reading frame as the PinPoint^{TM} Xa-1 T vector (Promega, Southampton, UK) sequence encoding the consensus biotinylation site. The reverse primers were designed with an artificial stop codon (cta) on the 5'end, with the exception of R10, which was designed to bind to a region downstream of the natural stop codon of the E1 gene. All primers were synthesised by MWG-Biotech.

Table 4.1 Primers

Forward Primer	Sequence 5'-3'	bp	Reverse Primer	Sequence 5' – 3'	bp
F1	cgaggaggctttcaccta	18	**R1**	ctaagaggagtaggcgttga	20
F2	cactggagcctgcattt	17	**R2**	ctacacgctcatcacggtgt	18
F3	caacgcctactcctctg	17	**R3**	ctagttgcagaacgggtgttc	21
F4	cgacaccgtgatgagc	16	**R4**	ctaatcggggccatggcaatt	21
F5	tgaacacccgttctgcaa	18	**R5**	ctaggggtcgtcggcgtc	18
F6	gaattgccatggccccgat	19	**R6**	ctagatccactcgggcattt	20
F7	cgacgccgacgacccc	16	**R7**	ctaaccgcactggtagcac	19
F8	cgaaatgcccgagtggat	18	**R8**	ctaggcggtgacgaacttc	19
F9	gtgctaccagtgcggta	17	**R9**	ctactccgacacagcagtg	19
F10	gaagttcgtcaccgccg	17	**R10**	tgggctagtgcgggtt	16

Figure 4.4 Schematic diagram of 10 overlapping cDNA fragments B to K spanning the RV E1 gene.



(ii) RNA extraction, reverse transcription and hemi-nested PCR

RNA was extracted from a vial of MMR vaccine (Merck, Sharpe and Dohme) containing the RA27/3 strain of RV using the Boom *et al.* (1990) method. For reverse transcription of RNA to cDNA, 40μl of extracted RNA was added to 60 μl of reaction mix containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 7.5 mM $MgCl_2$, 1.5 mM of each dNTP (Life Technologies), 25 ng of each random primer [$(pdN)_6$; Pharmacia], 1.6 U of Rnasin (Promega) and 200 U of Moloney murine leukaemia virus reverse transcriptase (Life Technologies). The reaction mix was incubated sequentially at RT for 10 min, 37°C for 45 min and 95°C for 5 min, and then quenched on ice.

RV E1 cDNA fragments were amplified by hemi-nested PCR using the primer sets shown in Table 4.2. PCR reactions were performed on the GeneAmp 9600 thermocycler (Perkin Elmer) in a final volume of 50 μl containing: Expand High Fidelity 1x reagent buffer with 1.5 mM $MgCl_2$ (Roche); 0.4 mM of each primer; 100 ng of cDNA, 1 M of Betaine (Sigma); 200 μM of each dNTP (Life Technologies), 2.5 U of Expand enzyme (Roche); and nuclease-free water (Promega). The thermal cycling conditions were an initial denaturation at 95°C for 5 min, followed by 30 amplification cycles of three steps: 95°C for 2 min; 55°C for 1 min; and 72°C for 2 min. About 1 μl of amplicon was re-amplified by hemi-nested PCR in the presence of the appropriate primers (Table 4.2) for another round of 30 cycles using the same thermal cycling conditions as the first round. Amplified PCR products were analysed on a 1% agarose gel. DNA fragments of the expected size were excised from the gel using a clean, sharp scalpel and purified using the QIAquick Gel Extraction Kit (Qiagen, Crawley, UK). Briefly, the excised gel slices were incubated with 3 gel volumes of Buffer QG (supplied with kit) for 10 min at 50°C, vortexing every 3 min, until the gel slices had dissolved. One gel volume of isopropanol was added to the samples and the mixtures were applied to QIAquick columns (supplied with kit). The columns were centrifuged for 1 min at 10,000 x g. The flow-throughs were discarded and the columns were washed with 0.5 ml of Buffer QG (supplied with kit), followed by 0.75 ml of Buffer PE (supplied with kit). Between washes the columns were centrifuged for 1 min at 10,000 g and the flow-throughs were discarded. The columns were placed into clean microfuge tubes and 50 μl of Buffer EB (supplied with kit) was added to the columns. The columns were incubated at RT for 1 min and then centrifuged for 1 min. Two microlitres of eluted material was analysed on a 1% agarose gel stained with ethidium bromide to estimate of the amount and purity of the DNA.

Table 4.2 Primer sets used to amplify the overlapping cDNA fragments B to K by hemi-nested PCR

E1 Fragment	1st round PCR	2nd round PCR	Expected Size (bp)
B	F1, R2	F1, R1	280
C	F1, R2	F2, R2	292
D	F3, R4	F3, R3	301
E	F3, R4	F4, R4	286
F	F5, R6	F5, R5	253
G	F5, R6	F6, R6	256
H	F7, R8	F7, R7	289
II	F7, R8	F8, R8	265
J	F8, R9	F9, R9	262
K	F9, R10	F10, R10	298

4.2.6 Ligation and transformation

Purified PCR fragments were ligated into the PV and ligation reactions were transformed into *E. coli* JM109 competent cells (Promega) according to the manufactures instructions with several modifications (Anon, 2001). Control DNA (a modified 368 bp fragment that encodes the *lacZ* α-peptide) supplied with the kit was used to verify the system components performed as expected and served as a control to allow estimation of background OD in ELISA. Ligation reactions were in a final volume of 10 μl containing: 1 x T4 Ligase Buffer, 50 ng of T-Vector, 1 - 3 Weiss units of T4 DNA, 50 ng of purified PCR product or PV control DNA, and nuclease-free water (Promega). Reactions were incubated overnight at 15°C. A 2 μl aliquot of each ligation reaction was gently mixed with 50 μl of thawed *E. coli* JM109 cells and left on ice for 30 min. The cells were heat shocked for 45 - 50 s in a water bath at 42°C and placed on ice for a further 2 min. Approximately 950 μl of SOC medium (Life Technologies) or LB (Luria-Bertani) broth was added to each tube and incubated for 1 h at 37°C with shaking at 200 rpm. The cells were centrifuged at 13,000 x g for 2 min, resuspended in 200 μl of SOC medium or LB broth and 100μl was spread over LB agar plates containing 100 μg/ml ampicillin (LB/amp). For control reactions LB/amp agar plates containing 0.5 mM of isopropyl-β-D-1-thiogalactopyranoside (IPTG; Promega) and 80 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-

galactopyranoside (X-Gal; Promega) were used. Clones with control vector in the correct orientation appeared blue. All plates were incubated overnight at 37°C.

4.2.7 Identification of recombinant clones

(i) PCR

PCR was used to determine the presence and orientation of the inserts within the recombinant clones. Colonies were inoculated into 100 μl of distilled H_20 and 1 μl was used as template for PCR using the forward primer specific for the fragment to be amplified and the reverse vector primer (gatttaggtgacactatag). PCR reactions were performed on the GeneAmp 9600 thermocycler (Perkin Elmer) in a final volume of 50 μl containing: 1 X Platinum® buffer (Life Technologies); 1 mM $MgCl_2$; 200 µM of each dNTP (Life Technologies); 0.4 units of Platinum® *Taq* DNA polymerase (Life Technologies); 0.5 µM of each primer; 1 μl of template; and nuclease-free water (Promega). Amplicons were run on a 1% agarose gel stained with ethidium bromide.

(ii) Sequencing

The identity of the selected recombinant clones was confirmed by sequencing. Single colonies were inoculated into 5 ml of LB broth containing 100 μg/ml of ampicillin and grown overnight at 37°C with shaking at 200 rpm. Plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen). Briefly, 1.5 ml of overnight cultures were centrifuged at 2000 x g for 5 min. The supernatants were discarded and the pelleted cells were resuspended in 250 μl of Buffer P1 (supplied with kit). A volume of 250 μl of Buffer P2 (supplied with kit) was added to the cells and the tubes were gently mixed by inversion 4 - 6 times. A volume of 350 μl of Buffer N3 (supplied with kit) was then added to the tubes and mixed by inverting gently 4 - 6 times. The mixtures were centrifuged at 10,000 x g for 10 min and the supernatants were applied to QIAprep spin columns (supplied with kit) within 2 ml collection tubes. The tubes were centrifuged at 10, 000 x g for 1 min and the flow-throughs were discarded. The columns were washed with 0.5 ml of buffer PB (supplied with kit), followed by 0.75 ml of Buffer PE (supplied with kit). After each wash the column was centifuged at 10, 000 x g for 1 min. A second spin was performed after the second wash to remove residual wash buffer. The columns were placed into clean microfuge tubes and 50μl aliquots of Buffer EB (supplied with kit) were applied to the centre of the columns, which were subsequently incubated at RT for 1 min and centrifuged

at 10,000 x g for 1 min. Eluted material was analysed on a 1% agarose gel stained with ethidium bromide to estimate the amount and purity of the plasmid DNA. The sequencing mix contained: 8 μl of CEQ^{TM} Dye Terminator Cycle Sequencing (DTCS) quick start mix (Beckman-Coulter, High Wycombe, UK), 3 μl of 3.2 pmol primer, 100 ng of PCR product which was made up to 20 μl with distilled water. The sequencing reaction was ampilfied on the GeneAmp 9600 thermocycler using the thermal cycling conditions: 95°C for 1 min; 55°C for 1 min; 60°C for 4 min. Amplicons were transferred to 1.5 ml microfuge tubes and the reactions were stopped by the addition of 5 μl of stop solution (Appendix A). Sixty microlitres of 95% cold ethanol was added to each tube and mixed. The tubes were centrifuged at 13,000 x g for 15 min at 4°C and the supernatants were removed. The pellets were washed twice with 200 μl of 70% cold ethanol (tubes were centrifuged at 13,000 x g for 2 min between washes). The pellets were dried in a Speedyvac for 15 min and then resuspended in 35 μl SLS loading buffer (Beckman Coulter). Reactions were loaded into wells of a 96-well sequencing plate and covered with a drop of mineral oil. Plates were placed into the CEQ 2000XL DNA analysis automated capillary sequencer (Beckman Coulter) for sequencing. The sequencing programme used was DCTS3, which allows long reads of up to 800 nucleotides. The sequences were compared with those known for the PV and the RV E1 (Pugachev *et al.*, 1997) using the software package Lasergene (DNAstar, Inc., Madison, WI, USA).

4.2.8 Production of recombinant biotinylated fusion proteins

Procedures for the expression of recombinant proteins were as described in the PinPoint technical manual (Anon, 2000). Briefly, *E. coli* carrying no vector (mock-infected), the PV *lacZ* α-peptide expression fusion (control) and the PV E1 expression fusions (pPVE1B to pPVE1K) were inoculated into 100 ml of LB broth containing 100 μg/ml ampicillin, and incubated overnight at 37°C with shaking. Ampicillin was not added to mock-infected cultures. Cultures were then diluted 1/100 with fresh LB broth containing 100 μg/ml ampicillin (except mock-infected cultures) and 2 μM D-Biotin (Sigma). After 1 h of culture at 37°C, the expression of the fusion proteins was induced by the addition of 1 mM IPTG and the cultures were further incubated for 4 - 5 hours at 37°C with shaking. Bacteria were pelleted by centifugation at 3000 x g for 10 min at 4°C and gently resuspended in 10 ml of cell lysis buffer (Appendix A). Cells were lysed by sonication for 2 min and cellular debris was pelleted by centrifugation at 10,000 x g for 15 min at 4°C. The supernatants (extracts) were removed and either stored at –70°C until testing or affinity purified.

4.2.9 Affinity purification of fusion proteins

Biotinylated fusion proteins were affinity purified either directly on streptavidin-coated microtitre wells (see section 4.2.13) or using Softlink™ Soft Release avidin resin (Promega). Prior to using the avidin resin the nonreversible binding sites were preabsorbed by incubating the resin with two-resin bed volumes of cell lysis buffer containing 5 mM biotin for 15 min at RT. After the incubation the resin was regenerated by washing with eight resin bed volumes of 10% acetic acid, followed by eight resin bed volumes of 100mM phosphate buffer (Appendix A). The resin was equilibritaed with cell lysis buffer and then incubated with the extracts containing the recombinant fusion proteins (3 ml of resin/L starting culture). The mixtures were incubated for 2 h at 4°C on a circular rotator. The resin was allowed to settle and unbound material was removed and discarded. The resin was washed with 10 volumes of buffer per ml of resin for 10 min at 4°C on a circular rotator. The wash step was repeated twice. The resin was centrifuged at 500 x g at 4°C for 5 min between washes to sediment it. The purified protein was eluted by incubating the resin with cell lysis buffer containing 5 mM biotin for 1 h at 4°C on a circular rotator. The resin was sedimented by centrifugation as described before and the supernatant containing the fusion protein was transferred to a clean tube and stored at –70°C until testing.

4.2.10 Immunoblotting

Extracts containing the recombinant proteins or avidin-purified recombinant proteins were separated by SDS-PAGE and blotted onto Hybond C nitocellulose (Amersham, Little Chalfont, U.K.) using the Minigel apparatus as described in section 3.2.12. Membranes were blocked with 1% skimmed milk powder in PBS for 1 h at 37°C and then incubated with either 7 ng/ml of alkaline phosphatase-labelled streptavidin (Strep-ALP; Promega) in PBST for 30 min at RT with gentle agitation or 10 μg/ml of RV specific HRP-labelled MAb in diluent D (Appendix A) for 30 min at RT with gentle agitation. After washing three times with PBST for 5 min each, bound strep-ALP was detected by incubation with Western Blue Stabilised Substrate (Promega) at RT with gentle agitation until purple bands appeared. RV-specific MAb-HRP was detected using electrochemiluminence (ECL; Amersham Pharmacia Biotech).

4.2.11 Detection of recombinant proteins using HRP-labelled RV-specific MAbs by ELISA

Microtitre plate wells (Nunc MaxiSorp^{TM}) were coated with extract or avidin-purified recombinant proteins diluted 1/100 and 1/5 respectively in PBS azide overnight at 4°C. The wells were washed three times with PBS and then blocked with 5% SoluPro for 2 h at 37°C. After washing, the wells were incubated with either 100 µl of 5 µg/ml RV-specific MAb-HRP or 1/2000 dilution of MAb-FITC in diluent B (Appendix A) for 30 min at 37°C. The wells were then washed and those that received MAb-FITC were further incubated with 100 µl of 1/2000 dilution of anti-FITC-HRP in diluent B for 30 min at 37°C and then washed. All wells were incubated with 100 µl of TMB and the enzyme reaction was stopped after 15 min with 100 µl of 0.5 M HCl and the absorbance was read at 450 nm (620 nm reference).

4.2.12 Competitive Binding Assays

Initially a 'checkerbox' titration of immobilised recombinant protein and HRP–labelled RV-specific MAb was performed to determine the concentrations required to produce an OD of approximately 1 - 1.5 for the competition assays. Dilutions of the recombinant proteins were immobilised on microtitre plates as described above (section 4.2.11). After the blocking step, the wells were incubated with dilutions of HRP-labelled RV-specific MAb in diluent B for 30 min at 37°C. After washing, the wells were incubated with 100 µl of TMB. The enzyme reaction was stopped after 15 min with 100 µl of 0.5 M HCl and the absorbance was read at 450 nm (620 nm reference). The dilutions of recombinant protein and MAb that gave an OD of 1 - 1.5 was recorded. Wells were then coated with the appropriate dilution of recombinant protein overnight at 4°C. Wells were washed, blocked and then incubated with 100 µl of 5 µg/ml unlabelled RV-specific MAb in diluent B for 1 h at 37°C. Control wells were incubated with diluent B only. After washing, wells were incubated with the appropriate HRP-labelled RV-specific MAb in diluent B for 30 min at 37°C. Bound enzyme conjugate was detected with TMB as described above. The percentage inhibition was calculated as follows: Percentage binding of HRP-labelled MAb in the presence of unlabelled (competitive) antibody = (OD of labelled MAb in the presence of competitive MAb/OD of HRP-labelled MAb in the absence of competitive MAb x 100. To determine the percent inhibition this percentage was subtracted from 100%.

4.2.13 Streptavidin capture ELISA for the detection of RV-specific IgG in serum

Streptavidin-coated wells (Labsystems) were blocked with 5% SoluPro at 37°C for 2 h. The wells were then incubated with 100 μl of extract containing recombinant protein diluted 1/100 in diluent D (Appendix A). To remove unbound proteins, the wells were washed thoroughly with PBST. Test sera were diluted 1/200 in diluent D and 100 μl were incubated in the appropriate wells for 30 min at 37°C. After washing, wells were incubated with 100 μl of anti-human IgG-HRP (Dako) diluted 1/3000 in diluent D for 30 min at 37°C, followed by 100 μl of TMB. The enzyme reaction was stopped after 15 min at RT with 100 μl of 0.5 M HCl and the absorbance was read at 450 nm (620 nm reference). Negative control wells contained extracts from mock-infected and control cultures. ELISA results were recorded as positive if the OD of the wells with extracts containing E1(F) were at least two times greater than the OD of the wells with control extracts.

4.3 Results

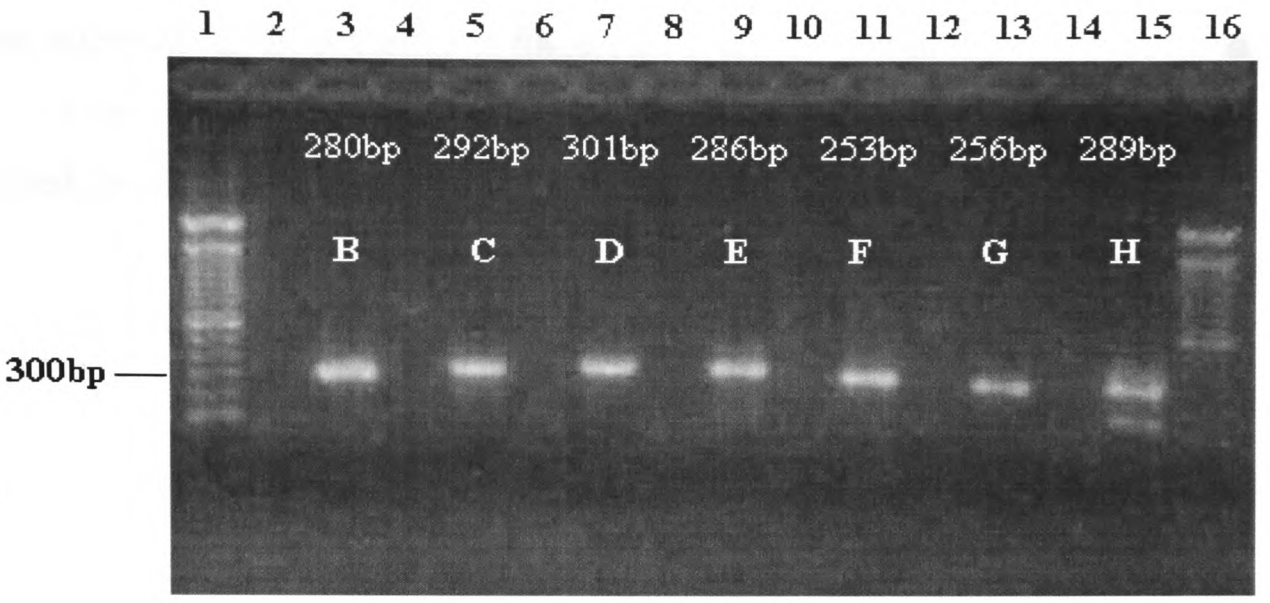
4.3.1 Generation of RV E1 sequences

A hemi-nested PCR approach was developed to amplify the RV E1 cDNA fragments, as in most cases single round amplification did not produce amplicons that could be visualised by gel electrophoresis and ethidium bromide staining. The primer sets used (Table 4.3) successfully generated PCR products of the expected size, ranging from 253 bp to 301 bp (Figure 4.5). In Figure 4.5b lanes 3 and 5, two bands were observed which represented the first and second round product of I and J respectively.

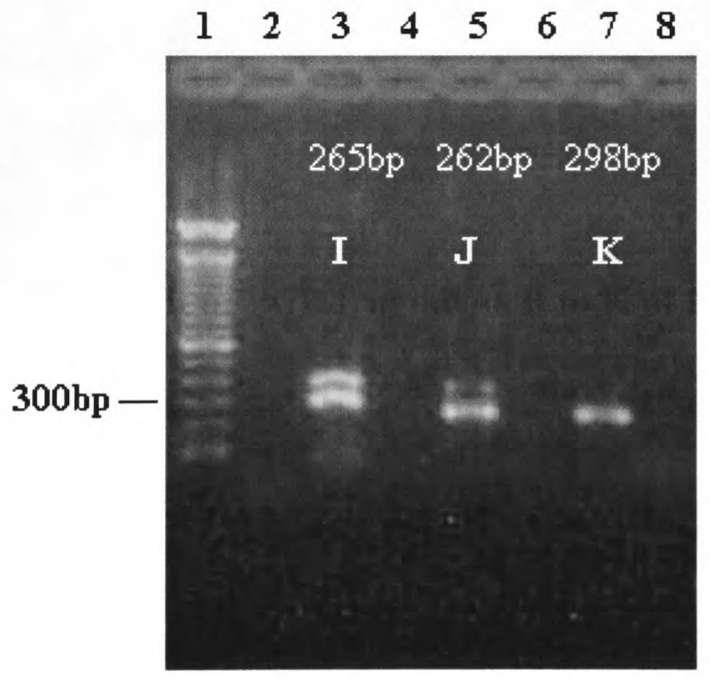
Initially, primer R10 was designed to bind to the last 16 bases of the E1 gene (tatagcgccgcgctag). However, no PCR product was generated when this primer was used, most likely due to primer-dimer formation. As a result a new primer was designed to bind to a sequence beyond the 3' end of the E1 gene. This did not effect the expression of fragment K as translation terminates at the natural E1 stop codon at the end of the gene.

Figure 4.5 Gel electrophoresis of PCR fragments B to K of E1 gene.

(a)



(b)

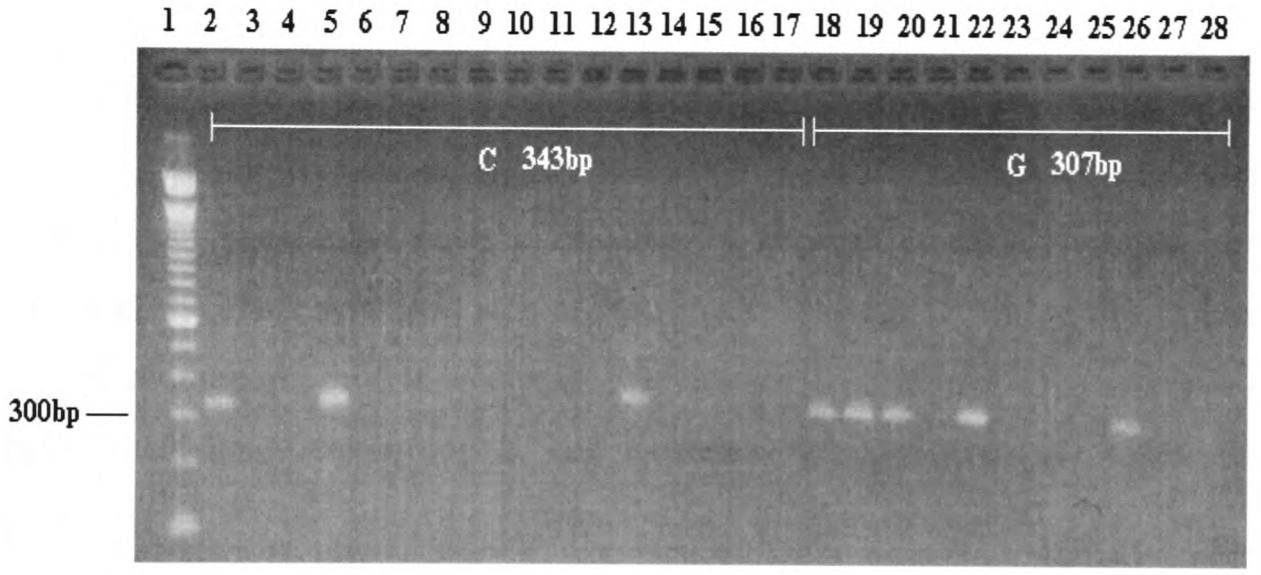


Amplified E1 cDNA fragments B to K were analysed on a 1% agarose gel stained with ethidium bromide. A 100 bp DNA molecular weight marker (Life Technologies) was run in lanes 1 and 16.

4.3.2 Ligation and transformation

To determine whether recombinant clones contained the E1 fragments in the proper orientation they were screened by PCR using the forward primer specific for the E1 fragment together with the reverse vector primer. Figure 4.6 shows the PCR results of clones screened for the presence of fragments C and G. Three out of 16 clones and five out of 11 clones contained fragments C and G in the correct orientation respectively. As expected the PCR products were 51 bp larger than those shown in Figure 4.5, lanes 5 and 13, as part of the vector was amplified; amplified fragments C and G were 343 bp and 307 bp respectively.

Figure 4.6 PCR to determine orientation of E1 fragments within the PinPoint Xa-1 T-Vector.



Clones were screened using PCR and the products were analysed on a 1% agarose gel stained with ethidium bromide. Lanes 2 to 17 contained amplified products from fragment C clones and lanes 18 to 28 contained amplified products from fragment G clones. A 100 bp DNA molecular weight marker was run in lane 1.

4.3.4 Sequencing

Sequencing was used to confirm that the E1 fragments were in the correct orientation and reading frame. In addition, mutations introduced during PCR amplification were identified. The sequences were compared with the published sequence of the RA27/3 vaccine strain. Some of the fragments had point mutations which resulted in an amino acid change, however only those that were identical to the published strain (RubNorP, accession number L78917) were used for protein expression (Appendix C).

4.3.5 Production and expression of recombinant proteins

The RV E1 sequences were inserted in-frame into the PV to produce recombinant plasmids pPVE1B to pPVE1K. The plasmids directed the expression of the recombinant biotinylated fusion proteins E1(B) to E1(K). The amino acid sequence and estimated size of the recombinant proteins are shown in Table 4.3.

Immunoblotting with Strep-ALP of extracts from *E. coli* containing the recombinant plasmids (Figure 4.7) showed bands of 23 to 25 kDa representing the biotinylated fusion proteins, which were not present in extracts from mock-infected (lane 2) and control (lane 3) cultures. The control (lane 3) directed the expression of biotinylated *lacZ* α-peptide of M_r 27 kDa. There was evidence of proteolytic cleavage or premature termination of some of the recombinant proteins suggested by the presence of lower M_r species. The 22.5 kDa band in all lanes containing *E. coli* extract corresponded to the biotin carboxyl carrier protein (BCCP), the only biotinylated protein produced naturally in *E. coli*. The 13.5 kDa band that was detected in all extracts, except when cells were mock-infected, was the vector portion of the fusion protein.

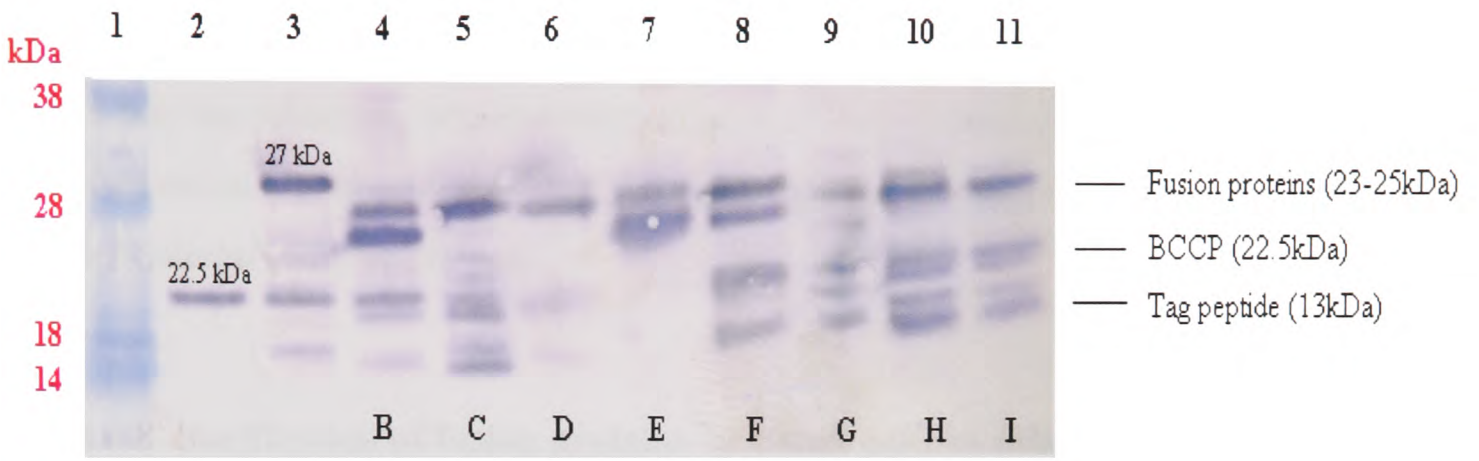
Table 4.3 Characteristics of fusion proteins E1(B) to E1(K)

Fusion protein	Number of Amino acids	Estimated M_r [a] (kDa)	Amino acid sequence
E1(B)	92	24	EEAFTYLCTAPGCATQAPVPVRLAGVRFESKIVDGGCFAPWDLEATGACICEIPTDVSCEGLGAWVP TAPCARIWNGTQRACTFWAVNAYSS.
E1(C)	96	24.5	TGACICEIPTDVSCEGLGAWVPTAPCARIWNGTQRACTFWAVNAYSSGGYAQLASYFNPGGSYYK QYHPTACEVEPAFGHSDAACWGFPTDTVMSV.
E1(D)	99	25	NAYSSGGYAQLASYFNPGGSYYKQYHPTACEVEPAFGHSDAACWGFPTDTVMSVFALASYVQHP HKTVRVKFHTETRTVWQLSVAGVSCNVTTEHPFCN.
E1(E)	94	24	DTVMSVFALASYVQHPHKTVRVKFHTETRTVWQLSVAGVSCNVTTEHPFCNTPHGQLEVQVPPDP GDLVEYIMNHTGNQQSRWGLGSPNCHGPD.
E1(F)	83	23	EHPFCNTPHGQLEVQVPPDPGDLVEYIMNHTGNQQSRWGLGSPNCHGPDWASPVCQRHSPDCSRL VGATPERPRLRLVDADDP.
E1(G)	84	23	NCHGPDWASPVCQRHSPDCSRLVGATPERPRLRLVDADDPLLRTAPGPGEVWVTPVIGSQARKCGL HIRAGPYGHATVEMPEWI.
E1(H)	96	24.5	DADDPLLRTAPGPGEVWVTPVIGSQARKCGLHIRAGPYGHATVEMPEWIHAHTTSDPWHPPGPLGL KFKTVRPVALPRALAPPRNVRVTGCYQCG.
E1(I)	88	23.5	EMPEWIHAHTTSDPWHPPGPLGLKFKTVRPVALPRALAPPRNVRVTGCYQCGTPALVEGLAPGGG NCHLTVNGEDVGAFPPGKFVTA.
E1(J)	86	23.5	CYQCGTPALVEGLAPGGGNCHLTVNGEDVGAFPPGKFVTAALLNTPPPYQVSCGGESDRASARVID PAAQSFTGVVYGTHTTAVSE.
E1(K)	98	25	KFVTAALLNTPPPYQVSCGGESDRASARVIDPAAQSFTGVVYGTHTTAVSETRQTWAEWAAAHW WQLTLGAICALLLAGLLACCAKCLYYLRGAIAPR.WAPARNPH.P

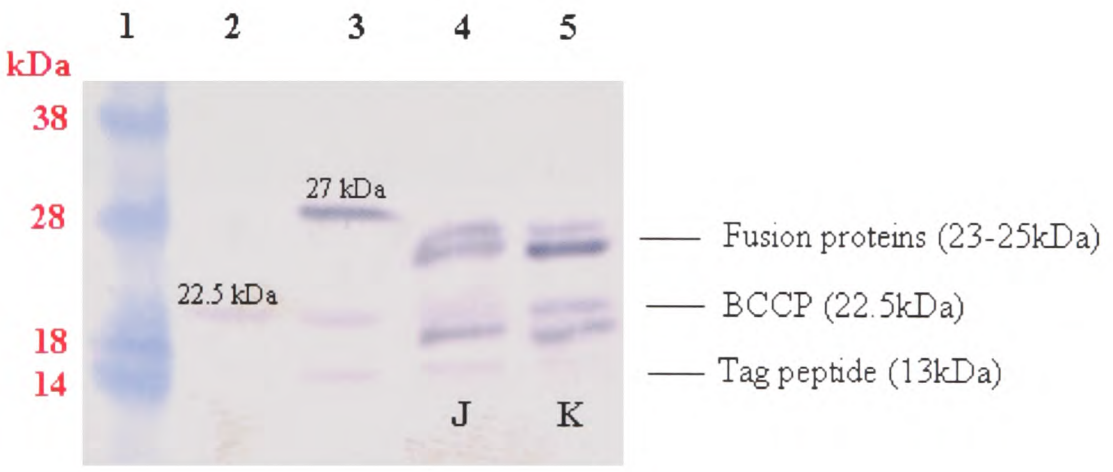
[a] The estimated molecular weight (M_r) was calculated as follows: number of amino acids x 120 kDa (average M_r of amino acid) + 13, 000 kDa (M_r of the fusion partner).

Figure 4.7 Detection of biotinylated fusion proteins E1(B) to E1(K) in extracts.

(a)



(b)

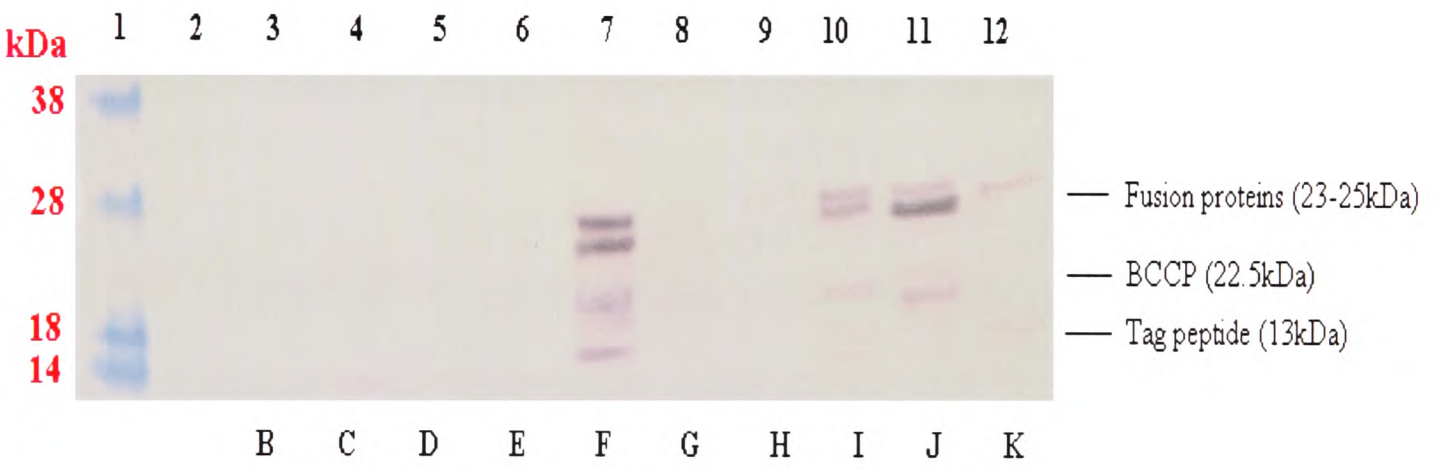


Western blot analysis with Strep-ALP of IPTG-induced *E. coli* JM109 cells producing the biotinylated fusion proteins: (a) lane 4, E1(B); lane 5, E1(C); lane 6, E1(D); lane 7, E1(E); lane 8, E1(F); lane 9, E1(G); lane 10, E1(H), lane 11, E1(I). (b) lane 4, E1(J); and lane 5, E1(K). In both Figure 4.7a and b, lane 1 contained SeeBlue™ Pre-stained Standard (Invitrogen), lane 2 contained extracts from mock-infected cells and lane 3 contained the *lacZ* α-peptide (control). The visible bands are the fusions proteins (23-25kDa), Biotin carboxyl carrier protein (BCCP; 22.5kDa) and the PinPoint™ tag peptide (13kDa).

4.3.6 Purification of recombinant proteins

The recombinant proteins and the controls were affinity purified by batch culture using Softlink™ Soft Release Avidin Resin. The material eluted from the affinity matrix by 5mM free biotin is shown in Figure 4.8. Good recovery of the fusion proteins E1(F), E1(I), E1(J) and E1(K) were observed. This suggests that these proteins were soluble and may indicate that the remaining proteins were largely locked in insoluble complexes. The yield of purified recombinant protein was in the range of 2 mg/L of culture medium, as measured spectrophotometrically at 280 nm.

Figure 4.8 Purification of fusion proteins by batch culture using Softlink™ Soft Release avidin resin.



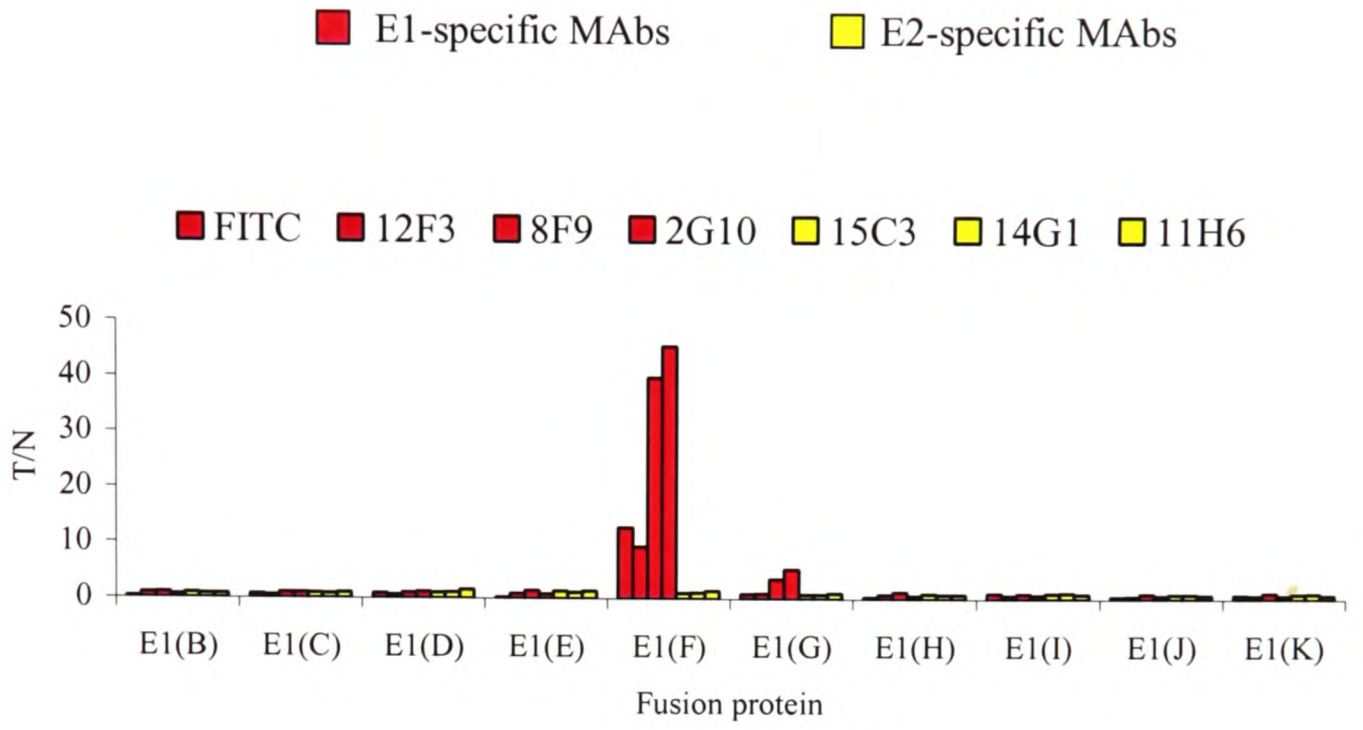
Western blot analysis with Strep-ALP of purified recombinant proteins: (a) lane 2, mock-infected; lane 3, E1(B); lane 4, E1(C); lane 5, E1(D); lane 6, E1(E); lane 7, E1(F); lane 8, E1(G); lane 9, E1(H), lane 10, E1(I). Lane 1 contained SeeBlue™ Pre-stained Standard (Invitrogen).

4.3.7 Characterisation of RV E1 recombinant proteins

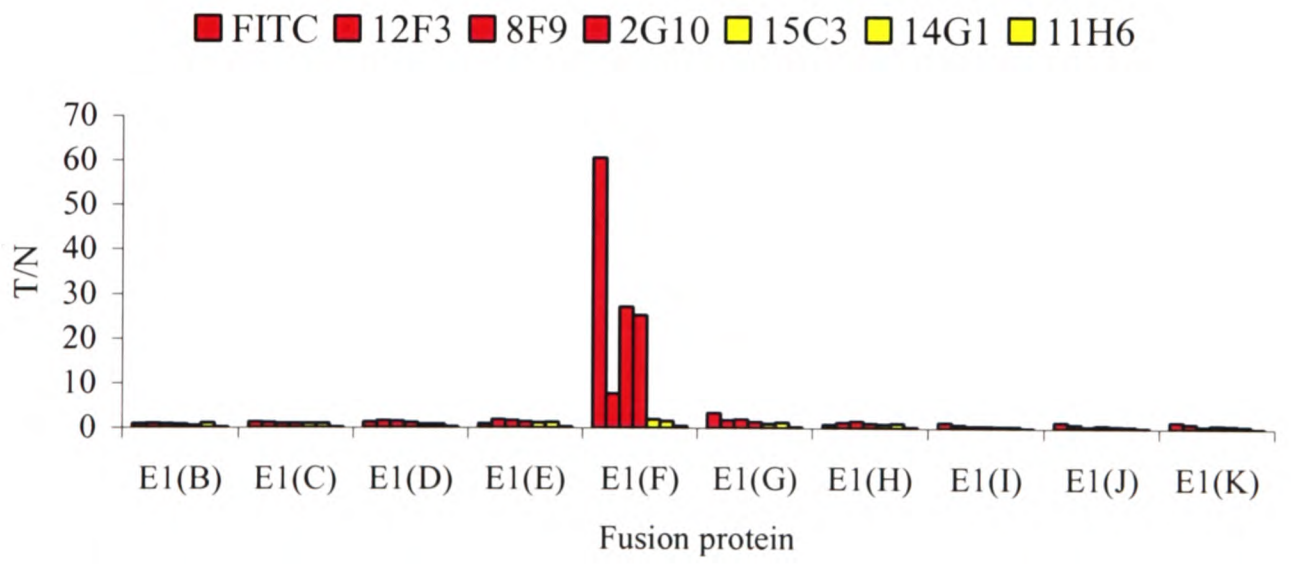
Immobilised extracts and avidin-purified proteins were reacted with MAb-FITC and a panel of HRP-labelled MAbs specific for either RV E1 or E2 glycoprotein to assess their immunoreactivity. As shown in Figures 4.9a and b, the four MAbs specific for E1 (FITC, 12F3, 8F9 and 2G10) reacted with E1(F). Several of the E1-specific MAbs (FITC, 8F9 and 2G10) also showed weak reactivity with E1(G). None of the proteins reacted with the E2-specific MAbs. Further analysis of E1(F) by Western blot with the E1-specific MAb 2G10 (Figure 4.10b) showed that it reacted with both the proteins represented by the two bands dentified by blotting with Strep-HRP (Figure 4.10a), suggesting the epitope for this MAb was present on both species.

Figure 4.9 Immunoreactivity of fusion proteins with RV-specific MAbs.

(a) immobilised extract diluted 1/100



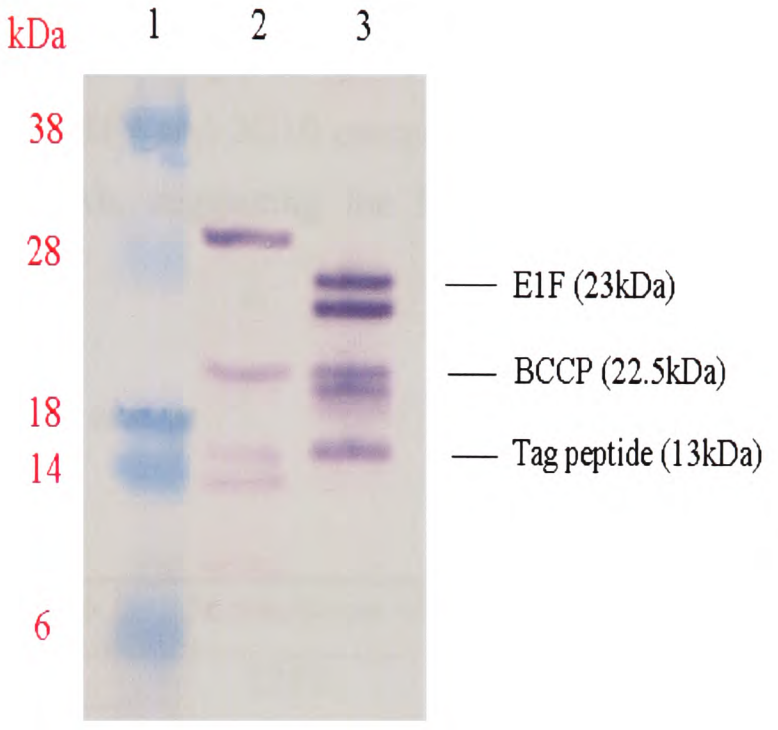
(b) immobilised avidin-purified protein diluted 1/5



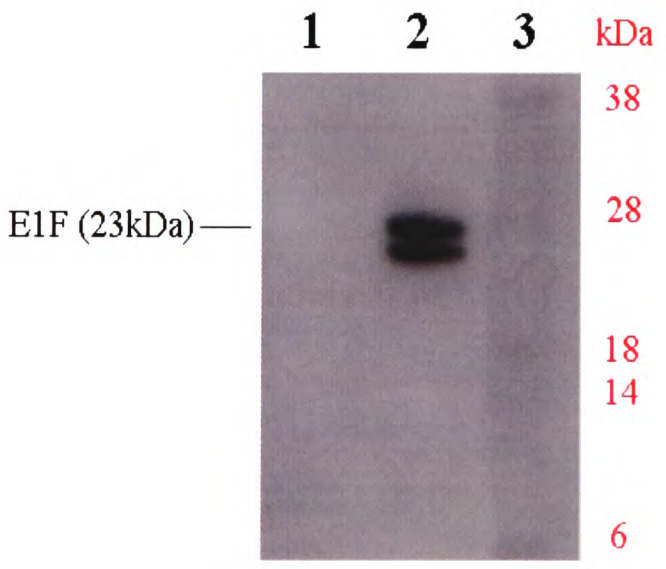
Microtitre plate wells coated with extract diluted 1/100 or avidin-purified protein diluted 1/5 were incubated with 5μg/ml of RV-specific HRP-labelled MAb or 1/200 dilution of RV-specific FITC-MAb for 30 min at 37°C. After washing, wells that had received FITC-MAb were incubated with anti-FITC-HRP (Microimmune Ltd.) diluted 1/2000 for 30 min at 37°C. Other ELISA conditions were as those described in section 4.3.7. T/N denotes OD in the presence of test recombinant protein divided by OD of the lac*Z* α-peptide control fusion.

Figure 4.10 Western blot analysis of E1(F).

(a)



(b)



Western blot analysis with (a) Strep-ALP and (b) RV E1-specific HRP-labelled MAb 2G10 of IPTG-induced *E. coli* JM109 cells producing the recombinant proteins: (a) lane 2, *lacZ* α-peptide; lane 3, E1(F); and (b) lane 1, *lacZ* α-peptide; lane 2, E1(F). Lanes 1 and 3 of (a) and (b) contain molecular weight marker respectively.

4.3.8 Identification of functional epitopes on the E1(F) protein by competitive binding assays

Competitive binding assays were used to determine whether MAb-FITC, 12F3, 8F9 and 2G10 recognised the same or different epitopes of the E1(F) fusion protein. As shown in Table 4.4, MAbs 12F3, 8F9 and 2G10 competed with one another, whereas they did not compete with FITC-MAb, suggesting the E1(F) fusion protein contains at least two antigenic epitopes.

Table 4.4 Competition assays

Labelled MAb	*% inhibition with unlabelled (competing) MAb*[a]		
	12F3	8F9	2G10
FITC	12	11	6
12F3	66	68	63
8F9	62	54	49
2G10	72	73	66

[a] Unlabelled MAb-FITC was not available.

4.3.9 Use of E1(F) as an antigen to detect RV-specific IgG antibodies in serum using ELISA

Initial experiments revealed false positive reactions with some of the test sera, independent of whether the recombinant proteins were avidin-purified or purified directly on streptavidin-coated ELISA plates. This was mainly due to reactions of serum antibodies with contaminating *E. coli* proteins, as the absorbance of the negative control wells was similar to that of the test wells.

Optimisation experiments revealed non-specific reactions were minimised when the commercial streptavidin-coated wells were pre-blocked with 5% SoluPro, and 1% skimmed milk was added to the assay diluent instead of BSA (data not shown).

A panel of serum samples submitted to the laboratory for rubella antibody screening were analysed using the streptavidin capture ELISA (Figure 4.3) and the results were compared with those obtained using alternative tests which employ native viral antigens (Rubagen latex agglutination [LA] or Behring ELISA). Twenty-four of the 32 serum samples positive by LA were positive by the streptavidin capture ELISA, while the other eight were negative (Table 4.5). One sample negative by LA was negative by streptavidin capture ELISA. Forty-two of the 51 serum samples positive by the Behring ELISA were positive by the streptavidin capture ELISA, while the other nine were negative. Five of the seven samples negative by the Behring ELISA were negative by the streptavidin capture ELISA, while the other two were positive. One serum equivocal by Behring was streptavidin capture ELISA positive. Overall seventeen serum samples gave false negative results and two samples gave false positive results in the Streptavidin capture ELISA, giving a sensitivity of 80% and specificity of 75%.

Table 4.5 Comparison of (a) latex agglutination and (b) Behring ELISA based on whole RV antigens with streptavidin capture ELISA using the recombinant antigen E1(F)

(a)

Latex agglutination	**Streptavidin capture ELISA**		
	Negative	Positive	Total
Negative	1	-	1
Positive	8	24	32
Total	9	24	33

(b)

Behring ELISA	**Streptavidin capture ELISA**		
	Negative	Positive	Total
Equivocal	-	1	1
Negative	5	2	7
Positive	9	42	51
Total	14	45	59

4.4 Discussion

For any immunoassay the selection of an appropriate antigen is important in the development of a sensitive and specfic test. Traditionally, cell culture-derived antigens have been used in ELISAs to detect RV-specific IgG. However, such antigens are difficult and expensive to produce, are potentially infectious and fail to define specific RV proteins and antigen determinants such as haemagglutination and viral neutralising epitopes. Recombinant antigens and synthetic peptides can mimic biologically relevant epitopes on proteins and have been increasingly used in immunoassays for the detection of specific viral antibodies.

In this study, the PV system was used to express RV E1 recombinant biotinylated fusion proteins. This is the first time it has been used to express RV proteins. The E1 glycoprotein was selected as a target as it bears the majority of the erythrocyte-binding and neutralisation sites and is thought to be the immunodominant antigen of the RV.

4.1 Expression of RV fusion proteins

The PV system was found to be a quick and effective way of expressing the RV PCR products. Primers were designed so that the correct reading frames for the expression of the fusion proteins were maintained between the purification tag and the PCR products, and also between the purification tag and stop codons. The E1 glycoprotein has a very high G/C content (69.5%) so selection of the primers was restricted. Despite this only one of the initial twenty primers designed failed to produce a PCR product. Recombinant clones containing PCR product that were not in-frame with the vector or incorrect coding sequences were easily detected by sequencing.

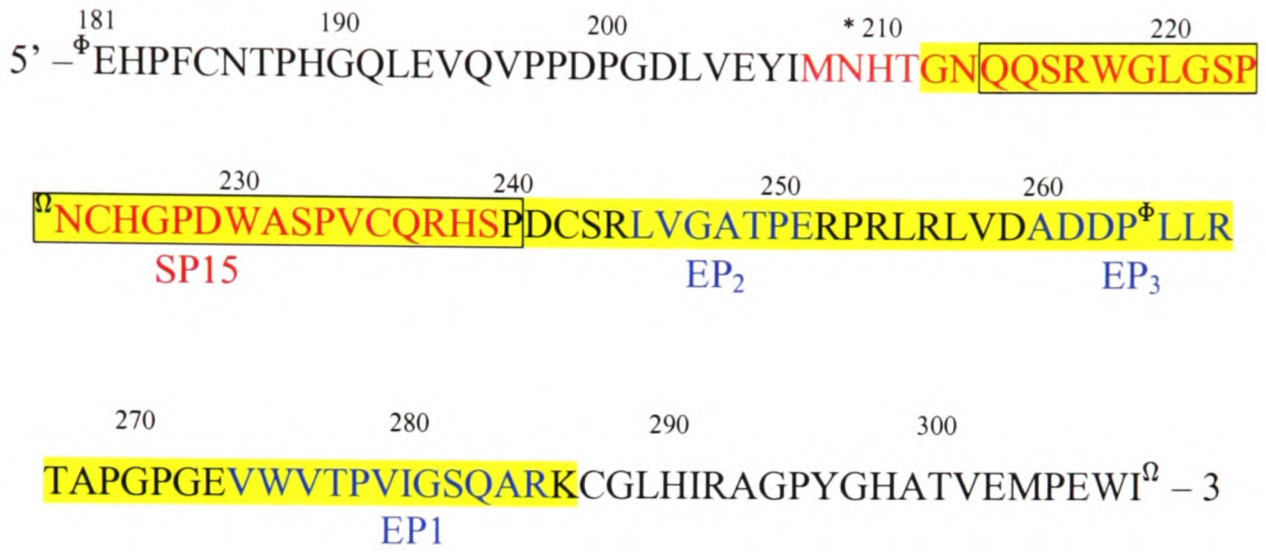
Ten overlapping fragments of E1 were expressed as biotinylated fusion proteins that contained between 83 and 99 amino acids. Despite all ten recombinant proteins being succesfully expressed, only four could be detected after affinity purification with avidin resin. One explanation for this was that the solubility of the recombinant proteins was variable. Starkey *et al.* (1995) expressed the entire E1 and truncated E1 proteins in *E. coli* as glutathione S-transferase (GST) fusion proteins and found the proteins could not be purified as soluble proteins by affinity chromatography on immobilised glutathione. They were able however to express smaller proteins of 44 and 75 amino acids in size which were soluble and suggested the larger proteins were present as inclusion bodies. Similarly, Londesborough (1992) expressed the E1 of the RV strain Judith using the *E. coli* expression vector LB03 and showed that the protein was found predominantly in inclusion

bodies. In the present study, procedures to solubilise the recombinant proteins, such as denaturation with 8 M urea, were not performed.

An alternative explanation for the lack of recovery of some of the recombinant proteins after affinity purification was that the proteins were not effectively eluted from the avidin resin. Evidence for this was obtained during repeat purifications of E1(F), where the recovery of the recombinant protein was inconsistent. To determine whether free biotin was successsfully eluting biotinylated protein from the avidin resin, 1 ml fractions of acetic acid (used for column regeneration) were applied to the resin and subsequently collected, coated onto ELISA plate wells and reacted with the E1-specific HRP-labelled MAb 2G10. The first two fractions collected from the column reacted very strongly with the MAb suggesting a proportion of the protein had not been eluted from the column using free biotin (data not shown). The avidin used for the resin was monomeric and binds biotin with a K_d value of 10^{-7} so should have allowed the reversible binding of the bound biotinylated proteins using mild elution conditions (as recommended by the manufacturers). This contrasts with native or tetrameric avidin, which binds biotin with a very strong affinity ($K_d = 10^{-15}$) and requires strong denaturing conditions to elute bound material. Although the reason(s) for the inconsistent recovery of the recombinant proteins was unclear, better recovery might have been achieved by using more stringent elution conditions.

MAbs specific for E1 recognised two of the ten fusion proteins. E1(F) was shown to react strongly, while E1(G) showed weak reactivity with the E1-specific MAbs. Since E1(F) contains a glycosylation site at residue 209 (Figure 4.11) the results suggest that glycosylation is not important for the antigens reactivity; proteins expressed in *E. coli* do not undergo post-translation modifications such as glycoslylation. This is consistent with others workers who have shown that the immunogenicity of E1 is independent of glycosylation (Ho-Terry and Cohen, 1984; Terry *et al.*, 1989). Competition experiments revealed E1(F) contained at least two independent antigenic epitopes. One of the epitopes represented the viral haemagglutinin as it was previously shown that MAb-FITC inhibits the haemagglutination of pigeon RBC's by RV (Tedder *et al.*, 1982). The other MAbs used in this study were only partly characterised and it is unknown whether they inhibit haemagglutination or neutralise RV infectivity. Further work is required to determine this.

Figure 4.11 Amino acid sequence of E1(F) and E1(G) overlapping fusion proteins (regions between the symbols $^{\Phi}$ ($E1_{181}$ to $E1_{263}$) and $^{\Omega}$ ($E1_{224}$ to $E1_{307}$) respectively).



The asterisk (*) marks a glycosylation site. The RV E1 epitope defined by Wolinsky *et al.* (1993) and Terry *et al.* (1986) and are shown in red and blue respectively. The boxed area contains the epitopes defined by Chaye *et al.* (1992). The yellow highlighted area represents the GST-fusion protein p1509 described by Starkey *et al.* (1995).

The results suggests the epitopes are located within the overlapping region of E1(F) and E1(G) (Figure 4.11). The observed weak recognition of E1(G) by the MAbs may have been due to solubility problems as mentioned earlier. Alternatively it may have been due to conformational differences resulting in inadequate presentation of the epitopes to the MAbs. E1(G) contains three of the four cysteines found in E1(F). Cysteines have the potential to form disulphide bonds and thus affect the way proteins fold. Wolinsky *et al.* (1993) found the replacement of two spaced cysteine residues at positions $E1_{225}$ and $E1_{235}$ with α-aminobutyric acid, a molecule homologous to cysteine but unable to form disulphide bonds, abrogated the binding of two neutralising MAbs (E1-18 and E1-20) to the synthetic peptide, SP15 ($E1_{208} - E1_{239}$). Other synthetic peptides that mimic antibody-defined neutralisation sites contain spaced cysteine residues that may ensure the stabilisation of the peptide and preserve critical conformations present in the native protein (LaRosa *et al.*, 1990; Manivel *et al.*, 1992).

An alternative explanation for the lack of reactivity of E1(G) with the MAbs is steric hindrance of the RV component of the fusion protein by the fusion partner. Removal of the biotinylated fusion partner by cleavage with Endoproteinase Factor Xa at the carboxyl terminus may have elucidated this.

The data presented here supports the findings of other investigators who have used synthetic or cleavage peptides to define antigenic determinants on E1. Ho-Terry *et al.* (1986) identified an antigenic domain of 40 amino acids between residues $E1_{245}$ to $E1_{285}$ of the E1 and further deduced three essential regions for MAb binding, EP_2 ($E1_{245}$ to $E1_{251}$), EP_3 ($E1_{260}$ to $E1_{266}$) and EP_1 ($E1_{273}$ to $E1_{285}$). MAbs recognising EP_1 and EP_2 showed HI and NT activity while MAbs recognising EP_3 showed only NT activity. All three epitopes were contained within E1(G) while only EP_2 and four residues of EP_3 were within E1(F) (Figure 4.11). Lozzi *et al.* (1990) showed however that the tetrapeptide ADDP (within E1F) were the critical residues of epitope EP_3.

Other workers have described a different region that also contains MAb-defined HI and NT epitopes of E1. Chaye *et al.* (1992a) using overlapping peptides and fusion proteins spanning 77 amino acids ($E1_{193}$ to $E1_{269}$) of E1 mapped a MAb-defined HA epitope to amino acid residues $E1_{214}$ to $E1_{240}$, and two MAb-defined NT epitopes to amino acid residues $E1_{214}$ to $E1_{233}$ and $E1_{219}$ to $E1_{233}$ respectively. Similarly, Wolinsky *et al.* (1993) used overlapping peptides and two NT MAbs to investigate 81 amino acids ($E1_{202}$ to $E1_{283}$) of E1. They found one of the peptides, SP15 ($E1_{208} - E1_{239}$), was an adequate ligand for their MAbs E1-18 and E1-20 in ELISA. They also deduced that the minimal amino-

terminal requirements for the MAbs binding were $E1_{221}$ and $E1_{223}$ respectively. All the epitopes defined by Chaye *et al.* (1992) and Wolinsky *et al.* (1993) were wholly contained within E1(F) but only partly within E1(G) (Figure 4.11). The residues of SP15 necessary for MAb recognition were not found in E1(G). Wolinsky *et al.* (1993) also showed that SP15 was immunogenic in rabbits and anti-rabbit-SP15 antibody had neutralising activity but lacked HI activity. However, the peptides SP7 ($E1_{244}$ to $E1_{266}$), which contained the epitopes EP_2 and EP_3, and SP13 ($E1_{262}$ to $E1_{283}$), which contained most of the residues of EP_3 and EP_1, were not immunogenic in mice. This suggests that the area between amino acids 245 and 285 may be less immunogenic than the neutralising domain.

Collectively the data suggests the epitopes are clustered in the mid-portion of E1. This area may be exposed and important in the early steps of the infectious process.

4.2 Use of E1(F) to detect RV-specific IgG in ELISA

The use of E1(F) as an antigen in ELISA was hindered by non-specific reactions (where a positive result was found in wells containing E1(F) and control antigen). This was mainly due to reactions of serum antibodies with contaminating *E. coli* proteins that co-purified with the fusion proteins. Previous workers have described false-positive reactions and problems arising from cross-reactivity to *E. coli* proteins when fusion proteins were used as antigens in serological assays (Zanoni *et al.*, 1991; Kwang *et al.*, 1992).

Non-specific reactions occurred independent of whether the fusion proteins were affinity purified using avidin resin or streptavidin-coated ELISA plates. The best results were obtained when extracts were directly applied to pre-blocked streptavidin-coated ELISA plates and milk was used as a blocking agent in the assay diluent. To avoid the misinterpretation of positve reactions with the E1(F) fusion protein the results were recorded as positive if the absorbance of the wells with E1(F) were at least twice the absorbance of control wells. Under these conditions a panel of 92 sera were tested, of which 80% reacted with E1(F). However this result was most likely an underestimate as some sera (3%) reacted strongly with the controls (OD > 1.0) which may have obscured specific reactions with E1(F). Also, in some cases higher OD values were observed in wells with extracts containing the *lacZ* α-peptide compared to wells with extracts from mock-infected cells, suggesting some sera reacted with either the biotinylated moeity and/or the *lacZ* α-peptide. A better control for the ELISA would have been the biotinylation moiety on its own rather than fused with the *lacZ*-α-peptide. As mentioned earlier the purification tag can be easily released by site-specific cleavage.

The failure of E1(F) to detect RV-specific antibodies in some sera may have been be due to conformational differences between the E1 moiety of E1(F) and of mature E1. It is unknown whether the epitopes of E1(F) are linear or conformational. MAb 2G10 reacted with E1(F) by Western blot analysis however one cannot conclude that the MAb was directed to the primary structure of E1(F) since partial renaturation including reformation of disulphide bonds may have occurred. As mentioned earlier, studies conducted by Wolinsky *et al.* (1993) suggested the neutralising domain of SP15 was conformational rather than linear. E1(F) contains 85 amino acids so one may expect the epitopes to be conformational, however whether they accurately mimic the three-dimensional structure of the mature E1 is unknown.

False-negative reactions may have also occurred because some individuals may develop antibody responses to other regions of E1 not present in E1(F). The neutralising epitope EP_1 defined by Terry *et al.* (1988) does not lie within E1(F). Also, Mitchell *et al.* (1993) found 15% of individuals reacted with the synthetic peptide $E1_{154}$ to $E1_{179}$. In this study, our MAbs did not react with E1(E), which contained amino acids 154 to 179 so this protein was not further investigated. It is possible, however, that some sera would have reacted with E1(E).

The performance of the ELISA described here was comparable to that of a previous study. Starkey *et al.* (1995) produced two GST fusion proteins containing 44 (p1503; $E1_{243}$ to $E1_{286}$) and 75 (p1509; $E1_{212}$ to $E1_{286}$) amino acid residues of E1 in *E. coli* and found they reacted with 76.8% and 85.7% of sera, respectively. p1503 contained the last 21 residues of E1(F) and the three neutralising and haemagglutinating epitopes defined by Terry *et al.* (1988) (Figure 4.11). p1509, like E1(F), additionally contained the putative neutralisation domain described by Wolinsky *et al.* (1993).

Synthetic peptides have also been successfully used as antigens in ELISAs to detect RV-specific IgG. Mitchell *et al.* (1992) investigated the immune responses of 44 females following RA27/3 vaccination using ELISAs employing whole RV and the synthetic peptide BCH-178 ($E1_{213}$ to $E1_{239}$), as well as HI and NT assays. They found the results of the synthetic peptide ELISA closely paralleled those obtained using the other assay methods. Similarly, Zrein *et al.* (1993) investigated the immune responses of 125 volunteers using ELISAs based on BCH-178 and whole-RV and found seroconversion rates were 96.8% and 95.2% respectively. More recently, Cordoba *et al.* (2000) compared the HI assay with an ELISA using the synthetic peptide SP15 for the determination of the immune status in 121 patients with remote natural infection. SP15 is nearly identical to

BCH-178C. They found the SPI5-ELISA was 100% specific and 98.2% sensitive compared to the HI assay. The sequences of SP15 and BCH-178 are wholly contained within E1(F), and within the fusion protein p1509 described by Starkey *et al.* (1995). The greater specificity and sensitivity observed with the SP15 ELISA may be associated with the synthetic peptide being a cleaner antigen compared with proteins expressed in *E. coli*.

The use of recombinant antigens in ELISAs has several advantages over conventional ELISA with whole RV antigens. The fusion proteins are relatively cheap to produce in large amounts and may be easily standardised. Also, the time frame for the production of recombinant protein is overnight instead of weeks for whole RV production. However, in this study reactions with *E. coli* proteins hindered the assessment of the recombinant proteins. Further or alternative purification steps are necessary to reduce the amount of non-specific reactions. The use of ion-exchange chromatography or high-pressure liquid chromatography might have been a better way of purifying the proteins. Alternatively, a non-*E. coli* expression system may be used to avoid cross reaction problems in human sera due to exposure to *E. coli* infections. Seppanen *et al.* (1991) have expressed a recombinant antigen consisting of the envelope polypeptides (E1 and E2) and a polyprotein precursor using a Baculovirus expression vector in insect cells and found the antigen was reactive with human sera by immunoblot analysis and the reactivity correlated well ($r = 0.861$) with that of whole virus antigen when tested by ELISA. The advantages of using Baculovirus expression are that the recombinant protein products appear to undergo normal posttranslational modifications and it is an "immunologically clean" system. Baculovirus and the insect cells used for their propagation have not been shown to cross react with human sera. However the method is more expensive and technically demanding.

In summary, ten RV fusion proteins, E1(B) to E1(K), were produced using the PV system. E1-specific MAbs reacted strongly with the E1(F) fusion protein, which was subsequently shown to contain at least two independent antigenic determinants. Preliminary experiments using E1(F) as an antigen to detect RV-specific antibodies were encouraging despite problems caused by contaminating *E. coli* proteins. Further purification of E1(F) is required before its suitablilty as an antigen can be adequately assessed. It is likely that the most appropriate recombinant antigen will be a mixture of the epitope-containing regions of RV or possibly a chimeric protein carrying these epitopes.

Chapter 5

Summary and Conclusions

The ability to identify individual and population susceptibility to infection is essential to the success of vaccination programmes. In this context the need for sensitive, convenient and robust assays for the determination of immune status is recognised. Preferably, specimens collected by cheap, simple, painless and non-invasive means should be applicable to the measurement of immune status, enabling the widest possible population sample. Studies have shown the relative ease and acceptibility of oral fluid compared with blood. Oral fluid samples are adequate for specific IgG determination for a range of viruses thanks to improved technologies that have increased the level of sensitivity of the assays.

In Chapter 2 of this thesis, the development and evaluation of a GACELISA for the detection of mumps-specific IgG in oral fluid was described. Highly purified and immunogenic reagents were employed and the assay was as sensitive and specific as a commercial ELISA for the detection of mumps-specific IgG in serum. A cut-off value for the assay, and the sensitivity and specificity associated with the cut-off selected was determined using two different approaches. Firstly, serum – oral fluid pairs were collected from volunteers and the serum samples were tested using a commercial ELISA to identify a negative (uninfected) population. The matching oral fluid samples were then tested by the GACELISA and the results from the negative population were used to define a cut-off value for the assay. This enabled the results to be classified as negative or positive and the performance charcteristics (i.e. sensitivity and specificity) of the assay to be determined (by comparing the results obtained using the commercial serum ELISA). This method has been widely used to evaluate newly developed oral fluid IgG assays. It is not, however, ideal as it requires the collection of blood samples. By contrast, the second approach of mixture modelling does not require an uninfected population to be identified prior to the analysis so blood collection is not necessary. Mixture models were fitted to the distribution of GACELISA results into seronegative and seropositive components and an optimal cut-off value and its sensitivity and specificity were estimated. The optimal cut-off value was very similar to that obtained using the conventional approach; however the sensitivity estimate associated with the selected cut-off was lower. This occurred because the results of the vaccinated population were widely distributed. This resulted in a large overlap with the negative population.

Defining a fixed cut-off is difficult when there is no clear separation between positive and negative results. For determining the immune status of individuals (i.e. for diagnostic purposes or pre-vaccination screening), samples need to be categorized as positive

(containing specific antibody) or negative (containing no specific antibody). However, for antibody prevalence studies it may be necessary only to determine accurately the proportion seropositive at each age. Mixture modelling has the advantage of allowing antibody prevalence to be derived from the distribution of results. Moreover, it has recently been used to improve the sensitivity of an oral fluid assay designed for antibody prevalence studies. Gay *et al.* (2003) demonstrated that when using a fixed cut-off, the sensitivity of a rubella IgG oral fluid assay relative to the serum assay decreased with increasing age of subject; 90% in those aged less than 10 years to 65% above 40 years. However, the prevalence estimates from an age-specific mixture model with three component distributions (negative, weak positive and strong positive) applied to the oral fluid data accurately reflected the results obtained using matching sera tested by a commercial assay.

The oral fluid GACELISA described in this study has potential application for assessing the immune status of individuals as a cut-off was defined, and for large-scale immunity surveillance studies.

In Chapter 3 of this thesis, the development and evaluation of a novel I-PCR assay for the detection of mumps-specific IgG was described. The approach was shown to be feasible for the detection of antibodies to mumps, but the sensitivity of the assay did not exceed that of a conventional ELISA due to high background signals. This was mainly due to non-specific binding of human IgG that could not be removed by blocking or washing the microtire plate wells; DNA-labelled antibodies with high specific activities can potentially improve assay sensitivity only if background signals can be limited. An alternative assay format might have served better to limit non-specific binding as in the indirect approach any non-specifically bound human IgG was detected by the anti-human IgG-DNA conjugate. Careful selection of the assay format is clearly important for the development of a sensitive and specific assay.

As well as the assay format, the quality of the antibodies used for the preparation of the signal-generating antibody conjugate and solid phase coating are important. Species-specific antibody preparations are commercially available from a variety of suppliers and selection of the best antibody should be done empirically. Further purification of these commercial reagents might be required to remove cross-reacting antibodies that may interfere with the assay. In the present study, cross-reacting antibodies were successfully removed from the commercial anti-human Ig preparation by affinity absorption on a mouse Ig column. By contrast, poor recovery of protein was observed after affinity purification on

a human IgG column. This was due to a failure to elute the antibodies with the highest affinity i.e. those critical for the assay.

A major advantage of the I-PCR approach described in this study was the linear amplification provided by real-time PCR. In previous assays based on conventional PCR, no direct correlation could be made between the amount of signal and the amount of antibody present in the sample. The LightCycler™ and the ABI PRISM® 7700 provided rapid and reproducible quantification of tDNA. The ABI PRISM® is a microplate-based system which renders the I-PCR technique compatible with established microplate ELISA in terms of high-throughput capabilities. This is important for immunity studies when large numbers of samples are being processed. The LightCycler™ is quick but additional steps are needed to transfer eluted material from microplates to the PCR capillaries, making the procedure more time consuming than the ABI PRISM® for high volume applications.

For I-PCR to be used routinely for widescale immunity surveillance studies a highly standardised assay is required that is sensitive enough to detect antibodies in oral fluid. Looking to the future, advances in conjugation chemistry, purification procedures and DNA label design should enable the development of better I-PCR assays. Microtitre plates that are suitable for antigen coating, real-time PCR and automated washing will simplify and advance the application of I-PCR.

Those immunoassays that use cell culture derived antigen and detect a variety of antibody specificities generally correlate well with neutralisation assays; however, a test that specifically detects protective antibodies (i.e. virus neutralising antibodies) would be preferable. Genetic manipulation offers the potential to produce antigens for antibody detection that have epitopes of defined antibody specificities (i.e. that might react with neutralising antibodies). Expression in *E. coli* is the cheapest and quickest way of producing recombinant antigens. In Chapter 4 of this thesis, 10 overlapping RV E1 biotinylated fusion proteins were produced in *E. coli*. One of the proteins (E1F) might be a suitable antigen for RV antibody detection as it reacted with the wild type RV E1-specific MAbs and a high percentage (80%) of the RV antibody positive sera tested. In addition, it spans a domain that others have shown to be important for viral infectivity and haemagglutination. Further work is required to assess fully the potential usefulness of E1F, and also E1G which was shown to react weakly with the E1-specific MAb's. This would involve removing the biotinylated tags using Factor Xa and further purifying the proteins i.e. by ion exchange chromatography to remove the contaminating *E. coli* proteins that were responsible for the false positive reactions. To improve the overall sensitivity of the

assay, the epitopes could be expressed as multimeric proteins, and/ or other RV epitope regions (i.e. in E2 and the capsid) could be expressed and incorporated in the antigen preparation.

Appendices

Appendix A - Buffers and solutions

PBS (pH 7.4)

8 g of NaCl

0.2 g of KCl

1.44 g of Na_2HPO_4

0.24 g of KH_2PO_4

Dissolve in 800 mL of distilled water. Adjust to pH 7.4 with HCl. Add distilled water to 1 L and autoclave.

PBST

Add 100 μl of Tween 20 to 1 L of PBS.

Transport medium

10 ml of foetal bovine serum (Life Technologies)

200 μl of Tween 20 (BDH)

200 μl of Gentamicin (Invitrogen)

200 μl of Fungizone (Invitrogen)

Add to 89.4 ml of PBS

0.1 M Sodium bicarbonate buffer (pH 8.3)

16.8 g of $NaHCO_3$

58 g of NaCl

Dissolve in 2 L of distilled water.

0.1 M Tris-HCl (pH 8.0)

12.1 g of Tris base

Dissolve in 800 mL of distilled water. Adjust to pH 8.0 with HCl. Add distilled water to 1 L and autoclave.

0.05 M Sodium carbonate buffer (pH 9.6)

21.2 g of Na_2CO_3 in 1 L of distilled water = solution A

16.8 g of $NaHCO_3$ in 1 L of distilled water =solution B

Add 16 ml of solution A to 34 ml of solution B. Add 150 ml of distilled water and 2 ml of 8% sodium azide. Adjust to pH 9.6 (using drops of 2N NaOH if required).

PBS azide

Add 5 ml of 8% sodium azide solution (Severn Biotech, Kidderminster, Worcester, UK) to 500 ml of PBS

0.1 M Glycine-HCl (pH 2.5)

0.75 g of glycine

Dissolve in 100 ml distilled water. Adjust to pH 2.5 using HCl.

Diluent A

10 ml of FCS

100 μl of Tween 20

Add to 100 ml of PBS

Diluent B

2 ml of BSA

100 μl of Tween 20

Add to 100 ml of PBS

Diluent C

2 g of skimmed milk powder (Marvel)

Dissolve in 100 ml of PBS. Add 10 ml of NGS and 100 μl of Tween 20.

Diluent D

2 g of skimmed milk powder (Marvel)

Dissolve in 100 ml of PBS. Add 100 μl of Tween 20.

0.5 M Dithiothreitol

0.5 g of DTT

Dissolve in 32 ml of distilled water.

Coomassie blue

0.25 g of Coomassie blue

Dissolve in 90 ml of methanol:distilled water (v/v) and add 10 ml of acetic acid.

Destain

45 ml of methanol

45 ml of distilled water

10 ml of acetic acid

Transfer buffer (pH 8.3)

1.5 g of Tris base

7.7 g of glycine

Dissolve in 400 ml of distilled water and add 100 ml methanol.

Blocking buffer

2 g of skimmed milk powder (Marvel)

Dissolve in 100 ml of PBS

Blocking solution

50 mg of 10 mg/ml Dextran sulphate, 10,000 MW

50 mg of 10 mg/ml Dextran sulphate, 100,000 MW

66 mg of 1,1-Diethylurea

Dissolve in 5 ml of 10 mM Citrate/EDTA buffer, pH 5.0

LB broth

10 g of tryptone

5 g of yeast extract

10 grams of NaCl

Dissolve in 1 L of distilled water. Autoclave.

LB agar plates

20 g of agar

10 g of tryptone

5 g of yeast extract

10 g of NaCl

Dissolve in 1 L of distilled water. Autoclave.

Stop solution

2 μl of 3 M NaOAc (Sigma)

2 μl of 100 mM EDTA (Sigma)

1 μl of glycogen (Beckman Coulter)

Cell lysis buffer (pH 7.5)

0.06 g of Tris

0.27 g of NaCl

0.5 ml of glycerol

One protease inhibitor cocktail tablet (Complete^{TM} Mini; Roche).

Dissolve in 10 ml of distilled water.

100 mM Phosphate buffer (pH 7.0)

3.12 g of $NaH_2PO_4 \cdot 2H_2O$ in 100 ml of distilled water = Solution A

7.17 g of $NaH_2PO_4 \cdot 12H_2O$ in 100 ml of distilled water = Solution B

Add 36.8 ml of Solution A and 13.3 ml of Solution B to 40 ml of distilled water. Adjust to pH 7.0 by adding Solution B.

Appendix B

Recruitment email

We are looking for volunteers to participate in a study to determine the performance characteristics of tests developed for the detection of antibodies to measles, mumps, rubella, hepatitis A and influenza in oral fluid.

Oral fluid has been increasingly used to test for viral antibodies for diagnostic or surveillance purposes as it is safe, cheap and easy to collect compared to blood. The only shortcoming of oral fluid is that while the antibody profiles reflect those in blood they are at lower concentrations. Hence before oral fluid tests can be routinely used they must be properly evaluated to determine whether they are reliable and will provide accurate data.

If you decide to participate in the study you will be asked to give one blood and six oral fluid samples. Blood will be collected by nursing or medical staff, whereas oral fluid will be self-collected using a sponge swab which should be used like a toothbrush.

The blood and oral fluid samples will be processed and tested for the presence the antibodies to the named viruses. The oral fluid test results will be compared with those obtained using a validated assay on serum separated from the blood samples. It will therefore be possible to assess the performance of the oral fluid assay compared to that of the validated serum test for the range of virus antibodies to be investigated.

Please note:

No other tests will be carried out on the samples.

Your decision to participate will not alter the relationship with your employer.

The study will be anonymised for patient confidential which means test results will not be available to you.

Please contact Anne M^{c}Kie (CPHL, Ext 3070, 3247) or Marianne Morris (CDSC, Ext 4439) if you would like to participate in the study.

Information sheet

Evaluation of oral fluid (saliva) assays for the detection of antibodies to measles, mumps, rubella, hepatitis A and influenza using paired serum-oral fluid samples.

You are being asked to take part in a research study. This information sheet explains why the research is being done and what it will involve. Please feel free to ask the researchers any questions. If you decide not to participate in this study please accept our apologies for taking up your time and please be assured that this decision will not alter the relationship with your employer.

Why is the study being done?

This is a study to determine the performance characteristics of tests developed to detect antibodies to measles, mumps, rubella, hepatitis A and influenza in oral fluid (saliva). The detection of antibodies to viral infections is important for diagnosing infection and determining immune status. Serum has traditionally been the specimen of choice for antibody detection as it contains high concentrations of antibodies. Blood collection however is invasive and expensive. It requires trained personnel and sterile equipment. Oral fluid by contrast is safe and cheap to collect and is more acceptable, especially for children and for population studies of immunity where large numbers of samples need to be easily and economically obtained. The only drawback of using oral fluid is that antibody levels are present in lower concentrations than in serum and special tests have to be devised and validated for the detection of low levels of antibody.

What does taking part in the study involve?

1. Giving a blood sample.
2. Self-collecting six oral fluid samples.

This is simple and painless. The paired samples will be tested for antibodies to measles, mumps, rubella, hepatitis A and influenza. **No other tests will be carried out on the samples.** The oral fluid test results will be compared with those obtained using a validated assay on serum separated from the blood samples. It will therefore be possible to assess the performance of the oral fluid assays compared to that of the validated serum tests for the range of virus antibodies to be investigated.

Will I benefit from taking part?

Participation in the study will not benefit you directly. However, results from the study will help assess current surveillance methods in place.

Is the study confidential?

Yes. No information will have your name on it so that you cannot be recognised from it. The results will be presented in a form that cannot identify you personally. As all data is being anonymised this also means the test results will not be available to you.

Who is organising and funding the study?

The study is being organised and funded by the Public Health Laboratory, which is the national body for monitoring infection.

THANK YOU VERY MUCH FOR YOUR HELP WITH THIS STUDY.

Consent Form

I have read and considered the information sheet about the study entitled "Evaluation of oral fluid (saliva) assays for the detection of antibodies to measles, mumps, rubella, hepatitis A and influenza using paired serum-oral fluid pairs".

I consent to give one blood sample and six oral fluid samples.

I understand that all information will be treated in the strictest confidence and the decision to participate will not alter the relationship with my employer.

I know that I may withdraw at any time

Signed: ..... Date:.....

Appendix C

Alignment of the overlapping E1 cloned sequences with the RA27/3 vaccine strain published sequence (RubNorP).

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                                        10        20        30        40
                                        |         |         |         |
                                        CGAGGAGGCTTTCACCTACCTCTGCACTGCACCGGGGTGCGCCACTCAA
B-stop.seq(1>277)                  →    CGAGGAGGCTTTCACCTACCTCTGCACTGCACCGGGGTGCGCCACTCAA
RubNorP E1.seq(1>1506)             →    cgaggaggctttcacctacctctgcactgcaccggggtgcgccactcaa

                                   50        60        70        80        90
                                   |         |         |         |         |
                                   GCACCTGTCCCCGTGCGCCTCGCTGGCGTCCGCTTTGAGTCCAAGATTG
B-stop.seq(1>277)                  →    GCACCTGTCCCCGTGCGCCTCGCTGGCGTCCGCTTTGAGTCCAAGATTG
RubNorP E1.seq(1>1506)             →    gcacctgtccccgtgcgcctcgctggcgtccgctttgagtccaagattg

                                  100       110       120       130       140
                                  |         |         |         |         |
                                  TGGACGGCGGCTGCTTTGCCCCATGGGACCTCGAGGCCACTGGAGCCTG
B-stop.seq(1>277)                  →    TGGACGGCGGCTGCTTTGCCCCATGGGACCTCGAGGCCACTGGAGCCTG
RubNorP E1.seq(1>1506)             →    tggacggcggctgctttgccccatgggacctcgaggccactggagcctg
C-stop.seq(1>289)                  →                                        CACTGGAGCCTG

                                  150       160       170       180       190
                                  |         |         |         |         |
                                  CATTTGCGAGATCCCCACTGATGTCTCGTGCGAGGGCTTGGGGGCCTGG
B-stop.seq(1>277)                  →    CATTTGCGAGATCCCCACTGATGTCTCGTGCGAGGGCTTGGGGGCCTGG
RubNorP E1.seq(1>1506)             →    catttgcgagatccccactgatgtctcgtgcgagggcttgggggcctgg
C-stop.seq(1>289)                  →    CATTTGCGAGATCCCCACTGATGTCTCGTGCGAGGGCTTGGGGGCCTGG

                                  200       210       220       230       240
                                  |         |         |         |         |
                                  GTACCCACAGCCCCTTGCGCGCGCATCTGGAATGGCACACAGCGCGCGT
B-stop.seq(1>277)                  →    GTACCCACAGCCCCTTGCGCGCGCATCTGGAATGGCACACAGCGCGCGT
RubNorP E1.seq(1>1506)             →    gtacccacagcccctttgcgcgcgcatctggaatggcacacagcgcgcgt
C-stop.seq(1>289)                  →    GTACCCACAGCCCCTTGCGCGCGCATCTGGAATGGCACACAGCGCGCGT

                                  250       260       270       280       290
                                  |         |         |         |         |
                                  GCACCTTCTGGGCTGTCAACGCCTACTCCTCTGGCGGGTACGCGCAGCT
B-stop.seq(1>277)                  →    GCACCTTCTGGGCTGTCAACGCCTACTCCTCT
RubNorP E1.seq(1>1506)             →    gcaccttctgggctgtcaacgcctactcctctggcgggtacgcgcagct
C-stop.seq(1>289)                  →    GCACCTTCTGGGCTGTCAACGCCTACTCCTCTGGCGGGTACGCGCAGCT
D-stop.seq(1>298)                  →                    CAACGCCTACTCCTCTGGCGGGTACGCGCAGCT

                                  300       310       320       330       340
                                  |         |         |         |         |
                                  GGCCTCTTACTTCAACCCTGGCGGCAGCTACTACAAGCAGTACCACCCT
RubNorP E1.seq(1>1506)             →    ggcctcttacttcaaccctggcggcagctactacaagcagtaccaccct
C-stop.seq(1>289)                  →    GGCCTCTTACTTCAACCCTGGCGGCAGCTACTACAAGCAGTACCACCCT
D-stop.seq(1>298)                  →    GGCCTCTTACTTCAACCCTGGCGGCAGCTACTACAAGCAGTACCACCCT

                                  350       360       370       380       390
                                  |         |         |         |         |
                                  ACCGCGTGCGAGGTTGAACCTGCCTTCGGACACAGCGACGCGGCCTGCT
RubNorP E1.seq(1>1506)             →    accgcgtgcgaggttgaacctgccttcggacacagcgacgcggcctgct
C-stop.seq(1>289)                  →    ACCGCGTGCGAGGTTGAACCTGCCTTCGGACACAGCGACGCGGCCTGCT
D-stop.seq(1>298)                  →    ACCGCGTGCGAGGTTGAACCTGCCTTCGGACACAGCGACGCGGCCTGCT
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                                    400        410        420        430        440
                                    GGGGCTTCCCCACCGACACCGTGATGAGCGTGTTCGCCCTTGCTAGCTA
RubNorP E1.seq(1>1506)      →       ggggcttccccaccgacaccgtgatgagcgtgttcgcccttgctagcta
C-stop.seq(1>289)           →       GGGGCTTCCCCACCGACACCGTGATGAGCGTG
D-stop.seq(1>298)           →       GGGGCTTCCCCACCGACACCGTGATGAGCGTGTTCGCCCTTGCTAGCTA
E-stop.seq(1>283)           →                   CGACACCGTGATGAGCGTGTTCGCCCTTGCTAGCTA

                                    450        460        470        480        490
                                    CGTCCAGCACCCTCACAAGACCGTCCGGGTCAAGTTCCATACAGAGACC
RubNorP E1.seq(1>1506)      →       cgtccagcaccctcacaagaccgtccgggtcaagttccatacagagacc
D-stop.seq(1>298)           →       CGTCCAGCACCCTCACAAGACCGTCCGGGTCAAGTTCCATACAGAGACC
E-stop.seq(1>283)           →       CGTCCAGCACCCTCACAAGACCGTCCGGGTCAAGTTCCATACAGAGACC

                                         500        510        520        530
                                         AGGACCGTCTGGCAACTCTCCGTTGCTGGCGTGTCGTGCAACGTCACCA
RubNorP E1.seq(1>1506)      →            aggaccgtctggcaactctccgttgctggcgtgtcgtgcaacgtcacca
D-stop.seq(1>298)           →            AGGACCGTCTGGCAACTCTCCGTTGCTGGCGTGTCGTGCAACGTCACCA
E-stop.seq(1>283)           →            AGGACCGTCTGGCAACTCTCCGTTGCTGGCGTGTCGTGCAACGTCACCA

                                    540        550        560        570        580
                                    CTGAACACCCGTTCTGCAACACGCCGCACGGACAACTCGAGGTCCAGGT
RubNorP E1.seq(1>1506)      →       ctgaacacccgttctgcaacacgccgcacggacaactcgaggtccaggt
D-stop.seq(1>298)           →       CTGAACACCCGTTCTGCAAC
E-stop.seq(1>283)           →       CTGAACACCCGTTCTGCAACACGCCGCACGGACAACTCGAGGTCCAGGT
F-stop.seq(1>250)           →        TGAACACCCGTTCTGCAACACGCCGCACGGACAACTCGAGGTCCAGGT

                                    590        600        610        620        630
                                    CCCGCCCGACCCCGGGGACCTGGTTGAGTACATTATGAACCACACCGGC
RubNorP E1.seq(1>1506)      →       cccgcccgaccccggggacctggttgagtacattatgaaccacaccggc
E-stop.seq(1>283)           →       CCCGCCCGACCCCGGGGACCTGGTTGAGTACATTATGAACCACACCGGC
F-stop.seq(1>250)           →       CCCGCCCGACCCCGGGGACCTGGTTGAGTACATTATGAACCACACCGGC

                                    640        650        660        670        680
                                    AATCAGCAGTCCCGGTGGGGCCTCGGGAGCCCGAATTGCCATGGCCCCG
RubNorP E1.seq(1>1506)      →       aatcagcagtcccggtggggcctcgggagcccgaattgccatggccccg
E-stop.seq(1>283)           →       AATCAGCAGTCCCGGTGGGGCCTCGGGAGCCCGAATTGCCATGGCCCCG
F-stop.seq(1>250)           →       AATCAGCAGTCCCGGTGGGGCCTCGGGAGCCCGAATTGCCATGGCCCCG
G-stop.seq(1>253)           →                                   GAATTGCCATGGCCCCG

                                    690        700        710        720        730
                                    ATTGGGCCTCCCCGGTTTGCCAACGCCATTCCCCTGACTGCTCGCGGCT
RubNorP E1.seq(1>1506)      →       attgggcctccccggtttgccaacgccattccccctgactgctcgcggct
E-stop.seq(1>283)           →       AT
F-stop.seq(1>250)           →       ATTGGGCCTCCCCGGTTTGCCAACGCCATTCCCCTGACTGCTCGCGGCT
G-stop.seq(1>253)           →       ATTGGGCCTCCCCGGTTTGCCAACGCCATTCCCCTGACTGCTCGCGGCT

                                    740        750        760        770        780
                                    TGTGGGGGCTACGCCAGAGCGTCCCCGGCTGCGCCTGGTCGACGCCGAC
RubNorP E1.seq(1>1506)      →       tgtgggggctacgccagagcgtccccggctgcgcctggtcgacgccgac
F-stop.seq(1>250)           →       TGTGGGGGCTACGCCAGAGCGTCCCCGGCTGCGCCTGGTCGACGCCGAC
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                                    740        750        760        770        780
                                    |          |          |          |          |
                                    TGTGGGGGCTACGCCAGAGCGTCCCCGGCTGCGCCTGGTCGACGCCGAC
G-stop.seq(1>253)             →     TGTGGGGGCTACGCCAGAGCGTCCCCGGCTGCGCCTGGTCGACGCCGAC
H-stop.seq(1>286)             →                                          CGACGCCGAC

                                    790        800        810        820        830
                                    |          |          |          |          |
                                    GACCCCCTGCTGCGCACTGCCCCTGGGCCCGGCGAGGTGTGGGTCACGC
RubNorP E1.seq(1>1506)        →     gacccccctgctgcgcactgccccctgggcccggcgaggtgtgggtcacgc
F-stop.seq(1>250)             →     GAcCCC
G-stop.seq(1>253)             →     GACCCCCTGCTGCGCACTGCCCCTGGGCCCGGCGAGGTGTGGGTCACGC
H-stop.seq(1>286)             →     GACCCCCTGCTGCGCACTGCCCCTGGGCCCGGCGAGGTGTGGGTCACGC

                                    840        850        860        870        880
                                    |          |          |          |          |
                                    CTGTCATAGGCTCTCAGGCGCGCAAGTGCGGACTCCACATACGCGCTGG
RubNorP E1.seq(1>1506)        →     ctgtcataggctctcaggcgcgcaagtgcggactccacatacgcgctgg
G-stop.seq(1>253)             →     CTGTCATAGGCTCTCAGGCGCGCAAGTGCGGACTCCACATACGCGCTGG
H-stop.seq(1>286)             →     CTGTCATAGGCTCTCAGGCGCGCAAGTGCGGACTCCACATACGCGCTGG

                                    890        900        910        920        930
                                    |          |          |          |          |
                                    ACCGTACGGCCATGCTACCGTCGAAATGCCCGAGTGGATCCACGCCCAC
RubNorP E1.seq(1>1506)        →     accgtacggccatgctaccgtcgaaatgcccgagtggatccacgcccac
G-stop.seq(1>253)             →     ACCGTACGGCCATGCTACCGTCGAAATGCCCGAGTGGATC
H-stop.seq(1>286)             →     ACCGTACGGCCATGCTACCGTCGAAATGCCCGAGTGGATCCACGCCCAC
I-stop.seq(1>262)             →                                CGAAATGCCCGAGTGGATCCACGCCCAC

                                    940        950        960        970        980
                                    |          |          |          |          |
                                    ACCACCAGCGACCCCTGGCACCCACCGGGCCCCTTGGGGCTGAAGTTCA
RubNorP E1.seq(1>1506)        →     accaccagcgaccccctggcacccaccgggcccccttggggctgaagttca
H-stop.seq(1>286)             →     ACCACCAGCGACCCCTGGCACCCACCGGGCCCCTTGGGGCTGAAGTTCA
I-stop.seq(1>262)             →     ACCACCAGCGACCCCTGGCACCCACCGGGCCCCTTGGGGCTGAAGTTCA

                                    990        1000       1010       1020
                                    |          |          |          |
                                    AGACAGTTCGCCCGGTGGCCCTGCCACGCGCGTTAGCGCCACCCCGCAA
RubNorP E1.seq(1>1506)        →     agacagttcgcccggtggccctgccacgcacgttagcgccaccccgcaa
H-stop.seq(1>286)             →     AGACAGTTCGCCCGGTGGCCCTGCCACGCGCGTTAGCGCCACCCCGCAA
I-stop.seq(1>262)             →     AGACAGTTCGCCCGGTGGCCCTGCCACGCGCGTTAGCGCCACCCCGCAA

                                    1030       1040       1050       1060       1070
                                    |          |          |          |          |
                                    TGTGCGTGTGACCGGGTGCTACCAGTGCGGTACCCCCGCGCTGGTGGAA
RubNorP E1.seq(1>1506)        →     tgtgcgtgtgaccgggtgctaccagtgcggtaccccgcgctggtggaa
H-stop.seq(1>286)             →     TGTGCGTGTGACCGGGTGCTACCAGTGCGGT
I-stop.seq(1>262)             →     TGTGCGTGTGACCGGGTGCTACCAGTGCGGTACCCCCGCGCTGGTGGAA
J-stop.seq(1>259)             →                    GTGCTACCAGTGCGGTACCCCCGCGCTGGTGGAA

                                    1080       1090       1100       1110       1120
                                    |          |          |          |          |
                                    GGCCTTGCCCCCGGGGGAGGGAATTGCCATCTCACCGTCAATGGCGAGG
RubNorP E1.seq(1>1506)        →     ggccttgccccgggggagggaattgccatctcaccgtcaatggcgagg
I-stop.seq(1>262)             →     GGCCTTGCCCCCGGGGGAGGGAATTGCCATCTCACCGTCAATGGCGAGG
J-stop.seq(1>259)             →     GGCCttgCCCCCGGGGGAGGGAATTGCCATCTCACCGTCAATGGCGAGG
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                                    1130       1140       1150       1160       1170
                                    ATGTCGGCGCCTTCCCCCCTGGGAAGTTCGTCACCGCCGCCCTCCTCAA

RubNorP E1.seq(1>1506)  ⟶  atctcggcgccttcccccctgggaagttcgtcaccgccgccctcctcaa
I-stop.seq(1>262)       ⟶  ATGTCGGCGCCTTCCCCCCTGGGAAGTTCGTCACCGCC
J-stop.seq(1>259)       ⟶  ATGTCGGCGCCTTCCCCCCTGGGAAGTTCGTCACCGCCGCCCTCCTCAA
K.seq(1>329)            ⟶                                GAAGTTCGTCACCGCCGCCCTCcTCAA

                                    1180       1190       1200       1210       1220
                                    CACCCCCCCGCCCTACCAAGTCAGCTGCGGGGGCGAGAGCGATCGCGCG

RubNorP E1.seq(1>1506)  ⟶  cacccccccgccctaccaagtcagctgcgggggcgagagcgatcgcgcg
J-stop.seq(1>259)       ⟶  CACCCCCCCGCCCTACCAAGTCAGCTGCGGGGGCGAGAGCGATCGCGCG
K.seq(1>329)            ⟶  CACCCCCCCGCCCTACCAAGTCAGCTGCGGGGGCGAGAGCGATCGCGCG

                                    1230       1240       1250       1260       1270
                                    AGCGCGCGGGTCATTGACCCCGCCGCGCAATCGTTTACCGGCGTGGTGT

RubNorP E1.seq(1>1506)  ⟶  agcgcgcgggtcattgaccccgccgcgcaatcgtttaccggcgtggtgt
J-stop.seq(1>259)       ⟶  AGCGCGCGGGTCATTGACCCCGCCGCGCAATCGTTTACCGGCGTGGTGT
K.seq(1>329)            ⟶  AGCGCGCGGGTCATTGACCCCGCCGCGCAATCGTTTACCGGCGTGGTGT

                                    1280       1290       1300       1310       1320
                                    ATGGCACACACACCACTGCTGTGTCGGAGACCCGGCAGACCTGGGCGGA

RubNorP E1.seq(1>1506)  ⟶  atggcacacacaccactgctgtgtcggagacccggcagacctgggcgga
J-stop.seq(1>259)       ⟶  ATGGCACACACACCACTGCTGTGTCGGAG
K.seq(1>329)            ⟶  ATGGCACACACACCACTGCTGTGTCGGAGACCCGGCAGACCTGGGCGGA

                                    1330       1340       1350       1360       1370
                                    GTGGGCTGCTGCCCATTGGTGGCAGCTCACTCTGGGCGCCATTTGCGCC

RubNorP E1.seq(1>1506)  ⟶  gtgggctgctgcccattggtggcagctcactctgggcgccatttgcgcc
K.seq(1>329)            ⟶  GTGGGCTGCTGCCCATTGGTGGCAGCTCACTCTGGGCGCCATTTGCGCC

                                    1380       1390       1400       1410       1420
                                    CTCCTACTCGCTGGCTTACTCGCTTGCTGTGCCAAATGCTTGTACTACT

RubNorP E1.seq(1>1506)  ⟶  ctcctactcgctggcttactcgcttgctgtgccaaatgcttgtactact
K.seq(1>329)            ⟶  CTCCTACTCGCTGGCTTACTCGCTTGCTGTGCCAAATGCTTGTACTACT

                                    1430       1440       1450       1460       1470
                                    TGCGCGGCGCTATAGCGCCGCGCTAGTGGGCCCCCGCGCGAAACCCGCA

RubNorP E1.seq(1>1506)  ⟶  tgcgcggcgctatagcgccgcgctag
K.seq(1>329)            ⟶  TGCGCGGCGCTATAGCGCCGCGCTAGTGGGCCCCCGCGCGAAACCCGCA

                                    1480
                                    CTAGCCCA

K.seq(1>329)            ⟶  CTAGCCCA
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Development of a quantitative immuno-PCR assay and its use to detect mumps-specific IgG in serum

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Abstract

Determination of the immune status of individuals to vaccine-preventable diseases requires an assay that can detect antibodies that may be present at very low levels, especially when natural or vaccine exposure may have been many years previously. Immuno-PCR (iPCR) has recently been described as an ultrasensitive method for the detection of antigens and we have adapted the method for the quantification of antibodies to mumps virus. The procedure used was similar to an indirect ELISA except that the detecting antibody (anti-human IgG) was chemically conjugated to a short capture oligonucleotide rather than an enzyme. The capture oligonucleotide was then detected by the addition of target DNA, which was designed to hybridise to the capture oligonucleotide and function as a template for real-time PCR. The quantity of target DNA detected by the PCR depended upon the level of specific antibody in the test sample. We found that the sensitivity (and specificity) of the iPCR assay did not exceed that of the conventional ELISA. The sensitivity was limited by nonspecific binding of human IgG to the solid phase. Further development of reagents and assay formats is necessary to fully exploit the potential of quantitative iPCR, so that potential improvements in the sensitivity of anti-mumps IgG detection can be realised. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Immuno-PCR assay; Real-time PCR; Mumps antibody detection

1. Introduction

The use of labelled antibodies in immunoassays offers the potential for developing highly sensitive and specific tests provided that labels of high specific activity are employed and nonspecific binding of labelled antibody is minimised (Ekins, 1981). In this context, Ekins has defined the specific activity of the labelled antibody as the number of observable events/unit time for each antibody molecule. Commonly used radioisotopic labels such as ^{125}I have relatively low specific activities, in the order of five disintegrations per minute per attomole (Ishikawa et al., 1989); but alternative nonisotopic labels such as chemiluminescent and fluorescent substances are capable of yielding higher specific activities, and circumvent the problems and hazards associated with handling radioisotopes. For example, the specific activity of mono-

Abbreviations: iPCR, immuno-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; MMR, measles, mumps and rubella; tDNA, target DNA; PBS, phosphate buffered saline; PBST, phosphate buffered saline containing Tween 20; SPDP, *N*-succinimidyl-3-(2-pyridyldithio) propionate; SMCC, succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate; BSA, bovine serum albumin; SD, standard deviation.

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clonal antibodies labelled with a chemiluminescent acridium ester was shown to be 50 photon counts per attomole of label (Weeks et al., 1984). Similarly, the use of europium labels in time-resolved fluorescence has permitted the development of sensitive immunoassays by reducing background noise and consequently increasing signal-to-noise ratios (Jackson and Ekins, 1986).

Enzymes such as horseradish peroxidase and alkaline phosphatase have also been widely used as labels. They have the potential for signal amplification since one molecule of enzyme may catalyse the conversion of many substrate molecules to detectable product. In practice, however, the colorimetric substrates used to monitor enzyme activity limit the sensitivity with which enzyme labels can be detected since relatively large concentrations of chromogens need to be generated for spectrophotometric detection. Enzyme assays have therefore not usually demonstrated much greater sensitivity than radioisotopic methods, though the use of fluorogenic and radioactive substrates or enzyme amplification methods have increased detection limits (Harris et al., 1979; Stanley et al., 1985; Johannsson et al., 1986; Ruan et al., 1993; Zhang et al., 2001).

Recently, DNA labels that are detected using PCR have been used in the development of ultrasensitive immuno-PCR (iPCR) assays (Sano et al., 1992; Ruzicka et al., 1993; Zhou et al., 1993; Hendrickson et al., 1995; Maia et al., 1995; Case et al., 1997; Wu et al., 2001). Sano et al. (1992) reasoned that since single target nucleic acid molecules can potentially be detected by PCR, the use of DNA labels would permit highly sensitive assays. Using this approach they were able to detect as few as 580 molecules of immobilised bovine serum albumin. To date, iPCR has been used for the sensitive detection of a variety of soluble protein molecules such as cytokines (Sanna et al., 1995; Saito et al., 1999), tumour markers (Suzuki et al., 1995) and hormones (Hendrickson et al., 1995). However, it has so far not been applied to the detection of antibodies to human pathogens. In this study we describe the development of a quantitative iPCR assay and assess its potential for the detection of antibody to mumps virus. Although the incidence of mumps in the United Kingdom has fallen to low levels since the introduction of measles, mumps and rubella (MMR) vaccination, serological surveillance remains important to provide early warning of any accumulation of susceptible individuals that could facilitate a resurgence of disease (Osborne et al., 2000).

2. Materials and methods

2.1. iPCR format

A schematic diagram of the iPCR assay developed for the detection of antibodies to mumps virus is shown in Fig. 1. First, serum samples were incubated in wells coated with recombinant mumps nucleoprotein antigen. After washing, wells were incubated with a mixture of conjugate (anti-human IgG covalently coupled to capture oligonucleotide) and target DNA (tDNA), which was designed to hybridise to capture oligonucleotide. After washing, hybridised tDNA was released into solution by restriction enzyme digestion and subsequently quantified by real-time PCR using the LightCycler™. The amount of tDNA released from the conjugate was proportional to the level of specific mumps IgG antibody in the test sample.

2.2. Design of target DNA, primers and probes

All oligonucleotides were synthesised by MWG-Biotech, UK (Milton Keynes, England) and are shown in Table 1. A single-stranded (ss) amplification target oligonucleotide (tDNA), 89 base pairs in length, was designed with six defined sequence regions: complementary sequence to the capture oligonucleotide; *Hind* III restriction site; 5′ primer sequence; complement sequence to the 3′ primer; and an internal probe sequence. Two primers (Table 1) of 20 base pairs in length were used to amplify a 67-bp region of the tDNA. They had a G+C content of 45–50%, similar melting temperatures and no complementary sequences. Two additional oligonucleotides were used. The internal probe was designed to hybridise to a sequence between the primer sites and was used to detect specific PCR product during amplification. It was labelled with Cy5 at the 5′ terminal and blocked with biotin at the 3′ terminal to prevent it acting as a primer during PCR. The capture oligonucleotide was complementary to the 5′ end of the tDNA and contained a *Hind* III restriction digest site to enable the release of

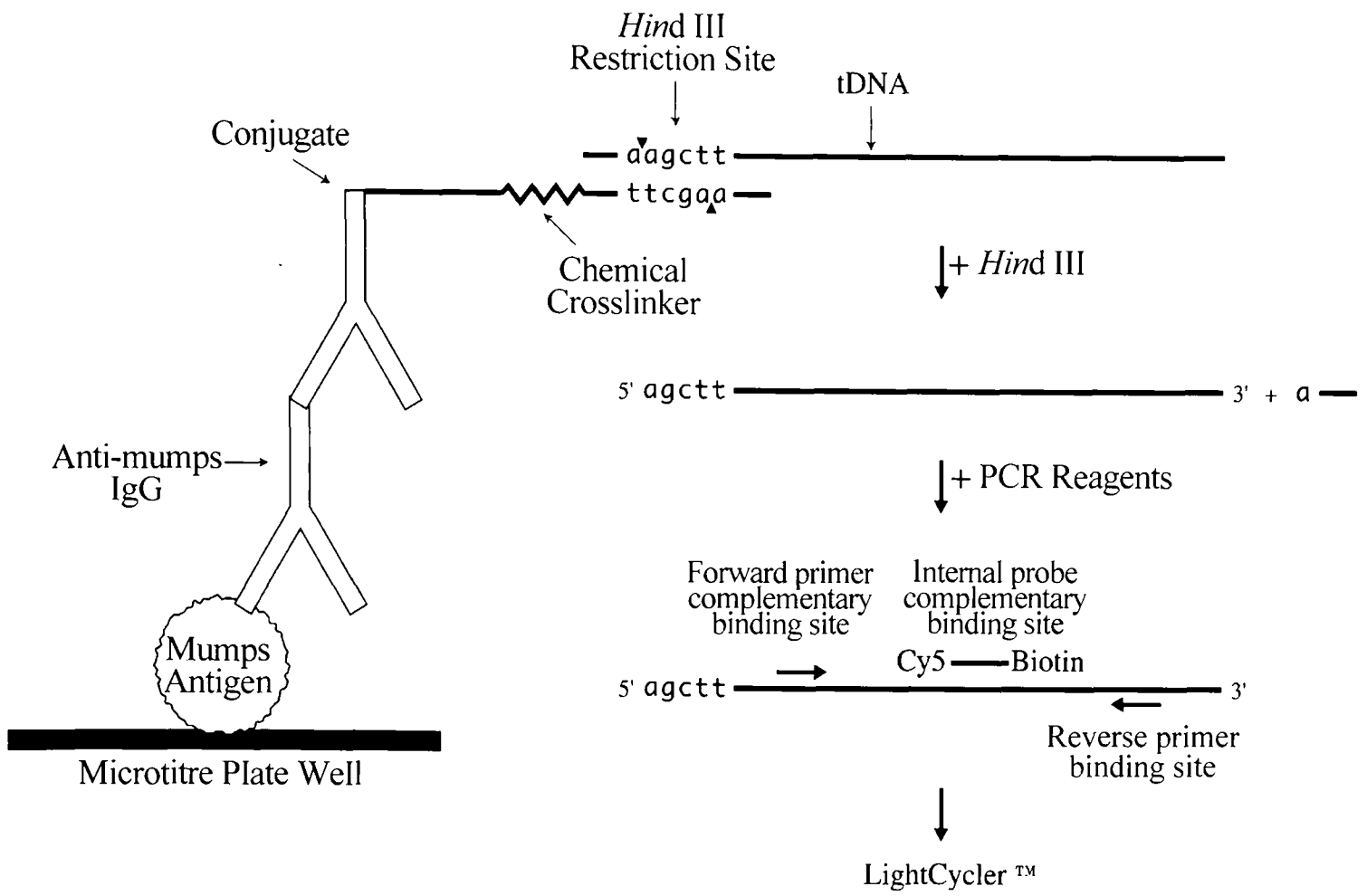

Fig. 1. Schematic diagram of the iPCR assay developed in this study. Refer to 'iPCR Format' in the Materials and Methods for explanation.

hybridised tDNA into solution prior to DNA quantification. In the assay development, the PCR was optimised first, followed by the antibody detection system, as described below.

2.3. Quantitative PCR using the LightCycler™

Five DNA standards, containing between 10^4 and 10^8 copies of tDNA, were tested in parallel with the samples to allow accurate quantification. The standards were prepared by making serial ten fold dilutions of tDNA of known concentration in nuclease-free water (Promega UK, Southampton, UK) containing 5 μg/ml of herring sperm DNA. In addition, at least one negative control containing no tDNA was included in each batch of PCR tests. PCR reactions were carried out using the LightCycler™ (Bio/Gene, Kimbolton, England) in a final volume of 10 μl containing 50 mM Tris–HCl, pH 8.3; 0.5 μg/ul bovine serum albumin; 3 mM $MgCl_2$; 200 μM deoxynucleoside triphosphates (Gibco, Paisley, Scotland); 0.4 units Platinum *Taq* DNA polymerase (Gibco); 1/10,000 SYBR Green I (Bio/Gene); 0.5 μM each primer; 0.25 μM internal probe; and 1 μl of tDNA or H_2O. The thermal conditions were an initial denaturation at 93 °C for 15 s, followed by 50 amplification cycles of four steps: 93 °C for 0 s; 55 °C for 0 s; 58 °C for 1 s; and 74 °C for 2 s. Fluorescence was measured once per cycle after the 58 °C step. Maximum ramp rates (20 °C/s) were used except for the transition from 55 to 58 °C when the ramp rate was 3 °C/s. Immediately after amplifi-

Table 1
Nucleotide sequences of tDNA, primers and probes

Region	Sequence (5′ →3′)
tDNA	tgc tca agc tta gga tcc ata gca tga cta cgt gac tcg gtt gga agc tat acg ttc gat ggc tgc tgc atc agt acg caa tcg gta tg
Forward primer	ca tga cta cgt gac tcg gtt
Reverse primer	cat acc gat tgc gta ctg at
Internal probe	Cy5-ata cgt tcg atg gct gct gc-biotin
Capture oligonucleotide[a]	cta tgg atc cta agc ttg agc att t

[a] Two forms of the capture oligonucleotide were synthesised, with either a biotin or amino link at the 3′ end.

cation, a two-step melting programme followed: 95 °C for 0 s; and 45 to 95 °C at a ramp rate of 0.2 °C/s with continuous fluorescent monitoring.

2.4. Capture and release of tDNA

Target DNA (3×10^{12} tDNA copies/μl) was incubated for 10 min at 50 °C, in the presence and absence of biotinylated capture oligonucleotide. Each mixture was diluted 1/100 (final concentration of tDNA approximately 1.5×10^{10} copies/μl) with phosphate buffered saline (PBS) and 40 μl was added to streptavidin-coated (Thermo Labsystems Oy, Helsinki, Finland) or negative control wells. After removal of unbound material by washing with PBS containing 0.05% Tween 20 (PBST), the well contents were incubated with 40 μl of buffer containing 1 U *Hin*d III, or mock digested, for 1 h at 37 °C. Eluted material was collected and 1 μl used as template for quantitative PCR using the LightCycler™.

2.5. Conjugation of anti-human IgG to capture oligonucleotide

Amino-modified capture oligonucleotide (Table 1) was chemically conjugated to affinity-purified rabbit anti-human IgG (Chemicon International, Temecula, CA) using the heterobifunctional cross linkers *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) and succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) by MicroImmune (Brentford, UK).

2.6. Preparation of human IgG- and bovine serum albumin-coated plates

To optimise and evaluate the binding of conjugate (anti-human IgG-capture oligonucleotide) to human IgG, microtitre plates were coated with human IgG or bovine serum albumin (BSA) as a control. Microtitre plate wells (MaxiSorb, Nunc, Life Technologies, Paisley, United Kingdom) were coated with 100 μl of 2 μg/ml affinity purified human IgG (Chemicon) or BSA in 0.05 M sodium carbonate–bicarbonate buffer (pH 9.6) and incubated at 37 °C overnight. After washing with PBS, nonspecific protein binding sites were blocked with 150 μl of 5% SoluPro (Dynagel, Calumet City, IL) in deionised water for 2 h at 37 °C. The wells were then washed, left to dry overnight at 37 °C and stored in a sealed container until use.

2.7. Serum samples

Mumps-specific IgG negative, weak positive and strong positive sera (as determined with a commercial ELISA Kit; Behring Enzygnost; Behringwerke, Marburg, Germany) were used to optimise the iPCR assay and subsequently used as controls in further experiments. A panel of 88 additional sera, which had been submitted for diagnostic testing by the same kit, was used to evaluate the iPCR assay.

2.8. Mumps-specific IgG iPCR

After experiments to optimise the assay conditions, the iPCR assay was performed as follows:

2.8.1. Coating

Microtitre plate wells were coated with 100 μl of 2 μg/ml recombinant mumps nucleoprotein antigen (Microimmune) in PBS containing 0.08% sodium azide, pH 7.2 and incubated at 37 °C overnight. After washing with PBS, the wells were blocked with SoluPro (Dynagel), dried and stored as described above.

2.8.2. Antibody capture

Sera were diluted 1/200 using assay diluent (PBS containing 10% normal goat serum, 2% skimmed milk, and 0.1% Tween 20). Twenty microlitres was added to wells and incubated at 37 °C for 30 min. The wells were then washed 12 times with PBST.

2.8.3. Detection of captured antibody

Conjugate/tDNA mixture was prepared by adding 1 μl of conjugate (anti-human IgG-capture oligonucleotide) and 50 μl of tDNA (10^{10} copies/μl) to 1 ml of assay diluent. Twenty microlitres were added to the wells and incubated at 37 °C for 1 h. Wells were washed 12 times with PBST and then digested with 1 U *Hin*d III (New England Biolabs (UK), Hitchin, Hertfordshire) for 1 h at 37 °C. Eluted material was collected and 1 μl was used as template for quantitative PCR using the LightCycler™. Results were quantified in terms of copies of amplified tDNA per microliter eluted from each well. All wash steps

were performed using an automated plate washer (Labsystems Wellwash 4 Mk 2).

2.9. Statistical methods

2.9.1. iPCR assay cut-off

A positive iPCR result was defined as any value greater than two standard deviations (SD) above the mean value of the negative control, which was tested in parallel with samples. The SD was determined by the analysis of a panel of 17 sera negative for mumps specific IgG by the Behring Enzygnost ELISA.

2.9.2. Reproducibility of iPCR assay

The reproducibility data were analysed in the BMDP program 8 V, which performs general mixed model analysis of variance for equal cell sizes (Dixon, 1992). The data were analysed for each serum sample separately to obtain serum specific estimates. In the analysis wells and PCRs were declared to be random effects.

3. Results

3.1. Sensitivity of the PCR using the LightCycler™

Under optimised amplification conditions, the PCR was able to detect 10^2 copies of tDNA/μl, with a wide dynamic range of >7 logs. Melting peak analysis revealed peaks at 63 and 83 °C, corresponding to the melting temperature of the internal probe/tDNA duplex and the full-length product, respectively (data not shown).

3.2. Capture and release of tDNA

Experiments were performed to determine whether the tDNA would hybridise to the capture oligonucleotide, and to determine the optimal conditions for the release of hybridised tDNA prior to PCR. Binding of tDNA to streptavidin-coated (Strep+) and uncoated (Strep−) wells, in the presence or absence of biotinylated capture oligonucleotide, was investigated. We found tDNA bound at very low concentrations to Strep− wells, both in the presence and absence of capture oligonucleotide (1×10^4 copies tDNA/μl out of 1.5×10^{10} copies tDNA/μl added bound to the wells). A similar level of binding was observed when tDNA was added to Strep+ wells in the absence of capture oligonucleotide. In contrast, in the presence of streptavidin and capture oligonucleotide, high levels of tDNA (1×10^9 copies tDNA/μl) were detected. This suggested that the tDNA was binding to the streptavidin-coated wells via the biotinylated capture oligonucleotide. The optimal release of hybridised tDNA from Strep+ wells was achieved by restriction digestion (1×10^9 copies tDNA/μl detected), however, the tDNA could also be eluted simply by incubating wells with buffer for a similar time period (3×10^8 copies tDNA/μl detected).

3.3. Binding of anti-human IgG-capture oligonucleotide conjugate to human IgG

The anti-human IgG-capture oligonucleotide conjugate was initially evaluated with respect to its capacity to bind to immobilised human IgG and to BSA as a control. As shown in Table 2, when a constant amount of tDNA was incubated with tenfold dilutions of conjugate, very low levels of tDNA were detected in the BSA-coated wells compared with the human IgG wells. The dilution of conjugate, which

Table 2
Binding of conjugate to human IgG and BSA

Conjugate dilution factor	tDNA (copies/well)	BSA-coated wells (tDNA/μl)	Human IgG-coated wells (tDNA/μl)	S/N
10^1	10^{11}	1.9×10^5	1.0×10^8	526
10^2	10^{11}	4.9×10^4	1.0×10^8	2041
10^3	10^{11}	1.3×10^4	4.6×10^7	3538
10^4	10^{11}	1.4×10^4	5.1×10^6	364
10^5	10^{11}	2.1×10^3	1.2×10^6	571
–	10^{11}	1.4×10^3	6.1×10^3	4
10^1	–	1.8×10^0	3.4×10^1	19
–	–	3.2×10^{-1}	2.9×10^1	91

Serial tenfold dilutions of conjugate were prepared in conjugate diluent (2% BSA; 0.05% Tween 20 in PBS) and 40 μl was added to microtitre wells coated with human IgG and BSA, where BSA served as a control for nonspecific binding. The wells were incubated for 1 h at 37 °C, washed, then incubated for 1 h at 37 °C with 40 μl of diluted tDNA (10^{11} copies/well). After washing, tDNA was eluted and quantified using the LightCycler™.
S/N denotes signal to noise ratio, which is the amount of tDNA eluted from the human IgG-coated wells divided by the amount eluted from the BSA-coated wells.
(−) denotes no conjugate or tDNA was added to the wells.

Table 3
Reproducibility of the iPCR assay

Control sera	Mean (log_{10})	SD (wells)[a]	SD (PCR)[b]	SD (all)[c]
Negative	4.86	0.08	0.09	0.13
Weak positive	6.09	0.04	0.08	0.1
Strong positive	6.69	0.07	0.10	0.13

[a] SD (standard deviation) for the averaged measurements from different wells.
[b] SD for the averaged measurements of different PCRs from the same well.
[c] SD obtained for all the measurements.

resulted in the highest signal-to-noise ratio, was 1/1000, hence this was used in subsequent experiments. At a conjugate dilution of 1/10,000, the signal obtained was approximately tenfold lower, but the level of nonspecific binding remained the same. In wells that received tDNA but no conjugate, only background levels of tDNA were detected. There was evidence of cross-contamination between wells, as traces of tDNA were detected in wells to which none had originally been added. A similar experimental approach was used to determine the optimal amount of tDNA for use in the iPCR assay, where the conjugate was fixed at a dilution of 1/1000 and the tDNA was varied. The optimal amount was found to be 10^{10} copies/well.

3.4. Reproducibility of the iPCR assay

The reproducibility of the iPCR assay was investigated by adding control sera to different wells on the same microtitre plate in quadruplicate. tDNA from each well was then quantified in four separate PCR assays. Table 3 shows the mean values (log_{10} scale) and standard deviations obtained. Both the inter-PCR and inter-well standard deviations were low, indicating that the assay was highly reproducible.

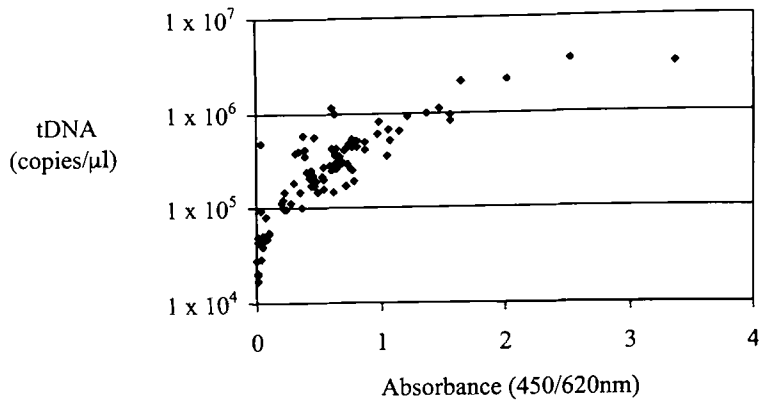

Fig. 2. Scatter plot comparing Behring Enzygnost ELISA and iPCR test results. Number of copies of tDNA/µl (log_{10} scale) plotted against the ELISA absorbance readings at 450/620 nm for the 88 sera tested.

3.5. Evaluation of the iPCR assay

The iPCR assay for mumps-specific IgG was evaluated in its optimised format using 88 serum samples previously tested in the commercial ELISA. Concordant results were obtained for 84 sera. Of the remaining four, one specimen was ELISA-positive and iPCR-negative, one was ELISA-negative and iPCR-positive, and two samples with equivocal ELISA results were iPCR-negative. For three of these discrepancies the ELISA result was very close to the cutoff (Table 4). Compared to the ELISA, the iPCR was 98.6% (71/72) sensitive and 92.9% (13/14) specific. When the ELISA absorbance readings were plotted against the iPCR results for the 88 sera, there was good correlation (correlation coefficient 0.88) between the values (Fig. 2).

Table 4
Data on discrepant samples

Sample number	Age (years)	Clinical details	Mumps-specific IgM[a]	Mumps-specific IgG (OD)[b]	iPCR
6	6	no information	negative	equivocal (0.106)	negative
14	25	hospital staff immunity check	not tested	positive (0.213)	negative
31	32	acute parotid swelling	negative	negative (0.041)	positive
73	37	? recent mumps	negative	equivocal (0.102)	negative

[a] Mumps-specific IgM antibody capture radioimmunoassay (Perry et al., 1993).
[b] Behring Enzynost ELISA (negative = < 0.1; equivocal=0.1 – 0.2; and positive = > 0.2).

4. Discussion

The detection of mumps-specific IgG plays an important role in immunity surveillance, monitoring the efficacy of vaccination programmes, identifying susceptible cohorts in the population and shaping future vaccination policies. Immunoassays are the method of choice for viral antibody detection, but the limited sensitivity of current mumps-specific IgG assays has restricted the value of the procedure (Pipkin et al., 1999). This poses a particular problem when measuring mumps-specific IgG prevalence in populations in which mumps virus infection has been controlled by MMR vaccination. Firstly, antibody responses to vaccination are lower than following infection with wild type virus and, secondly, levels of antibody in the population are no longer boosted by continuing circulation of the virus. In a recent ELISA-based study of pre-school children in the United Kingdom, 15% were mumps antibody negative, compared to 8% and 10% for measles and rubella, respectively (Gay et al., 1997). It is unclear whether this was due to the mumps test being less sensitive or to the mumps virus component of the vaccine being less antigenic than the other two components.

To improve assay sensitivity, we developed an indirect, quantitative iPCR assay for the detection of mumps-specific IgG in serum. The distinctive features of our assay were: (a) the conjugate, which had the dual function of binding to any immobilised human IgG and capturing tDNA; (b) the use of restriction enzyme digestion for the specific release of hybridised tDNA; and (c) the quantification of tDNA by real-time PCR using the LightCycler™.

Several versions of iPCR that deliver improvements in assay sensitivity have been published. These methods differ mainly in the way target DNA is linked to detecting antibody (Sano et al., 1992; Ruzicka et al., 1993; Zhou et al., 1993; Hendrickson et al., 1995). Sano et al. (1992) used a recombinant chimeric protein that contained two moieties, protein A and streptavidin, which bound to the Fc fragment of antibodies and biotin-modified DNA, respectively. However, the lack of availability and limited application of the chimera led others to use one of the biotin-binding proteins, avidin or streptavidin, to link biotinylated DNA and biotinylated antibody (Ruzicka et al., 1993; Zhou et al., 1993). More recently, directly labelled conjugates have been described where DNA is covalently coupled to detecting antibody (Hendrickson et al., 1995; Joerger et al., 1995). The use of such conjugates permits simple assay formats to be adopted since fewer washing and incubation steps are required. In addition, multiple analytes can be simultaneously detected.

In this study, a novel approach was used to link detecting antibody to target DNA. Secondary antibody was covalently conjugated to a short oligonucelotide, which was partly complementary to the 5′ end of tDNA. Restriction digestion was then used to release hybridised tDNA from the solid phase, though an alternative way of releasing tDNA would have been to use heat treatment (Sanna et al., 1995). Initially, we reasoned that restriction digestion would improve the specificity of the test; however, our experiments indicated that the release of tDNA could be achieved simply by incubating wells with restriction digestion buffer at 37 °C. The nature of this release is unclear; the buffer would not be expected to denature the tDNA from the capture oligonucleotide, but it may be that the antigen or antibodies desorb from the solid phase over time.

Amplification on the LightCycler™ proved very useful in the development of the assay by providing rapid and reproducible quantification of tDNA. Once tDNA samples were ready for PCR, amplification and analysis were completed within 30 min, considerably faster than by conventional PCR. Quantitative PCR was essential to allow the relative levels of nonspecific binding of tDNA, conjugate and human IgG to be accurately determined. In previous studies using conventional PCR, based on the detection of amplicons after gel electrophoresis, it was not possible to determine accurately the contribution of the specific signal compared to the nonspecific signal. We found that nonspecific binding of human IgG was the main limiting factor affecting the sensitivity of the assay, compromising the advantages gained by PCR amplification. Hence, it was necessary to define the experimental results on the basis of signal-to-noise ratios rather than the presence or absence of specific product.

To minimise nonspecific binding extensive optimisation experiments were performed. Most critical were the working concentrations of conjugate and tDNA. We also found that signal-to-noise ratios improved

when milk was used as a blocking agent in the assay diluent. This is consistent with the results of a study investigating the efficacy of different blocking agents for suppressing background caused by nonspecific protein adsorption to the solid phase (Vogt et al., 1987). To minimise the level of nonspecific binding of target DNA, sheared sperm DNA has been used by several groups (Sano et al., 1992; Maia et al., 1995; Sperl et al., 1995; Kakizaki et al., 1996). However, addition of sheared sperm DNA to the assay diluent did not improve the performance characteristics of our assay. As reported by others (Sano et al., 1992; Kakizaki et al., 1996) and confirmed in the present study (data not shown), extensive washing of wells with detergent-containing buffer to remove unbound reagents was essential to minimise background noise.

When the optimised iPCR was compared with the ELISA, concordance was good. Discrepant findings were obtained in four cases, two of which were attributable to equivocal values in the commercial ELISA. A possible explanation was the use in the two assays of different antigen preparations for capturing the anti-mumps antibody. We employed a recombinant mumps nucleoprotein antigen in contrast to the whole virus preparation containing a mixture of native antigens used in the Behring Enzygnost ELISA. The use of a single purified antigen has the potential to give fewer false-positive results, but may contribute to false-negative results since antibodies to other mumps antigens will not be detected. In the clinical setting, a false-negative result is relatively less important than a false-positive result, as in the latter case the individual would not be vaccinated, leaving them susceptible to wild-type mumps infection.

The present report demonstrates the feasibility of using iPCR for the detection of antibodies in human serum. In contrast to previously published reports, we were unable to show an enhanced sensitivity over the ELISA but we have shown (in a way that was not possible in previous studies based on qualitiative PCR endpoints) that the main limitation in iPCR is nonspecific binding of assay components to the solid phase. Also, the wide dynamic range of the assay gives a more accurate assessment of high level antibody responses and may therefore be useful in longitudinal studies.

Improvements in reagents and assay formats, leading to enhanced performance characteristics, will be required before iPCR can be routinely used for diagnosis of infection or determination of immune status. In addition, a solid phase more compatible with antigen coating, automated washing and quantitative PCR would greatly simplify and advance the applications of iPCR.

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Novel methods for the detection of microbial antibodies in oral fluid

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Compared with blood, oral fluid has several advantages as a sample for antibody detection. It is simple, safe, painless, and cheap to collect. The only drawback is that while the antibody profiles indicate those in blood, they are at lower concentrations. Antibody capture assays are the method of choice for the detection of microbial antibodies in oral fluid, but their relative lack of sensitivity when based on conventional immunoassay techniques has mostly limited their use to epidemiological applications. Immuno-PCR and time-resolved fluorescence offer more sensitive detection systems that could be applied to oral fluid specimens. We review antibody detection in oral fluid and discuss immuno-PCR and time-resolved fluorescence as candidate systems. Both have the potential to broaden the applications of oral fluid testing to clinical diagnostics.

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Traditionally, serum has been the specimen of choice for detecting antibodies to diagnose infection or establish immune status. Blood collection is, however, invasive, risky, and expensive. It needs trained staff and sterile equipment. Oral fluid and urine, by contrast, are safe and cheap to collect and are more acceptable, especially for population immunity studies where large numbers of specimens need to be easily and economically obtained, often from children.[1]

Oral fluid and urine are feasible alternatives to serum because both contain immunoglobulins. Urine is not an ideal specimen because production on demand is not always possible and can cause the patient embarrassment. Also, difficulties have been encountered in storing urine samples. Oral fluid, however, is simple, safe, and cheap to collect (figure 1). It is defined as the fluid obtained by the insertion of absorptive collectors into the mouth.[2] Depending on the location and material used, the fluid collected is enriched by specific components of whole saliva. Fluid in the oral cavity consists of secretions from the salivary glands (mainly the parotid, submandibular, and sublingual glands) and transudate from the capillary bed, in particular, crevicular fluid that constantly flows from the crevice between the gum margin and the teeth.[2] Crevicular fluid is the component in saliva that contains the highest concentrations of IgA, IgG, and IgM. Hence an oral fluid sample that is well represented with crevicular fluid is most suitable for antibody detection. The mean immunoglobulin concentrations in several salivary components and plasma is shown in table 1. The salivary concentrations of plasma-derived immunoglobulins vary between individuals depending on the collection methods used and levels of oral hygiene. For example, patients with gingivitis (characterised by inflammation and bleeding of the gums) might be expected to show high concentrations of plasma-derived antibody in their saliva.

Figure 1. Collection of oral fluid from an infant with an Oracol sponge swab.

A drawback of using oral fluid samples is that although specific antibody concentrations indicate concentrations in plasma, they are much lower. This means that sensitive assays are needed to detect antibodies in oral fluid; antibody capture assays have been extensively used for this purpose. However, there is concern that present protocols lack the sensitivity needed for certain applications, especially for detection of past infection. There is a need to develop a new generation of more sensitive immunoassays. Immuno-PCR (I-PCR) and time-resolved fluorescence (TRF) are two techniques that could be used to improve antibody detection. We discuss the collection of oral fluid and compare the advantages of antibody capture assay, I-PCR, and TRF, particularly for use on oral fluid specimens.

Collection of oral fluid

Saliva testing has been widely applied, including in the detection of immunoglobulins, hormones, metabolites, electrolytes, and pharmceuticals.[5,6] Most early developmental work on the detection of microbial antibodies in saliva used whole saliva that was collected by

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Table 1. Mean immunoglobulin concentrations (mg/L) in plasma and salivary components.

Specimen	IgG	IgM	IgA*
Plasma	14730	1280	2860
Parotid saliva	0·36	0·43	39·5
Crevicular fluid	3500	250	1110
Whole saliva	14·4	2·1	19·4

*Although high concentrations of IgA are present in crevicular fluid, the diagnostic usefulness of this class of antibody is relatively limited compared to specific IgM and IgG.
Sources: Brandtzaeg et al[3] and Roitt and Lehner[4]

being dribbled into a sterile, wide-mouthed container.[7,8] However, some patients found dribbling distasteful, and sputum specimens or samples with insufficient volume were frequently received. Also, pipetting untreated saliva was difficult. This led to the development of proprietary devices designed to collect oral fluid, four of which are featured in table 2 and figure 2. Studies that compared three oral-fluid-collection devices (OraSure, Omni-SAL, and Oracol) for use in the surveillance of virus diseases identified Oracol as the preferred collection device.[9,10] It was cheap, acceptable to participants, and produced the highest quality oral fluid samples for antibody testing in terms of total antibody concentrations, as well as rubella-specific IgG concentrations. The results were not surprising since Oracol was designed to specifically target the gums, the area of the oral cavity most likely to be rich in crevicular fluid. However, the efficiency of antibody elution from the absorbent material could also play a part, as OraSure also targets the gums albeit less vigorously.

Although oral fluid collection has many advantages compared with venepuncture, investigators should not become complacent when collecting and analysing oral fluid samples. Few problems may arise when health professionals in the clinical environment collect oral fluid samples; however, samples collected by untrained personnel in a nonmedical setting should be checked for adequate quality and quantity. A study involving the collection of 4000 Salivette specimens from injecting drug users participating in a multi-site survey of HIV and hepatitis B virus core antibodies in the UK revealed 8·6% were inadequate for testing, either due to insufficient volume or inadequate total IgG concentrations.[11]

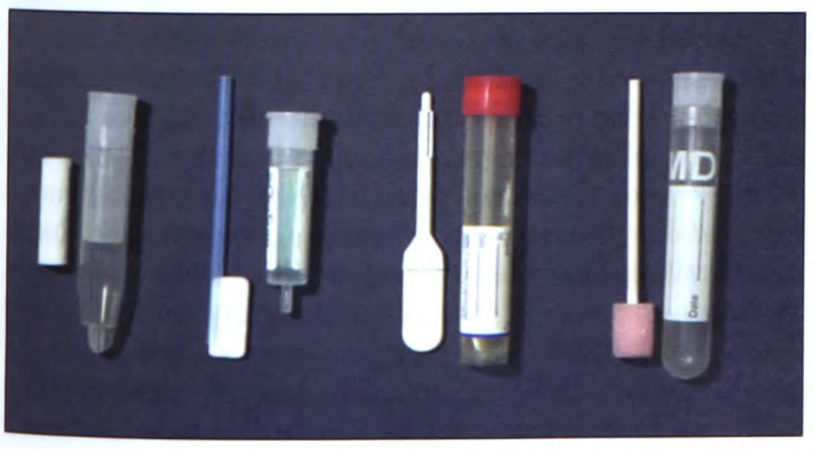

Figure 2. Four oral fluid collection devices. From left to right: Salivette, OraSure, OmniSAL, and Oracol.

Antibody-capture assay

One of four configurations—indirect, competitive, antigen sandwich, or class-specific antibody-capture assays—is generally chosen to detect specific antibodies.[11] The choice depends to a large extent on the type of clinical specimen being tested and the concentration of immunoglobulin that it can be expected to contain.

The basis of the "antibody capture" technique is that a single class of human immunoglobulin (ie, IgG, IgM, or IgA) in the specimen is captured by an immobilised anti-immunoglobulin. Specific antibody is then detected by the addition of an antigen and, for example, a mouse monoclonal antibody to the antigen and a labelled anti-mouse immunoglobulin. The label could be an enzyme that produces a colour signal in the presence of substrate or a radioisotope such as I^{125} (figure 3).

The antibody-capture assay differs from other assay configurations in that the strength of the signal is not dependent on the concentration of a specific antibody in the sample, but on the proportion of the captured immunoglobulin that is specific for the antigen. Because oral fluid includes plasma transudate as the predominant source of immunoglobulin, the ratio of specific to total immunoglobulin is similar in both serum and oral fluid.[12] Hence, as long as the test specimen contains sufficient IgG to saturate the anti-IgG binding sites, the signal obtained should be similar whichever sample is used.[11–13]

Antibody capture assays were first described by Flehmig et al[14] and Duermeyer et al[15] for the detection of IgM antibodies against hepatitis A virus (anti-HAV). They are now widely used in the diagnosis of virus infection. Originally, these assays were intended for use with serum. However, when the advantages of oral fluid sampling were realised, assay protocols were modified appropriately. Parry et al[7] were the first to describe oral fluid-based antibody capture assays for the detection of IgG antibodies to HIV, rubella virus, HAV, and the core antigen of hepatitis B virus (HBV). Subsequently, antibody capture assays for oral fluid were developed for the detection of virus-specific IgG to measles,[16] Epstein-Barr virus (EBV),[17] and parvovirus B19,[18] and for specific IgM in patients with recent hepatitis A or

Table 2. Characteristics of four oral fluid collection devices.

Collection device	Description	Manufacturer	Unit cost*
Salivette	Chew cotton wool for 30 to 45 s or place under tongue for 1 min	Sarstedt	£0·36
OraSure	Move pad gently along gum twice. Place against lower gum on one side and keep stationary for 2 min	OraSure technologies	£3·76
Omni-SAL	Place pad under tongue and keep until indicator of device turns blue	Saliva Diagnostic Systems	£2·20
Oracol	Rub sponge firmly along the base of the gums (inside and out) of the upper and lower jaw, for 1 min, using an action similar to tooth brushing	Malvern Medical Developments	£0·50

*Unit costs at November 2001

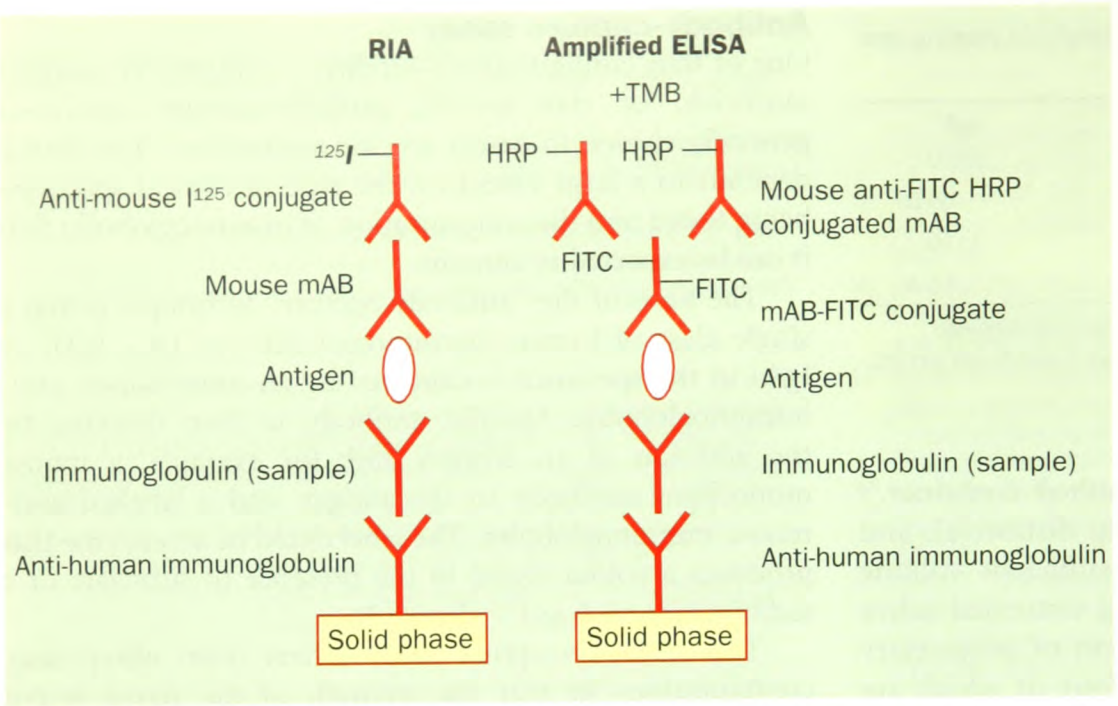

Figure 3. Radioimmunoassay (RIA) and amplified ELISA formats used in antibody capture assays for use with oral fluid samples. Immunoglobulin (IgG, IgM, or IgA) in the test sample is captured by immobilised anti-immunoglobulin. Specific antibody is then detected by the addition of an antigen, followed by either: mouse monoclonal antibody (mAB) and I^{125}-labelled anti-mouse immunoglobulin for the RIA; or fluorescein isothiocyanate (FITC)-labelled mAB, horseradish peroxidase (HRP)-labelled mouse anti-FITC mAB, and the chromogenic substrate tetramethylbenzidine hydrochloride (TMB) for the ELISA.

B,[19] measles, mumps, rubella,[16] and parvovirus B19 infection.[20] Many of these assays were developed as radioimmunoassays, and they have the advantages of a wide dynamic range, quantification, and sensitive detection of radioactivity by a gamma counter.

There are practical disadvantages associated with radioimmunoassay. Not only does radio-labelling make technical demands and produce hazardous radioactive waste, but it is not easily transferable between laboratories.[21] Unfortunately, however, the substitution of an enzyme-labelled conjugate for a I^{125}-labelled conjugate has not in most cases resulted in an ELISA with the same sensitivity as radioimmunoassay when testing oral fluid.[5] It is to address this issue of sensitivity, that amplification-based ELISAs have been investigated. For example, oral fluid IgG capture ELISA (GACELISA) assays that use an FITC/anti-FITC amplification system have been developed to detect measles and rubella antibodies.[21,22]

Oral fluid testing has been most useful for epidemiological surveillance, especially for HIV infection.[8] As a result, several oral fluid anti-HIV screening assays have been developed and commercial anti-HIV test kits have become available that have been reported to be sensitive and specific.[23] These have begun to be used diagnostically in some settings. Oral fluid testing has also been used to monitor the measles, mumps and rubella (MMR) programme. With the implementation of the national vaccination campaign in the UK in 1988, and the subsequent achievement of high vaccine coverage, it has been shown that the predictive value of clinical notifications of MMR declines with the decreasing incidence of disease. To provide accurate surveillance data, clinical notifications have been confirmed by laboratory testing[24] and, since November 1994, the Public Health Laboratory Service (PHLS) has improved its MMR surveillance by offering an oral fluid test for cases notified to the Office for National Statistics (ONS).[25–28] Samples collected between 2 and 6 weeks after the onset of symptoms are tested for virus-specific IgM by antibody capture radioimmunoassays[16] to confirm recent infection. These assays have been shown to be greater than 90% sensitive and specific.[24,29]

Although oral fluid has been widely used for epidemiological purposes, it has not been routinely used in clinical laboratories to diagnose infection or confirm immune status in individual patients, due mainly to concerns about sensitivity. For example, the FITC/anti-FITC GACELISA developed for rubella-specific IgG was seen to be more sensitive when testing samples from children compared with samples collected from adults,[21] suggesting that in an immunised population rates of specific antibody decline with age, probably due to lack of boosting. This trend has not, however, been seen with anti-HIV assays, which could indicate the differences in immune response to persistent infections such as HIV in comparison with acute self-limiting infections such as measles and rubella.

Present technology for the detection of specific antibody in oral fluid using antibody capture assays has therefore mostly been limited to surveillance rather than diagnosis, and there remains a need to improve sensitivity so that the benefits of oral fluid testing can be more fully realised. Two approaches to this, I-PCR and TRF, are described.

Immuno-PCR

I-PCR is a modification of the ELISA where the enzyme conjugate used to detect an antigen-antibody reaction is replaced by a specific antibody that is either directly or indirectly coupled to a DNA molecule. This DNA can be used as a template for PCR amplification. The technique offers both the specificity of antigen-antibody interactions and the large amplification capability of PCR, potentially achieving antibody sensitivity far higher than by conventional assays.

The feasibility of I-PCR was first assessed for the detection of an antigen by Sano et al.[30] Bovine serum albumin (BSA) was immobilised on the surface of microtitre plate wells and subsequently detected with anti-BSA IgG linked to biotinylated DNA via a recombinant streptavidin-protein A chimera. Streptavidin binds specifically to biotin residues on modified DNA and protein A binds to the Fc portion of detecting antibody. The biotinylated DNA was used as template for PCR, the product of which was analysed by gel electrophoresis and ethidium bromide staining. The assay detection limit was a few hundred molecules of antigen, roughly fivefold more sensitive than the equivalent ELISA.

Since then several modified I-PCR formats have been described for the detection of a wide range of proteins. One of the main drawbacks of the I-PCR developed by Sano et al[30] was that the chimera was not commercially available. This led others to develop a modified protocol that could be universally adopted. Instead of the chimera, a biotin binding protein, either avidin or streptavidin, was used to link biotinylated DNA with biotinylated detecting antibody (figure 4). Zhou et al[31] used this process to detect anti-lipoprotein IgG immobilised on microtitre plate wells, and Ruzicka et al[32] used it to detect recombinant human proto-oncogene EST1 immobilised on PCR tubes. In both cases, the I-PCR enabled detection of very low concentrations of immobilised target. This approach has since been used to detect bovine herpesvirus 1,[33] the fish pathogen, *Pasteurella piscicida*,[34] and various structurally diverse antigens such as oligomeric pyruvate dehydrogenase complex.[35]

In the previous examples either a direct or indirect assay was used. However, with the (strept)avidin/biotin system other assay formats can be adopted. The sandwich-type assay, in which immobilised antibody captures the antigen, has been used by Numata and Matsumoto[36] and Sperl et al[37] to detect α-human atrial natriuretic peptide and soluble T cell receptors in plasma, respectively. Effective development of such assays would have been difficult with the chimera protein, because the protein A moiety would bind to the Fc region of immobilised capture antibodies, which increases the background and compromises assay specificity. Furthermore, protein A is known to have varied affinities for different types of antibodies. Its use would thus be restricted depending on the class or subclass of antibodies used.

Direct chemical conjugation of antibodies with marker DNA has been the most recent advance in I-PCR. Both ss-DNA and ds-DNA have been shown to function effectively as I-PCR labels when covalently coupled to antigen-specific antibodies.[38,39] Although there have been few published reports of DNA-antibody chimeras, these chimeras could offer advantages over the (strept)avidin/biotin system. Preparation of the conjugates before the assay avoids the in vitro assembly of components, which has been shown to lack homogeneity and may in turn affect accuracy and reproducibility.[40] In addition, the requirement for fewer incubation and wash steps would simplify and speed up the assay procedure, which has obvious advantages for clinical diagnostic applications.

As yet I-PCR has not been used to detect specific antibody in oral fluid, but Mweene et al[33] have shown it to be a very sensitive method for the detection of bovine herpesvirus IgG in serum of experimentally infected calves. The innate sensitivity of I-PCR would be expected to be useful for salivary antibody detection and the usefulness of the technique for detecting viral antibodies is being studied in our laboratory.

One of the major drawbacks to I-PCR is the amplification of background signals and the limit this could place on assay sensitivity. As for conventional ELISA, a degree of non-specific binding of assay components to the solid phase is unavoidable. However, since the

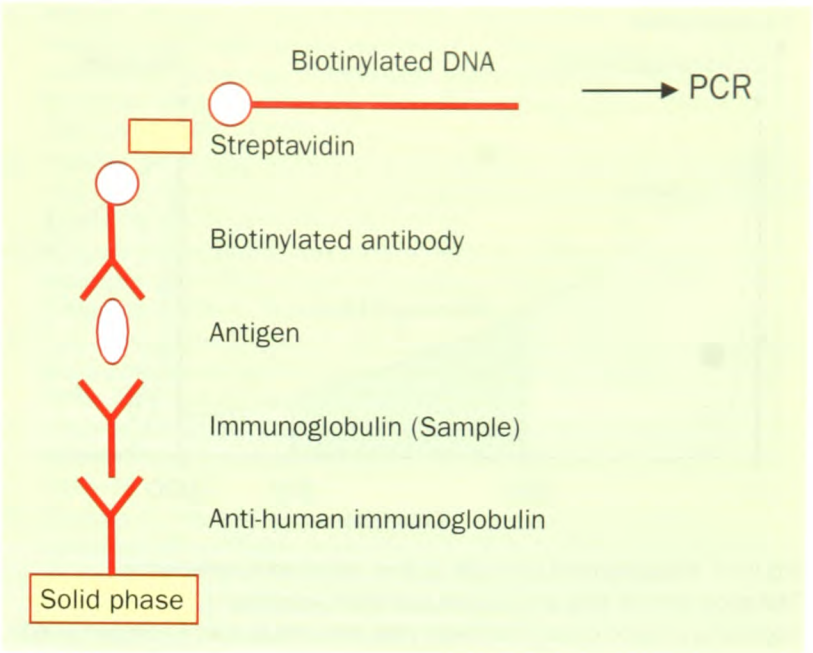

Figure 4. Immuno-PCR using free streptavidin to link biotinylated DNA and detecting antibody. Immunoglobulin (IgG, IgM or IgA) in the test sample is captured by immobilised anti-immunoglobulin. Specific antibody is then detected by the sequential addition of an antigen, biotinylated antibody, strepatvidin, and biotinylated DNA. PCR is used to detect the DNA.

detection method of I-PCR is so sensitive, even low rates of non-specific binding could lead to appreciable "noise" after DNA amplification. This means that for accurate results, quantitative assays are needed where positive samples are defined on the basis of a signal to noise ratio, rather than the absence or presence of a signal.

There are several ways of reducing background noise in immunoassays, such as washing with detergent-containing buffers and blocking reactive sites with denatured proteins and sheared sperm DNA. Sano et al[30] showed it necessary to wash wells extensively to reduce non-specific binding, and Maia et al[41] included a 3-h blocking step before the addition of marker DNA. Another potential problem with I-PCR is the contamination, by the marker DNA, of assay components, and/or of adjacent microtitre wells during the assay procedure. However, careful and clean laboratory practice should minimise this risk and if signal to noise ratios deteriorated due to contamination, marker DNA could be changed because it is purely arbitrary.

We conclude that further refinement and also standardisation and automation, is needed before I-PCR can be adopted in diagnostic laboratories. For example, a solid phase that is compatible with antigen coating, automated washing and quantitative real-time PCR would both simplify and expand the applications of I-PCR.

Time-resolved fluorescence (TRF)

Fluorescence has been used in diagnostic laboratories for many years,[42] either in the direct fluorescent antibody format or the indirect fluorescent antibody format. In the direct technique virus antigen is detected after reaction with specific fluorescein conjugated antibody. This technique has had wide application, especially in detecting specific virus in respiratory secretions. The indirect technique offers greater sensitivity than the direct technique and commonly uses fluorescein-

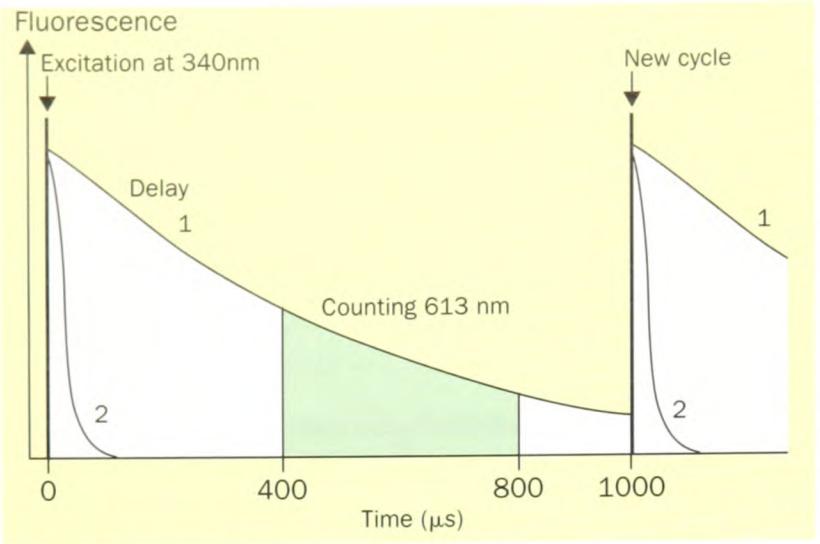

Figure 5. Measurement principle in time-resolved fluorescence
The cycle time is 1ms and pulsed excitation less than 1μs occurs at the beginning of each cycle. The delay time after the pulsed excitation is 400 μs and the actual counting time within the cycle has the same duration. The total measurement time is 1s. Curve 1 represents the fluorescence of the europium chelate and curve 2 the background fluorescence (actual decay time less than 1μs). Adapted from Lovgren and Hellstrand.[44]

labelled anti-species (eg, anti-rabbit) globulin to detect a specific reaction between virus antigen and specific antibody (eg, rabbit antiserum). These, or more complex formats, can be used to create highly sensitive detection systems with the application of TRF technology.

To appreciate how time resolution technology enables highly sensitive detection systems to be developed it is necessary to consider the theoretical basis for fluorescence-based assays. Certain molecules, known as fluorophores, can absorb light and emit it at a longer wavelength. At a particular wavelength of light there is maximum absorption, which in turn gives the maximum degree of excitation and emission. The difference between the excitation and emission wavelength is called the Stokes shift. The efficiency at which light is re-emitted is termed the quantum yield and can never be greater than one because there is always more energy (quanta) absorbed than is emitted in the form of fluorescence. Fluorescein is a commonly used fluorophore. It has a quantum yield of 0·7 and is most often used in its conjugated form, fluorescein isothiocyanate (FITC). Unfortunately, its wavelengths of maximum absorption (490 nm) and maximum emission (517 nm) are very close, making it difficult for any detection system to completely screen out incident light.

Immunoassay methods that use conventional fluorescent labels have been used, but they lack the high sensitivity of radioimmunoassays because of significant concentrations of background fluorescence due to materials—eg, plastic—and the presence of intrinsic fluorescence from many proteins—eg, in serum.[43] Such background fluorescence rapidly decays, and with lanthanide fluorescent chelates that have exceptionally large Stokes' shifts (>200 nm), decay times (>500 ns), and quantum yields (0·3–1·0), it is possible to distinguish between specific fluorescence and non-specific fluorescence and interference from incident light or light scattered due to proteins or colloids. Coupling the high resolution of specific fluorescence with high quantum yields has led to the development of TRF assays, which have sensitivity similar to, or greater than, radioimmunoassays and have a large dynamic range. The principle of TRF is illustrated in figure 5.

TRF assays have been applied to HIV antibody detection,[45–47] and have been shown to be highly specific and sensitive. There has also been interest in using such assays for the detection of rubella antibody[48,49] and diphtheria and tetanus antitoxins;[50,51] in all cases they have been shown to be highly sensitive and specific. TRF assays have also been used to detect various viral antigens,[52–54] but so far there are few data relating to the detection of viral antibodies. There are many examples of the use of TRF assays for detecting non-viral antibodies, enzymes, and hormones in oral fluid. TRF assays have also been used to measure concentrations of specific salivary IgA antibodies against mycobacterial heat shock protein 65,[55] levels of testosterone in saliva,[56] and to quantify carbonic anhydrase VI in saliva.[57]

Search strategy and selection criteria
Data for this review were identified by searches of Medline, references from relevant articles, and book chapters, and unpublished reports from colleagues within the PHLS. Search terms were "saliva", "oral fluid", "immuno-PCR", and "time-resolved fluorescence". We reviewed papers in English language only.

Conclusion

Specific antibody is routinely assayed in the microbiology laboratory for diagnosis and to detect evidence of past infection. In recent years assays have been modified for application to oral fluid. The main advantages of this are that sample collection is simple, economical, and non-invasive.

Antibody capture assays have so far mainly been used for this purpose and have been effective in surveillance and screening. However, the relative lack of sensitivity of these assays has mostly prevented them from being used diagnostically. Therefore, before oral fluid testing can be more widely adopted in diagnostic laboratories, technical improvements leading to tests with enhanced sensitivity are needed. This could have a major effect on the type of specimen that is used for diagnostic and reference work in the microbiology laboratory in the future, and would especially benefit patients because specimens could be obtained with minimum discomfort and distress.

I-PCR and TRF have been shown to extend limits for the detection of various microbial antigens and antibodies, but as yet, they have not been fully exploited for oral fluid diagnosis. Further work is needed to establish whether these approaches will improve existing methods based on antibody-capture assays.

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Protocol

A quantitative immuno-PCR assay for the detection of mumps-specific IgG

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Abstract

Sensitive assays are required for seroprevalence studies of measles, mumps and rubella (MMR)-vaccinated populations where many may have low levels of antibodies. This protocol describes a quantitative immuno-PCR assay to detect mumps-specific IgG antibodies. The purpose of the protocol is to determine the immune status of individuals to mumps. Mumps-specific IgG from a dilution of patients serum is bound by recombinant mumps nucleoprotein coated on the surface of microtitre plate wells. Bound antibody is detected by PCR using a conjugate of anti-human IgG covalently coupled to an oligonucleotide. The oligonucleotide is detected by the addition of target DNA, designed to hybridise to the oligonucleotide and serve as a template for real-time PCR using the LightCycler. The quantity of target DNA detected by the PCR depends upon the level of specific antibody in the test sample.
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Keywords: Mumps; Antibody detection; Immuno-PCR; Real-time PCR

1. Type of research

(i) The detection of human IgG antibodies to mumps virus is important for immunity surveillance to monitor the efficacy of vaccination programmes, to identify susceptible cohorts in the population and to shape future immunisation policies (Gay et al., 1997; Osborne et al., 2000). Immunoassays are the method of choice for viral antibody detection; however, current protocols lack the sensitivity required for certain protocols, such as measuring mumps-specific antibodies in oral fluid.

(ii) Immuno-PCR (iPCR) offers the potential for highly sensitive assays and has been used for the detection of a variety of antigens including cytokines (Saito et al., 1999; Sanna et al., 1995), tumour markers (Ren et al., 2001; Suzuki et al., 1995), hormones (Hendrickson et al., 1995; Joerger et al., 1995), toxins (Wu et al., 2001), microbial antigens (Kakizaki et al., 1996; Maia et al., 1995; Mweene et

Abbreviations: MMR, measles, mumps and rubella; iPCR, immuno-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; LC, LightCycler; TaqMan, ABI 7700 Sequence Detection System; tDNA, target DNA; rNP, recombinant mumps nucleoprotein; PBS, Phosphate-buffered saline; PBST, phosphate-buffered saline containing Tween 20; BSA, bovine serum albumin; SD, standard deviation.

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al., 1996b) and antibodies (McKie et al., 2002a). Instead of an enzyme as in enzyme-linked immunosorbent assay (ELISA), the technique utilises a DNA label that is detected by PCR. Several different ways of linking the DNA label to specific antibody have been described including: streptavidin–protein A chimeras, where streptavidin binds specifically to biotin residues on modified DNA and Protein A binds to the Fc portion of antibody (Sano et al., 1992); streptavidin or avidin, to link biotinylated DNA and biotinylated antibody (Case et al., 1997; Kakizaki et al., 1996; Maia et al., 1995; Mweene et al., 1996a; Numata and Matsumoto, 1997; Ren et al., 2000; Ruzicka et al., 1993; Saito et al., 1999; Sanna et al., 1995; Zhang et al., 1998; Zhou et al., 1993) and: antibody–DNA chimeras, where antibody is covalently bound to DNA, usually via heterobifunctional crosslinking agents (Hendrickson et al., 1995; Joerger et al., 1995; McKie et al., 2002a; Wu et al., 2001). In this protocol, an antibody–DNA chimera is used to detect mumps-specific antibodies (Fig. 1).

(iii) Detection of DNA is usually by PCR amplification followed by agarose gel electrophoresis. However, real-time PCR allows PCR amplification and analysis to be performed in a single reaction tube. The method uses fluorogenic probes to detect specific nucleic acid amplification products as they accumulate in real-time. A variety of probe systems have been described, including Bi-Probes (Gibson et al., 1999), hybridisation probes (Wittwer et al., 1997), hydrolysis probes (Heid et al., 1996) and molecular beacons (Tyagi and Kramer, 1996). All systems rely on the specific binding of probe to its target sequence, resulting in an increase in fluorescence that is monitored by a real-time PCR instrument, such as the Roche LightCycler (LC), the ABI 7700 Sequence Detection System (TaqMan) or the Bio-Rad iCycler Real-Time PCR Detection System.

2. Time required

The time required for the whole protocol is approximately 26 h:

(i) Preparation of antigen-coated wells (22 h)
(ii) Reaction with test sera, washing, followed by antibody detection (2 h)

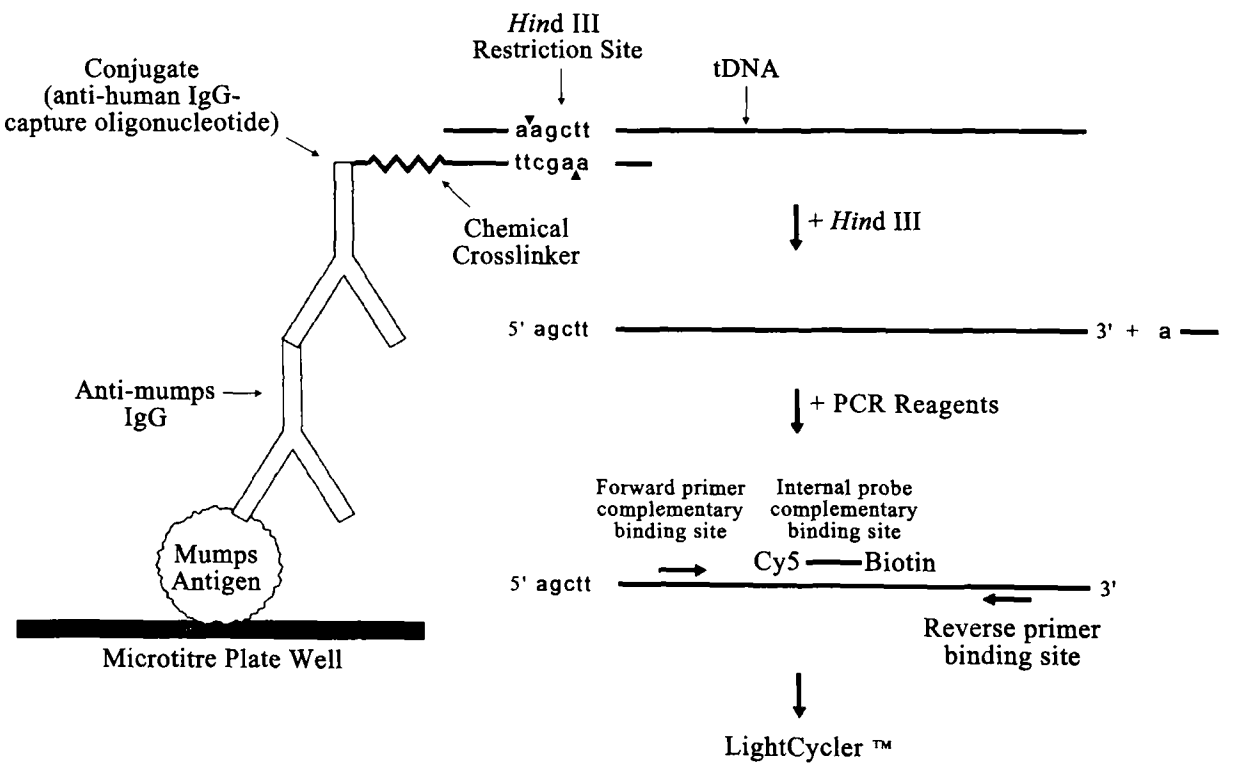

Fig. 1. Schematic diagram of the iPCR assay. Serum samples are incubated in wells coated with recombinant mumps nucleoprotein antigen. After washing, wells are incubated with a mixture of conjugate (anti-human IgG covalently coupled to a capture oligonucleotide) and tDNA, which is designed to hybridise to capture oligonucleotide. After washing, hybridised tDNA is released into solution by restriction enzyme digestion and subsequently quantified by real-time PCR using the LightCycler™. The internal probe is used to detect specific PCR product during amplification. It is labelled with Cy5 at the 5′ terminal and blocked with biotin at the 3′ terminal to prevent it acting as a primer during PCR.

(iii) Elution of target DNA (tDNA) by restriction digestion (1 h)
(iv) Preparation of PCR mastermix and standards (20 min)
(v) Real-time PCR on the LC (30 min).

3. Materials

3.1. Special equipment

Microtitre plate washer (Wellwash 4 Mk 2, Thermo Labsystems, Basingstoke, UK)
LightCycler instrument (Roche Diagnostics, Mannheim, Germany)
An incubator

3.2. Chemicals and reagents

Microtitre plate wells (MaxiSorp, Life Technologies, Paisley, UK)
Recombinant mumps nucleoprotein (Microimmune Ltd., Brentford, UK)
Anti-human IgG-capture oligonucleotide conjugate (Microimmune Ltd.)
Herring sperm DNA (Sigma, Poole, Dorset, UK)
*Hin*dIII (New England Biolabs (UK), Hitchin, Hertfordshire, UK)
Phosphate-buffered saline
8% w/v Sodium Azide buffer (Severn Biotech, Park Lane, Kidderminster, Worcestershire, UK)
Dried skimmed milk (Marvel, Cadbury's, UK)
Tween 20
SoluPro (Dynagel, Calcument City, IL)
Goat serum (Life Technologies)
Mumps IgG positive and negative control serum
Tubes for diluting reagents and serum
Capillaries (Roche Diagnostics)
Target DNA, capture oligonucleotide, primers and internal probe (Table 1) (MWG-Biotech, Milton Keynes, UK).
Platinum Taq DNA polymerase (Life Technologies)
SYBR Green I (Bio/Gene, Kimbolton, UK)
Bovine serum albumin
dNTPs (Life Technologies)
Nuclease-free water (Promega, Southampton, UK)

4. Detailed procedure

(i) Coat microtitre plate wells with 100 μl of 2 μg/ml recombinant mumps nucleoprotein (rNP) in phosphate-buffered saline containing 0.08% sodium azide, pH 7.2 (PBS azide). Incubate at 4 °C overnight (~18 h) and then at 37 °C for 2 h. Wash wells once with phosphate-buffered saline (PBS). Wells should be placed in a moist container during incubations and the wells should be washed using an automated plate washer. Add 250 μl of 5% SoluPro to wells and incubate at 37 °C for 2 h. Wash wells once with PBS. The wells can be used immediately or left at 37

Table 1
Nucleotide sequences of target DNA, primers and probes

Oligonucelotide sequence (5′→3′)[a]	Length	GC%	T_m (°C)
Target DNA TGCTCAAGCTTA**GGATCC**ATAGCATGACTA CGTGACTCGGTTGGAAGCTATACGTTCGATG GCTGCTGCATCAGTACGCAATCGGTATG	89	49.4	>75
Capture oligonucleotide CTAT**GGATCC**TAAGCTTGAGCATTT	25	40	59.7
Forward primer CATGACTACGTGACTCGGTT	20	50	57.3
Reverse primer CATACCGATTGCGTACTGAT	20	45	55.3
Internal probe Cy5-ATACGTTCGATGGCTGCTGC– biotin	20	55	59.4

[a] *Hin*dIII restriction digestion sites are shown in bold. The internal probe is labelled with the fluorescent dye, Cy5, and blocked with biotin to prevent extension during PCR.

°C overnight to dry. Store wells in a dry, sealed container.

(ii) Dilute test sera and controls 1/200 by adding 5 μl of serum to 995 μl of assay diluent (PBS containing 10% goat serum, 2% skimmed milk, and 0.1% Tween 20). Add 20 μl of test sera and controls to wells and incubate at 37 °C for 30 min. Wash wells 12 times with PBST.

(iii) Prepare the conjugate/tDNA mixture by adding 1 μl of 1/1000 dilution of conjugate (~150 ng/ml) and 50 μl of 10^{10} copies/μl of tDNA to 1 ml of assay diluent. Add 20 μl to wells and incubate at 37 °C for 1 h. Wash wells 12 times.

(iv) Add 1 U of *Hin*dIII in buffer to wells and incubate at 37 °C for 1 h.

(v) Transfer eluted tDNA to a fresh eppendorf and store at 4 °C until ready for testing.

(vi) Prepare external standards (10^4 to 10^8 copies/μl) by performing a 10-fold serial dilution of tDNA.

(vii) Prepare mastermix reactions for real-time PCR in a final volume of 10 μl containing 50 mM Tris–HCl, pH 8.3; 0.5 μg/μl bovine serum albumin (BSA); 3 mM $MgCl_2$; 200 μM dNTPs; 0.4 units Platinum *Taq* DNA polymerase; 1/10,000 SYBR Green I ; 0.5 μM each primer; and 0.25 μM internal probe. The standards and mastermix reactions for real-time PCR can be prepared during the incubation steps described above.

(viii) Add 1 μl of each of the external standards, test samples and nuclease-free water as a negative control, to 9 μl of mastermix.

(ix) Load reaction mixtures into glass capillaries and centrifuge at 1000 rpm for 5 s. Seal capillaries with the plastic plug and place in the LC for fluorescent monitoring.

(x) Programme the LC to perform an initial denaturation at 93 °C for 15 s, followed by 50 amplification cycles of four steps: 93 °C for 0 s; 55 °C for 0 s; 58 °C for 1 s; and 74 °C for 2 s. Measure fluorescence once per cycle after the 58 °C step. Use maximum ramp rates (20 °C/s) except for the transition from 55 to 58 °C when the ramp rate is 3 °C/s. Immediately after amplification perform a two-step melting programme: 95 °C for 0 s; and 45 to 95 °C at a ramp rate of 0.2 °C/s with continuous fluorescent monitoring (about 30 min).

5. Results

5.1. Real-time PCR

Data analysis is performed using the LC software. For quantification, a standard curve is constructed by plotting the log input copy numbers of the external standards (10^4–10^8 copies tDNA) against the crossing point cycle number (e.g. Fig. 2). The crossing point cycle number is calculated by determining the point at which the fluorescence exceeds a background level. The quantity of tDNA in test samples is determined by plotting the corresponding crossing point cycle numbers on the standard curve.

PCR product is identified by performing a melt after quantification. Plotting fluorescence as a function of temperature ($-dF/dT$) as the LC heats through the dissociation temperature of the product gives a

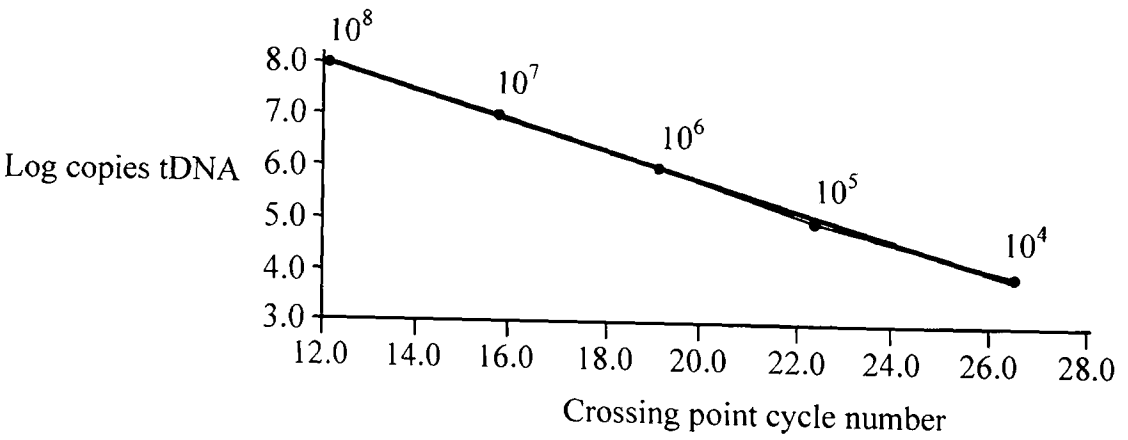

Fig. 2. Standard curve showing the correlation between the concentration of target DNA and the crossing point cycle number. The graph was derived by plotting the log of the standards containing 10^4–10^8 copies of tDNA against the crossing point cycle number.

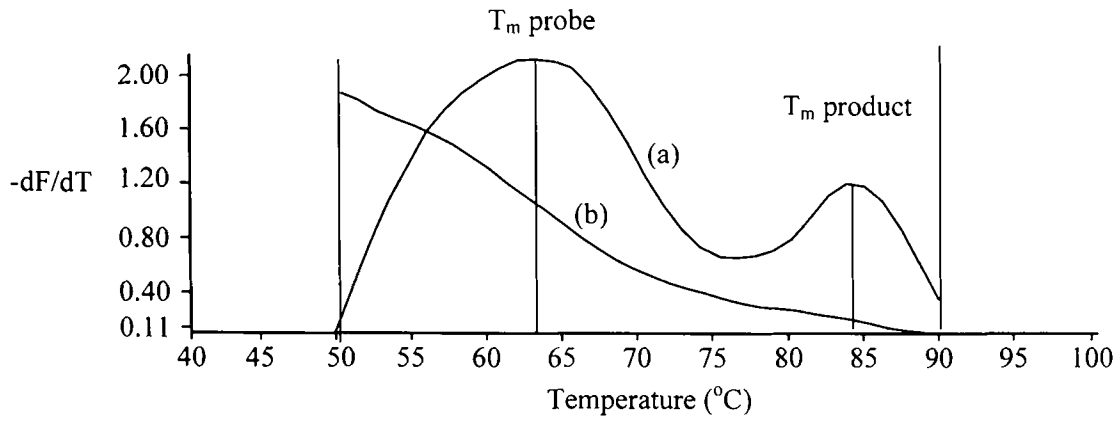

Fig. 3. Melting curve analysis of (a) an external standard (10^8 copies tDNA) and (b) the negative control. Melting peaks were derived by plotting the negative derivative of fluorescence ($-dF/dT$) with respect to temperature.

DNA melting curve. Fig. 3 shows melting curves of an external standard and the negative control. For the external standard, two peaks can be observed at 63 and 84 °C, corresponding to the melting temperature (T_m) of the internal probe/tDNA duplex and the full-length product, respectively. No peaks can be seen in the negative control.

5.2. Immuno-PCR

The iPCR results are quantified in terms of copies of amplified tDNA per μl eluted from each well. Runs can be accepted when the controls give the expected results and the standards are within 0.5 log of their target.

A cut-off for the assay can be determined by testing a panel of sera negative for mumps-specific IgG using a validated assay. The SD obtained can then be multiplied by a factor of 2 or 3 and added to the negative control tested in parallel with patient's samples to determine a positive cut-off for the run.

6. Discussion

6.1. Trouble-shooting

6.1.1. Low signal/noise ratios

The most common cause of problems is the use of a reagent at sub-optimal concentration, especially the conjugate and tDNA. Reagents should be titrated and used at their optimal concentration.

6.1.2. Nonspecific binding

Inadequate washing or blocking of wells can cause high levels of nonspecific binding and lead to false-positive results. Wells should be thoroughly washed between incubation steps. The use of blocking agents is essential to limit nonspecific binding of assay components. We found nonfat dried milk was the best blocking agent tested.

6.1.3. Contamination

A signal detected in the PCR negative control may be due to contamination of reagents or equipment with tDNA. If this occurs, fresh reagents should be used and the working area should be thoroughly cleaned. Ideally, separate areas should be used for antibody detection, preparation of the PCR master-mix, addition of tDNA to mastermix and real-time PCR. If the problem persists, the tDNA may be changed, as the nucleotide sequence is random.

6.2. Alternative/support protocols

6.2.1. Real-time PCR

In this protocol, a Bi-probe system (SYBR Green I/Cy5) was used to detect tDNA on the LC. However, an alternative probe system and/or real-time PCR machine may be used. For example, a hydrolysis assay could be developed for the TaqMan. The main advantage of the TaqMan is that 96 samples can be simultaneously tested in one run compared to 32 on the LC.

6.2.2. Linking antibody to capture oligonucleotide

An alternative to using the anti-human IgG-capture oligonucleotide conjugate would be to use streptavidin or avidin to link biotinylated capture oligonucleotide and biotinylated antibody, all of which can be obtained commercially.

6.2.3. Detection of mumps-specific IgG

In-house (Pipkin et al., 1999) and commercial ELISAs (Behring Enzygnost and Microimmune) are available for the serological detection of mumps-specific IgG. These are quick, easy to perform and less technically demanding than the iPCR assay described here. Alternatively, virus neutralising and haemaglutination inhibition assays may be used. These however are time consuming and are therefore unsuitable for population studies where large numbers of samples need to be tested.

7. Essential literature references

Original papers: Sano et al. (1992), Ruzicka et al. (1993), Zhou et al. (1993), Wittwer et al. (1997).

Review papers: Kim (2001), McKie et al. (2002b).

8. Quick procedure

(i) Coat microtitre plate wells with 100 μl of 2 μg/ml rNP in PBS azide.
(ii) Incubate at 4 °C overnight then at 37 °C for 2 h.
(iii) Block wells using 5% SoluPro at 37 °C for 2 h.
(iv) Incubate wells with 20 μl of 1/200 dilution of test samples and controls at 37 °C for 30 min.
(v) Wash wells 12 times with PBST.
(vi) Incubate wells with 20 μl of conjugate/tDNA mix (1/1000 dilution of conjugate and 10^{10} copies/μl of tDNA in assay diluent) at 37 °C for 1 h.
(vii) Wash wells 12 times with PBST.
(viii) Incubate wells with 20 μl of buffer containing 1 U *Hin*dIII at 37 °C for 1 h.
(ix) Prepare external standards (10^4 to 10^8 copies tDNA) and PCR mastermix reactions.
(x) Add 1 μl template to samples and load reactions into capillaries.
(i) Spin capillaries and place in the LC for fluorescence monitoring.

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