

The Development, Implementation And Evaluation Of A Real-Time PCR-Based Diagnostic Service For Viral Causes Of Infectious Intestinal Disease

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Declaration of Originality

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This thesis is submitted in partial fulfilment for the degree of Doctor of Philosophy. I declare that it has been composed by myself, and the work described is my own research.

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Abstract

Outbreaks and sporadic cases of viral Infectious Intestinal Disease (IID) are a major public health issue resulting in significant morbidity and sometimes mortality each year. The economic costs associated are substantial.

Laboratory diagnosis of viral IID is important as the many infectious and non-infectious causes cannot be reliably differentiated using clinical or epidemiological characteristics alone. An accurate diagnosis can aid patient management, infection control procedures and reduce health care costs by preventing unnecessary treatments, testing for alternative causes and hospital stay. It also aids public health surveillance.

At the start of the research described in this thesis the West of Scotland Specialist Virology Centre (WOSSVC) used Electron Microscopy (EM) as the frontline test for outbreaks and sporadic cases of IID. However, although rapid on a small number of samples, this technique has been shown to be insensitive, laborious and is not suited to testing large numbers of samples.

The research presented in this thesis sought to examine whether molecular diagnostic techniques such as conventional gel-based or real-time Polymerase Chain Reaction (PCR) assays could be a viable replacement for EM as the frontline test(s) for viral IID in a routine laboratory service of this type, and whether their implementation could bring benefits to the laboratory service in terms of improved rapidity, sensitivity and throughput. The aim was to adapt published PCR methods for use in routine diagnostic work rather than for research purposes, an approach that distinguishes this research from previous work in this area.

In order to achieve this aim, the appropriate PCR techniques were first selected from the literature, based on a combination of clinical and laboratory requirements, and were adapted for use in the laboratory service. A series of laboratory experiments was then carried out in order to compare the sensitivity of the adapted methods to existing techniques such as EM and antigen detection assays (EIAs) and to other methods that emerged during the period of study including alternative PCR assays. Where found to be suitable, the selected PCR tests were implemented in the routine diagnostic service for viral IID. The effects of these changes on the laboratory service were then examined.

The results show that since the introduction of molecular tests at WOSSVC for the detection of viral pathogens in cases of gastroenteritis the number of samples tested has risen steadily, as have the detection rates for each of the main viral causes of IID. Furthermore, this has been achieved at the same time as a substantial reduction in sample turn-around-times. Such improvements will have a positive impact in several areas of public health relating to viral IID and are discussed fully, including patient management, infection control and national surveillance.

List of Publications

Miller I, Gunson R, Carman WF. (2002). Norwalk like virus by light cycler PCR. J Clin Virol. Aug;25(2):231-2.

Gunson R, Miller J, Carman WF. (2003(i)). Comparison of primers for NLV diagnosis. J Clin Virol. Apr;26(3):379-80.

Gunson RN, Miller J, Carman WF. (2003(ii)). Comparison of real-time PCR and EIA for the detection of outbreaks of acute gastroenteritis caused by norovirus. Commun Dis Public Health Dec;6(4):297-9.

Gunson RN, Mackie P, Leanord A, Carman WF. (2003(iii)). First rotavirus, now astrovirus: the evolving benefits of RT-PCR. Commun Dis Public Health. Apr;6(1):66-7.

Gunson RN, Miller J, Leonard A, Carman WF. (2003(iv)). Importance of PCR in the diagnosis and understanding of rotavirus illness in the community. Commun Dis Public Health. Apr;6(1):63-5.

Gunson RN, Carman WF.(2005). Comparison of two real-time PCR methods for diagnosis of norovirus infection in outbreak and community settings. J Clin Microbiol. Apr;43(4):2030-1.

Gunson RN, Collins TC, Carman WF. (2006(i)). The real-time detection of sapovirus. J Clin Virol. Mar;35(3):321-2. Epub 2006 Jan 4.

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Abbreviations

A&E: Accident and Emergency

BMT: Bone Marrow Transplant

CSF: Cerebrospinal Fluid

Ct: Cycle Threshold

DNA: Deoxyribonucleic Acid

EIA: Enzyme immunoassay

FeCV: Felince calicivirus

HIV: Human immunodeficiency virus

HPA: Health Protection Agency

HPS: Health Protection Scotland

HSV: Herpes Simplex Virus

ICG: ImmunoChromatoGraphy

IEM: Immune Electron Microscopy

IID: Infectious Intestinal Disease

LA: Latex Agglutinations

mCMV: murine cytomegalovirus

MGB: Minor Groove Binding

ORF: Open Reading Frame

PAGE: Polyacrylamide Gel Electrophoresis

PCR: Polymerase Chain Reaction

QC: Quality Control

RNA: Ribonucleic Acid

RT: Reverse transcriptase

RT-PCR: Reverse transcriptase polymerase chain reaction

SCIEH: Scottish centre for infection and environmental health.

TRT: turn around time

VLP: Virus Like Particles

VTM: Viral Transport Medium

WOSSVC: West of Scotland Specialist Virology Centre

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Chapter 1

Introduction

1.1. Introduction

The thesis expounded in this research is that the implementation of the molecular method Polymerase Chain Reaction (PCR) for the diagnosis of the viral causes of outbreaks and sporadic cases of gastroenteritis can bring significant benefits to a routine laboratory service in terms of increased sample throughput, increased detection rates and rapidity of results.

Outbreaks and sporadic cases of Infectious Intestinal Disease (IID) are a major public health issue, resulting in significant morbidity and sometimes mortality each year. Outbreaks affect more than one person, whilst sporadic cases are individual cases that cannot be confirmed as belonging to an outbreak. The associated economic costs are substantial. A recent study estimated that IID costs the United Kingdom greater than £743 million each year (Roberts *et al*, 2003). This total was based on health-care costs alone, including the cost of hospitalisation, GP consultation, treatments and laboratory investigations. It did not include the cost to business in terms of lost workdays, the costs due to missed education or leisure time or the costs relating to public health interventions. Furthermore, the costs of outbreaks occurring in closed settings such as hospitals, residential homes and the food and leisure industry were not included. Few studies incorporate these factors, all of which are likely to increase the overall economic burden of IID significantly.

Laboratory diagnosis of IID is important, as its agents cannot be reliably differentiated using just clinical or epidemiological characteristics alone. Accurate diagnosis can aid patient management, infection control procedures and reduce health care costs by preventing unnecessary treatments, testing for alternative causes and hospital stay. Accurate diagnosis also aids public health surveillance. In the UK a number of NHS laboratories provide routine diagnostic services for IID. In general these laboratories offer testing for bacteria, parasites and viruses. The exact nature of the types of tests offered and the pathogens tested for tend to differ between laboratories. Specialist centres are also available that offer a more comprehensive testing service for some pathogen types.

Viral pathogens have been shown to be responsible for a significant proportion of the IID burden, particularly in sporadic cases in children and outbreaks in all age

groups. From a clinical standpoint, the ideal diagnostic test for viral IID in a routine diagnostic setting is one that gives an accurate diagnosis in a timeframe rapid enough to be useful to clinicians and public health officials. Ideally the test should also be high throughput (i.e. able to process a large number of samples simultaneously) as this will contribute to the overall test rapidity and is also more likely to mean that the test is robust when faced with the fluctuations in sample submissions seen with seasonal infections such as viral IID. From a laboratory standpoint the test should also be cost effective and easy to use.

Many methods exist for the diagnosis of viral causes of IID, which differ in sensitivity, rapidity, ease of use and throughput. Electron Microscopy (EM), for example, is a commonly used method in many diagnostic virology laboratories, mainly for historical reasons. However, although rapid on a small number of samples, it is limited in terms of sensitivity, is laborious and is not suited to testing large numbers of samples. Antigen detection methods such as enzyme immunoassays (EIA) and Immunochromatography (ICG) offer similar sensitivity to EM but with the potential for improved rapidity, ease of use and throughput. These methods however, are not available for all the main viral causes of IID.

In recent years, the molecular method Polymerase Chain Reaction (PCR) has been increasingly used in research on the main causes of viral IID. PCR assays are not currently offered by commercial companies but can often be more sensitive than either EM or antigen detection methods. Research studies utilising conventional gel-based PCR have determined that viral pathogens are the main causes of IID in children and contribute significantly to IID in adults and immunocompromised patients. Furthermore, use of this method has established that norovirus is the principle cause of outbreaks of IID. However, early versions of this assay were technically challenging to implement as they consisted of numerous steps, which in turn reduces the test rapidity, robustness and throughput. Furthermore, such assays require a strict laboratory set-up and as a result are expensive to implement. Thus, although it is now possible to reduce the number of steps involved in conventional PCR, the assay is rarely used as a frontline diagnostic test for IID in routine laboratory services and is still mainly used as a research tool.

Real-time PCR has the potential to overcome many of the pitfalls associated with the early PCR methods. Using real-time PCR, all or the majority of the numerous steps associated with PCR can be carried out in a single step. Reducing the number of

separate steps results in improved rapidity, ease of use and favours high throughput and automation. The costs of implementation, equipment and reagents have also reduced significantly in recent years as an increasing number of companies compete to sell to an increasing number of users. As a result, real-time PCR has characteristics that make it more likely to be of value in a routine diagnostic laboratory.

The West of Scotland Specialist Virology Centre (WOSSVC) is one such diagnostic laboratory. The WOSSVC is based at Gartnavel General Hospital in Glasgow and it is one of only two specialist virology centres in Scotland. It provides a virology service for the whole of Glasgow. In addition, over 40% of its workload is from health boards outside Glasgow. The users include hospital-based users, users in primary care and general practice and private industry. Each year over 200 000, predominantly viral, investigations are performed.

At the start of the research described in this thesis the WOSSVC used EM as the frontline test for outbreaks and sporadic cases of IID. Outbreak testing was carried out on samples from all age groups whilst for sporadic cases testing focused on samples from children less than 10 years of age and immunocompromised individuals.

The research presented in this thesis sought to examine whether conventional gel-based or real-time PCR tests could be a viable replacement for EM as the frontline test for viral IID in a routine laboratory service of this type, and whether their implementation could bring benefits to the laboratory service in terms of improved rapidity, sensitivity and throughput. The aim was to adapt published PCR methods for use in routine diagnostic work rather than for research purposes, an approach that distinguishes this research from previous work in this area.

First, the laboratory methods to be used for each step of the molecular service were selected from published scientific data based on the combination of clinical and laboratory requirements described earlier and knowledge of the equipment available in the laboratory. Following this the chosen PCR assays were adapted for use in the routine setting and evaluated against existing assays such as EM and commercial antigen detection methods. Since this is an evolving field of research, the assays were also compared to tests that emerged during the period of study including new EIA methods and alternative conventional and real-time PCR methods. Studies aimed at amending each assay, in order to reduce the turn-around-times and cost of each without affecting their sensitivity were also carried out. Based on this research several

changes to the routine service were implemented. The outcomes of these changes on the laboratory service in terms of sample throughput, detection rate and turn-around-times were then examined retrospectively.

This work will have wide applicability, as it will influence other diagnostic laboratories in Scotland and in other countries. It will also influence disease surveillance and patient management and form the basis for new services in future.

1.2. Thesis Outline

The structure of the thesis is as follows.

Chapter 2 provides a background to the research carried out in this thesis. It begins with an overview of the burden of IID in developing and developed countries and discusses the economic costs of IID. This is followed by a discussion of the five main viral causes of IID: adenovirus, astrovirus, norovirus, rotavirus and sapovirus. Aspects of these pathogens discussed include their virology, classification, elicited immune response, epidemiology, the clinical illness in which they result, methods of preventing this illness, infection control and treatment. The contribution of viral pathogens to the overall burden of IID in sporadic cases of IID in adults and children, outbreaks and asymptomatic individuals is then outlined, which provides the rationale for decisions taken later in the work concerning the patient groups tested.

The next section begins by outlining the importance of laboratory diagnosis in cases of IID and then discusses the ideal characteristics of a diagnostic test. Following this is a review of the available diagnostic assays such as EM, antigen detection, and conventional and real-time PCR. The chapter concludes with a description of the diagnostic service used at the WOSSVC for viral IID at the start of the research and outlines the aim of replacing this EM-based service with a PCR-based service in two parts: a norovirus RT-PCR as the frontline test for outbreaks of IID and PCR tests for both norovirus and the other viruses as the frontline test for sporadic IID in children less than 10 years of age and immunocompromised patients.

Chapter 3 discusses the PCR and associated methods that were selected for evaluation as the proposed replacements for the EM-based service, including the sample preparation methods stool extraction and nucleic acid extraction. This is followed by a detailed review of the published PCR methods for adenovirus, astrovirus, norovirus, rotavirus and sapovirus. This chapter then concludes by

outlining which of these PCR tests were adapted for routine use in the proposed molecular service and explains the factors that influenced their choice including published evaluation data, the clinical and laboratory characteristics of an ideal test and the equipment available in the laboratory at the time of development.

Chapter 4 begins with an explanation of how the new PCR assays were then assessed. The assessments are split into two parts: those that focussed on the development of a molecular service for outbreaks of IID; and those carried out with the development of a molecular service for sporadic cases of IID in mind. Each section contains a progressive series of controlled experiments comparing the adapted PCR methods (described in Chapter 3) to existing diagnostic methods such as Electron Microscopy (EM) and antigen detection methods, and to newer methods that emerged as the project progressed.

The outbreak section begins by comparing the selected norovirus real-time PCR method to EM for testing samples from outbreaks of IID. This PCR method is then compared to two EIA methods and then two alternative PCR methods for norovirus: a conventional gel-based method recommended by a European collaboration in 2003, and a real-time PCR method using dual-labelled probes (published in 2003). The results of each comparison are discussed and the resultant changes to the WOSSVC diagnostic service are described.

The section on the development of a routine sporadic service begins with an experiment attempting to multiplex gel-based methods for the remaining four viral pathogens into a smaller number of assays. The resultant assays are then compared to antigen detection methods for rotavirus and astrovirus before being compared to four different dual-labelled probe-based PCR methods. The results of each comparison and the resultant changes to the diagnostic service are then discussed. This section concludes with the description of a number of experiments aimed at reducing the turn-around-times and cost of the resulting service without affecting the sensitivity of the assays.

Chapter 5 contains an analysis of the effects of these test developments on the WOSSVC diagnostic service. The chapter begins by summarising the changes made to the service between 1999-2006 as a result of the research described above. Various measures are then used to examine the outcomes of the service developments, including the number of samples submitted for testing (examined by total, by health board of origin and by age group), the number of detections made, the number of

outbreaks classified as either positive or negative, and the turn-around-times of the results. These data are examined for both the outbreak and sporadic service and are cross referenced with the service changes to determine whether these resulted in improvements in sample throughput, test sensitivity and rapidity of results. Contributing to this analysis are the results of a questionnaire, which asked laboratories in Scotland to outline their testing protocols for outbreaks and sporadic cases of IID. This questionnaire examined how other laboratories use the WOSSVC service and examined whether the developments described have resulted in changes to their own services.

Finally, a thorough discussion of all the research findings is presented in Chapter 6. Included in this are the likely outcomes of the service developments on non-laboratory aspects such as patient management, infection control and national surveillance. A discussion of the potential role of the molecular methods in new services for the prevention and investigation of outbreaks based on testing non-patient samples such as environmental samples is also included.

Chapter 2

Background To The Research

2.1. Overview

This chapter outlines the importance of Infectious Intestinal Disease (IID) in public health. Its burden in terms of morbidity and mortality worldwide is discussed followed by an in-depth review of the economic cost of IID in developed countries. The main viral causes of IID are then reviewed with particular focus on each pathogen's virology, classification, elicited immune response, epidemiology, the clinical illness in which they result, methods of preventing this illness, infection control and treatment. The contribution viral pathogens make to overall IID in children, adults and outbreaks is also discussed. The next section begins by describing the characteristics of an ideal diagnostic test for viral IID before outlining the different methods available. Electron Microscopy (EM), antigen detection assays, and Polymerase Chain Reaction (PCR) methods such as conventional gel-based and real-time PCR are all described. The advantages and disadvantages of each test type are described, together with data on their relative performance. The chapter concludes with a description of the West of Scotland Specialist Virology Centre (WOSSVC) diagnostic service for viral IID at the start of the research followed by a description of the thesis aims for the improvement of this service using molecular methods.

2.2. Importance Of Infectious Intestinal Disease (IID)

2.2.1. Mortality And Morbidity Of IID Worldwide

Infectious Intestinal Disease (IID) results in a substantial burden of morbidity and mortality worldwide. It is responsible for approximately 4 billion cases of diarrhoea and 3 to 4 million deaths each year (Farthing, 2000; World Health Organisation 1996; World Health Organisation 1994).

In both the developing and the developed world the majority of illness and death is borne by infants and children, with the risk increasing as the weaning stage ends (Kosek, Bern and Guerrant, 2003). Children in developing countries experience most of the total annual burden of IID (Parashar *et al*, 2003). In economically deprived areas such as the Indian subcontinent, Africa and Latin America children can experience up to 10 episodes of IID per year. Repeated cases in children leads to malnutrition, which can result in impaired growth and development, and death. Most

deaths are a result of excessive fluid and electrolyte loss leading to severe dehydration and acidosis (Farthing, 1998) although deaths can easily be avoided by the prompt administration of fluid and electrolytes. The economic burden of treating IID in developing countries often takes up a substantial proportion of the nation's health-care budget.

The elderly are also at increased risk of severe IID. This is thought to be a result of decreasing immune and gastric acid protection. Others at risk of severe IID include the immunocompromised (e.g. HIV positive individuals, solid organ transplant and bone marrow transplant recipients), individuals with congenital abnormalities and travellers (Faruque *et al*, 2004; Mattila *et al*, 1992).

2.2.2. Morbidity And Mortality Of IID In The Developed World

In developed nations sporadic cases and outbreaks of IID result in significant morbidity and sometimes mortality. Sporadic cases are those that cannot be identified as belonging to a particular outbreak. In the US an estimated 79 million cases of sporadic IID occur each year resulting in approximately 325,000 hospitalisations and around 5000 deaths (Frenzen, 2003; Mead *et al*, 1999). The mortality rate is significant when compared to the mortality rates of other infection related deaths in the US. For example, cervical cancer, rheumatic fever and hepatitis B virus result in approximately 4400, 4800 and 4400 deaths per year respectively (American Cancer Society, 2001; American Heart Association, 2001; Kane, 1995). Studies carried out in other developed countries have also highlighted a substantial burden of sporadic IID. For example, each year Australia experiences an estimated 15 million cases of sporadic IID resulting in more than 1 million GP visits and more than 1 million missed workdays (Hellard *et al*, 2003). In the Netherlands an estimated 4.5 million episodes of sporadic IID occur each year (Wit MAS *et al*, 2001). As a result of these, more than 80,000 patients require hospitalisation and approximately 300,000 workdays are missed. A study of sporadic IID in England estimated there to be approximately 9.4 million cases each year resulting in 1.5 million GP visitations (Wheeler *et al*, 1999). A telephone study in Ireland concluded that an estimated 3.2 million cases of IID occurred in 2003 (Acute Gastroenteritis in Ireland, North and South - A Telephone Study, 2003).

Outbreaks of IID (where multiple individuals are affected) are also an increasingly recognised problem in developed countries (Meakins *et al*, 2003;

Fankhauser *et al*, 2002; Lopman *et al*, 2003). An assessment of outbreaks reported to the Health Protection Agency (HPA) from 1991-2000 showed that there were more than 5000 outbreaks in England and Wales during this time. This total is likely to be a huge underestimate of the total number of outbreaks as many are not detected or reported each year. Over 50% of the outbreaks that were reported occurred in hospitals and residential care homes. Due to the general poor health of many of the patients present in hospitals and care homes the risk of significant morbidity and death in these settings as a result of IID is increased (Goller *et al*, 2004). Outbreaks in the food and leisure industry are also common with large outbreaks reported in holiday camps, hotels, restaurants and cruise ships (Koopmans and Duizer, 2003; Lawrence, 2004).

Few studies to date have examined the short and long term sequelae in patients with clinically diagnosed IID. Cumberland *et al* followed up a large cohort of patients with sporadic IID and found that a significant proportion had symptoms that persisted for longer than previously described (Cumberland *et al*, 2003). For example, over 25% of patients had persistent diarrhoea 3 weeks after onset, approximately 12% had persistent vomiting and around 33% of adults and children had continued abdominal pain. As a result patients were more likely to request a repeat consultation with their GP within 3 months of the initial episode. This was especially true in children (those aged under 5 years) and the elderly (aged over 60 years). There was also an increased rate of hospitalisation and subsequent diagnosis of irritable bowel syndrome in patients who had suffered IID. Other studies have also shown an increased risk of irritable bowel syndrome after IID (Parry *et al*, 2003). Helms and colleagues followed a cohort of 48,547 patients in Denmark with diagnosed IID (Helms *et al*, 2002). Patients with IID were shown to have an increased risk of mortality both in the short and long term. The increased risk was mainly as a result of complications such as invasive illness (septicaemia, endocarditis, vasculitis, septic arthritis, etc), intestinal perforation, abscesses, and complications of surgery. These complications occurred weeks or months after the initial IID episode.

2.2.3. The Economic Cost Of IID In Developed Countries

The substantial morbidity caused by IID in developed countries has a significant economic impact. Many studies have attempted to measure the overall economic burden of sporadic IID. Most have based their estimates on the cost of

medical treatment (hospitalisation, Accident & Emergency visitation, GP/physician visitations and prescriptions/treatments), missed workdays (due to illness or having to care for a relative), laboratory investigations, and transport (of the affected individual to the GP/hospital). Garthright *et al* estimated the cost of sporadic IID in the US to be greater than \$23 billion per year (Garthright, Archer and Kvenberg, 1988). Similar high totals have been estimated in Australia (more than \$1000 million Australian dollars per annum), Canada (\$1.1 billion US dollars per annum), the Netherlands (345 million euros per year) and England (£743 million) (Hellart *et al*, 2003; Van den Brandhof *et al*, 2004; Roberts *et al*, 2003; Todd, 1989). No study has yet measured the cost of sporadic IID in Scotland but it is likely to be substantial.

Most of the published studies described above are likely to be underestimates as many do not include the costs to business, the costs of missed education (e.g. at school/university), lost leisure time (e.g. interrupted evenings, weekends and holidays), and public health interventions (the costs of which are likely to be substantial). Furthermore these studies do not include costs relating to post IID complications such as repeat GP consultation or hospital admission (described above).

The cost of outbreaks of IID moreover, has not been included in many of the aforementioned studies. Outbreaks of IID in hospitals are a substantial burden and can often affect multiple wards, resulting in ward closure, cancelled operations and even hospital closure. Few studies have attempted to measure these costs. A recent study by Lopman *et al* estimated the cost of health-care associated outbreaks of norovirus by monitoring 3 hospital sites in Avon, England during 2002 (Lopman *et al*, 2004). In total, outbreaks of IID caused by norovirus were found to have cost more than £115 million-12.5% of the total Health care Associated Infection (HAI) budget or 1% of the total NHS expenditure on in-patient service.

Zingg *et al* estimated the cost of a single nosocomial outbreak of norovirus (Zingg *et al*, 2005). The costs were estimated based on various factors including nursing care, staff sick leave, laboratory testing and the infection control team costs. In total, this single outbreak was estimated to cost the US health care system over \$40,000. A recent study in England estimated the cost of a single norovirus outbreak to be greater than £280,000 (Cooke, Goddard and Golland, 2002).

The cost of outbreaks of IID to the food and leisure industry is also likely to be substantial. Large outbreaks result in mass cancellations, emergency treatment, refunds and compensation proceedings. Any accompanying bad publicity is likely to

affect future bookings. However, no studies have so far attempted to measure these costs.

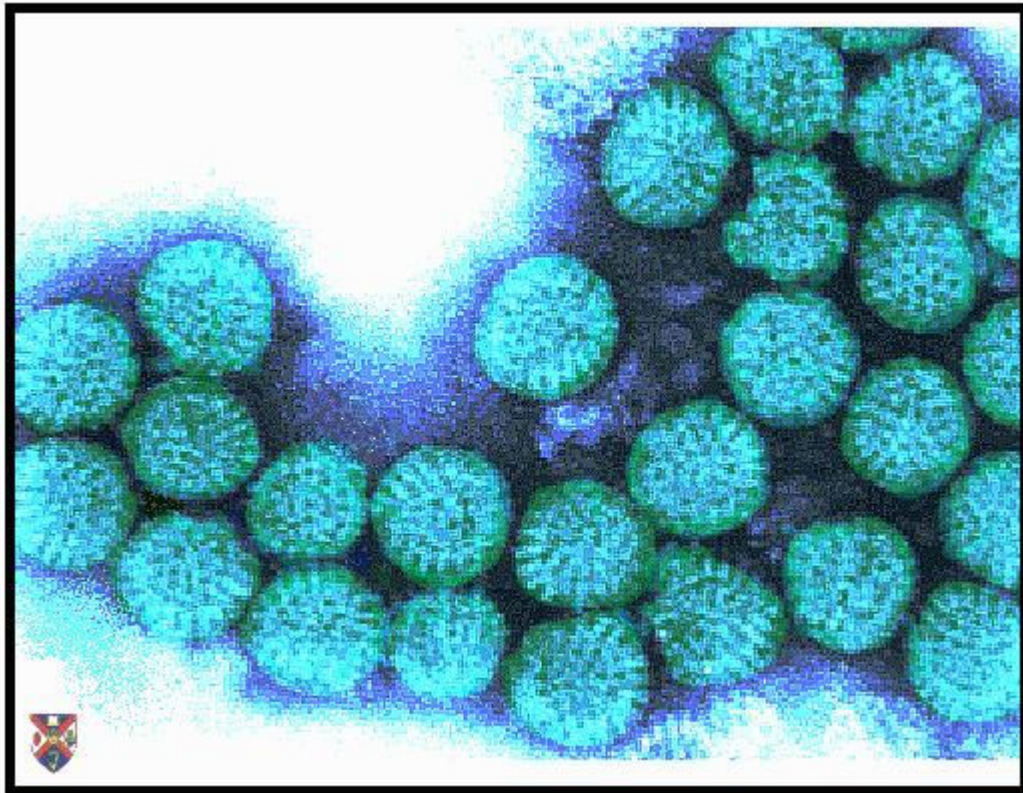
2.3. Viral Causes Of IID

There are at least 5 viral causes of IID: rotavirus, norovirus, sapovirus, astrovirus and adenovirus. Below is a detailed review of each of the pathogens virology, their classification, their elicited immune response, their epidemiology, the clinical illness they cause and various methods aimed at preventing, controlling and treating these infections due to these causes. This review will show that each of these pathogens is genetically complex with genomes that contain areas of significant variability between the many types and subtypes. This is important when considering the design of molecular assays, which is a major part of the next chapter. The complex immune responses elicited are highlighted to show that repeat infections can occur with each virus and that infections can occur in all age groups. Their numerous sources and transmission routes are also described in order to highlight how difficult these pathogens are to control and prevent. Together, these factors ensure that these viral pathogens contribute significantly to sporadic illness and outbreaks in all age groups.

2.3.1. Rotavirus

Rotaviruses were first discovered as a cause of human illness in 1973 by Bishop and colleagues using electron microscopy (EM) to examine duodenal biopsy specimens from children with gastroenteritis (Bishop *et al*, 1973). Rotaviruses are members of the *Reoviridae* family. They are icosahedral, non-enveloped viruses of 70-75nm in diameter. The rotavirus capsid is composed of 3 concentric protein layers that enclose the viral genome. Viewed under the EM rotavirus particles have a characteristic “wheel-like” appearance (Figure 1) from which their name is derived (the latin for wheel is “rota”).

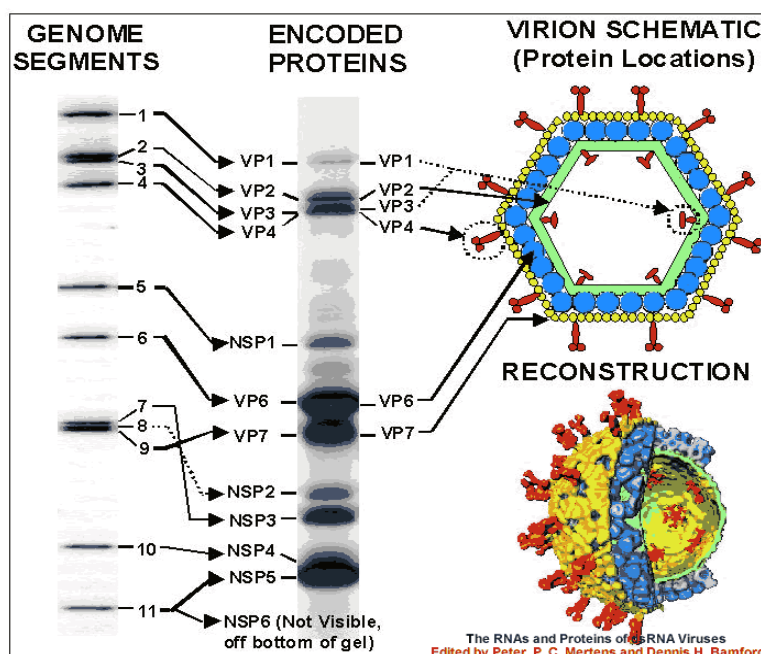
Figure 1: Rotavirus as viewed under electron microscopy (Copyright 1994 Veterinary Sciences Division)



2.3.1.1. Genome Organisation and Function

Rotaviruses possess a genome made up of 11 segments of double stranded RNA of approximately 18,850 base pairs in length. Each segment codes for 1 protein except segment 11, which codes for 2. Of the 12 proteins, 6 are structural (VP1-4, 6 and 7) and 6 are non-structural (NSP1-6) (Figure 2). At present the role of a number of these proteins are unclear.

Figure 2: The genome segments and encoded proteins of rotavirus.



Structural Proteins

VP1 is coded for by gene segment 1. It is a minor component of the central core making up 2% of total virion mass. Comparisons of different rotaviruses have shown VP1 to be highly conserved both at amino acid and protein levels. It is the largest polypeptide and shares consensus sequences with several well-characterised RNA dependent RNA polymerases (Valenzuela *et al*, 1991).

VP2 is encoded by gene segment 2 and alongside VP1 and VP3, makes up the inner core of the rotavirus. It is the most abundant protein of the inner core and it encloses the VP1, VP3 and genome (Jayaram *et al*, 2004). It is highly conserved and of the 3 proteins that make up the inner core it is the only one that has nucleic acid binding activity. VP2 interacts with VP6 in order to transport metabolites in and nascent RNA out during transcription (Prasad *et al*, 1988). VP2 is also thought to have a role in the assembly of the rotavirus particles and is necessary for replicase and transcription activity (Mansell and Patton, 1990).

VP3 is encoded by gene segment 3. It is a minor component of the central core. It has sequence homology to other viral polymerases and is thought to have guanyltransferase and methyl transferase activity (Chen *et al*, 1999; Liu *et al*, 1992).

The VP4 protein is 775 amino acids long and is the product of gene sequence 4. Cryo-electron microscopy studies have shown that for every 780 copies of VP7

present in the outer layer, there are 120 copies of VP4 (Jayaram *et al*, 2004). It appears as spikes on the capsid surface and is implicated in cell entry, attachment, haemagglutination, neutralisation and virulence (Ciarlet and Estes, 2001). The VP4 region contains several variable regions that define the P serotype (Gorziglia *et al*, 1990).

The VP6 protein is encoded by gene segment 6. It is the major constituent of the intermediate layer which contains 260 trimers of VP6 arranged as hexamers on a T=13 icosahedral lattice (Mathieu *et al*, 2001; Jayaram *et al*, 2004). It is sandwiched between the outer T=13 layer (VP4/7) and the inner T=1 (VP2) layer. The VP6 is essential for transcription of the genome. It also has a structural and immunological role and is responsible for the wheel-like appearance of the rotavirus (Greenberg *et al*, 1983; Kohli *et al*, 1987). Although VP6 is more conserved than either VP7 or VP4, this region contains variable regions that are used to determine the group (A-G) and subgroup antigens (Sg1, Sg2, non Sg 1 or 2, and Sg1+2).

VP7 is encoded by gene segment 9 and is the only structural glycoprotein. It forms the outer layer of the viral capsid and comprises 30% of total virion mass (Jayaram *et al*, 2004). Cryo-electron microscopy reconstructions have identified that the outer layer comprises of 780 copies of VP7 which are located at the local and strict fold axis at T=13 (Prasad *et al*, 1990; Jayaram *et al*, 2004). The VP7 may modulate the VP4 function during cell attachment and entry and may also interact with cell surface molecules after VP4 attachment (Beisner *et al*, 1998; Mendez *et al*, 1996; Mendez *et al*, 1999). Comparative studies of various rotaviruses have identified at least 6 (A-F) or 9 discrete regions within the VP7 (VR1-9) that show significant amino acid divergence (Gouveau *et al*, 1990; Green *et al*, 1987). These areas are the target of type-specific and cross-reactive neutralisation antibodies and are used to determine the rotavirus serotype (G type). Genotypic classification based on the VP7 region has been shown to match serotype (Gouveau *et al*, 1990).

Non-Structural Proteins

NSP1 is coded for by gene segment 5. At present its role is unknown but it is thought to have a role in modulating the innate immune response by degrading IFN-3 (Barrow and Patton, 2005). Sequence analysis shows that this region contains extensive sequence variation, although there is a 88 amino acid region present that is conserved amongst all rotaviruses (Xu *et al*, 1994).

NSP2 is encoded by gene segment 8. As with NSP1 its role is currently unclear although it is proposed to have a role in RNA replication and packaging (Gombold *et al*, 1985). Studies have also showed it to have nucleoside triphosphate activity. Sequence comparisons of mammalian rotaviruses have shown a high level of amino acid conservation of over 83% (Patton *et al*, 1993). The last 75 nucleotides at the 5' end and 23 nucleotides of the 3' end are nearly identical among rotaviruses.

NSP3 is coded for by gene segment 7. It has shown to be an RNA binding protein and therefore it has been proposed that it may have a role in re-initialisation of translation protein synthesis (Boyle and Holmes, 1986). Others have suggested that it may have a role in viral replication in the gut and then subsequent spread to other tissues (Mossol and Ramig, 2002). Sequence analysis of the NSP3 shows that more than 75% is conserved amongst mammalian rotaviruses (Rao *et al*, 1995). However, using this method, rotaviruses can be split into 3 evolutionary groups: SaII, Wa and S2. Of particular interest are the eighty nucleotides in the 3UTR region, which are highly conserved (Pang *et al*, 2004).

The NSP4 is coded for by gene segment 10. It is the most studied NSP. Studies have shown it to interact with both VP6 and VP4 and it has been suggested that it may be involved in outer capsid assembly (Lynn *et al*, 2005). Several studies have suggested that NSP4 is also a viral enterotoxin (Parr *et al*, 2006; Ball *et al*, 2005). The proposed mechanism of action is via the activation of a signal transduction pathway resulting in altered intestinal epithelial transport, membrane destabilisation and altered calcium levels in the endoplasmic reticulum (Tian *et al*, 1995; Ball *et al*, 1996; Horie *et al*, 1999). Studies using fusion proteins, containing 86-175 aminoacids of the murine NSP4 (expressed in E-coli), were found to cause severe diarrhoea when given to CD1 mice. Furthermore, sequence differences in the NSP4 have been observed between symptomatic and asymptomatic rotavirus infections (Kirkwood *et al*, 1996; Ward *et al*, 1997). However, others have failed to confirm either of these findings (Angel *et al*, 1998). Sequence analysis of the NSP4 shows there to be 2 hyper variable regions allowing rotaviruses to cluster into 2-5 groups (Cunliffe *et al*, 1997; Kirkwood *et al*, 1999). There are two main groups for human rotaviruses (A and B). These correlate with the VP6 subgroups-VP6 Sg1 matches with NSP4 A and VP6 Sg2 matches with NSP4 B (Iturriza-Gomara *et al*, 2003).

NSP5 is encoded by gene segment 11. It is the only segment with more than one Open Reading Frame (ORF) (Mitchell and Both, 1988). These are overlapping.

The ORF1 is 198 amino acids in length and the ORF2 is 92 amino acids. ORF2 codes for NSP6. At present their roles are unclear. Transient expression of this protein in uninfected cells has provided evidence for its participation in the formation of “viroplasms”, which are thought to be key structures for the replication of rotavirus (Lopez *et al*, 2005).

2.3.1.2. Classification

Serological cross reactivity of the inner capsid protein VP6 has determined there to be 7 groups of rotavirus (A-G) (Greenberg *et al*, 1983; Kapikian *et al*, 1981). Groups A-C are human and animal pathogens whereas the remainder are pathogens of animals only (Estes, 2001). Group A rotaviruses can be further divided into four subgroups (subgroup 1, subgroup 2, subgroup 1+2, subgroup non 1 and non 2) based on reactivities with two VP6 specific monoclonal antibodies. Subgroup 1 includes mostly animal and some human rotaviruses. Subgroup 2 includes mostly human rotaviruses, whereas subgroup 1+2 and subgroup non 1 and non 2 are mostly animal pathogens and are rarely associated with infection in humans.

Strains of rotavirus are classified by the analysis of VP4 and VP7 surface proteins using a similar method to the one that is used to classify influenza. Using this method rotaviruses are attributed G types (based on the VP7 Glycoprotein) and P types (based on the VP4 Protease sensitive protein). So far, at least 14 G types and 20 P types have been recognised (Rao, Gowda, and Reddy, 2000). This enormous antigenic and genetic diversity is the result of a number of mechanisms. The principle method of generating rotavirus diversity is thought to be re-assortment (known as antigenic shift) (Iturriza-Gomara *et al*, 2001). VP4 and VP7 are coded for on different RNA segments. Dual infection of a host by different rotavirus strains can lead to re-assortment and the subsequent emergence of new G and P type combinations. Dual rotavirus infections are common. A recent study in the UK found that 2% of rotavirus infections were of mixed type (Iturriza-Gomara *et al*, 2000). Rates of mixed infection in other countries are thought to be higher (Ahmed *et al*, 1991; Unicomb *et al*, 1999; Jain *et al*, 2001). Although intra-group re-assortment has been described on a number of occasions it has yet to be documented for different rotavirus groups. One hypothesis is that rotavirus groups differ in their genome end terminal RNAs and this may prevent inter group re-assortment.

Other methods of generating rotavirus diversity are thought to include the accumulation of point mutations (known as antigenic drift) and recombination (Blackhall *et al*, 1996; Desselberger, 1996; Ramig, 1997).

The introduction of animal rotaviruses or animal/human strain re-assortments has also been raised as possible contributors to rotavirus diversity (Cook *et al*, 2004; van der Heide *et al*, 2005; Palombo, 2002). Studies have demonstrated that animal rotaviruses can cause symptomatic infection in humans (Desselberger *et al*, 2001; Theil, 1990; Kojima *et al* 1996; El-Attar *et al*, 2001). For example, Nakagomi and Nakagomi found a feline G3 rotavirus infection in a child with a pet cat (Nakagomi and Nakagomi, 1989). Molecular studies on human rotavirus type G9 have shown it to share genetic similarities to porcine strains thus raising the possibility that it may have been recently introduced from pigs (Iturriza-Gomera *et al*, 2000; Hoshino *et al*, 2005; Teodoroff *et al*, 2005). The clinical and epidemiological characteristics of G9 infection compliment this view as it tends to occur in older age groups and cause more severe infection when compared to common rotavirus strains (Widdowson *et al*, 2000; Cubitt, Steele and Iturriza, 2000; Iturriza-Gomara, Kang and Gray, 2004). Furthermore this type had not been detected in the UK prior to 1995. Dual rotavirus infection with animal and human types has also been demonstrated (Nakaomi *et al*, 1994; Holmes *et al*, 1999).

2.3.1.3. Immune Response

After primary infection, the immune response to rotavirus is mainly humoral. This response is thought to be homotypic although there is partial protection against other serotypes (Gorrell and Bishop, 1999). This has been confirmed by volunteer transmission and vaccination studies that have shown that natural infection or vaccination protects from severe disease in subsequent infections (Davidson, Hogg and Kirubakaran, 1983; Bishop *et al*, 1983; Hjelt *et al*, 1985; Bhan *et al*, 1993; Ward and Bernstein, 1994). However, although partial protection is elicited after primary infection subsequent infections occur throughout life. Valazquez and colleagues examined 200 Mexican infants over a 5-year period (Velazquez *et al*, 1996). By 2 years of age approximately 96% of infants had had a primary rotavirus infection. Over the same period approximately 70% had a second rotavirus infection and by 5 years of age over 10% had suffered greater than 5 episodes of rotavirus. Cases of rotavirus in adults are being increasingly recognised, again highlighting partial protection

(Gonzalez-Losa *et al*, 2001; Wenman *et al*, 1979; Echeverria *et al*, 1983; Cubitt and Holzel, 1980; Holzen *et al*, 1980; Dupuis *et al*, 1995; Linhares *et al*, 1981). Some studies have shown that protection correlates with the formation serum IgA antibodies targeting VP4 and VP7 antigens and the VP6 capsid antigen (Ward, Bernstein and Shukla, 1989; Hjelt *et al*, 1987). Other studies have highlighted the importance of IgG, IgA and IgG or neutralising antibody (Clemens *et al*, 1992; O’Ryan *et al*, 1994; Velazquez *et al*, 2000). Cytotoxic T Lymphocytes (CTL) responses have been detected but their exact role has yet to be determined (Chiba *et al*, 1986; Chiba *et al*, 1993).

2.3.1.4. Epidemiology

Group A rotavirus has a worldwide distribution and is the predominant cause of childhood gastroenteritis in the developed and developing world. In temperate regions rotavirus infections occur predominantly during the winter months (Cook *et al* 1990). However, a recent study highlighted that the seasonality of rotavirus infections in Japan has shifted from winter to early spring (Suzuki *et al*, 2005). The reason for this is unclear. The seasonality is less defined in tropical areas with infections occurring all year round (Iturriza-Gomarra *et al*, 2000; Kapikian *et al*, 1976).

Sources of rotavirus infection include individuals with asymptomatic infection. A recent case control study by de Wit *et al* detected rotavirus in 0.6% of control samples (de Wit *et al*, 2001). More recent studies using increasingly sensitive tests have detected higher rates (Amar *et al*, 2007). Individuals with long term shedding (4-57 days) and severe illness (as they have been shown to have a higher viral load in faecal samples (Richardson *et al*, 1998; Kang *et al*, 2004)) are another source of illness. Recent evidence also suggests interspecies transmission of rotavirus as numerous animal and human strains have been shown to share genetic and antigenic features (see section 2.3.1.2).

Group A rotavirus has a low infectious dose (Goldman, 1992; Bishop, 1996). As a result transmission is person to person and occurs mainly via the faecal oral route (Al Frayh *et al*, 1987; Dennehy, 2000; Vipond, 2001). Evidence also points towards spread via the respiratory route (Price *et al*, 1986; Goldwater, Chrystie and Banatvala, 1979). Studies using aluminium surfaces have shown rotavirus to remain stable for up to 60 days, suggesting transmission via contaminated fomites (Sattar *et al*, 1986;

Koopmans and Duizer, 2004; Abad, Pinto and Bosch, 1994). Other forms of transmission include contaminated food and water.

Most rotavirus infection occurs in infants greater than 3 months of age (Sharma *et al*, 2002). Infants under 3 months can have symptomatic infection but most seem to be protected from severe infection, probably as a result of maternal antibody (Crawley *et al*, 1993).

Adults are also at risk of rotavirus infection. However, although adults may obtain infection from the sources described above, the greatest risk is from rotavirus-infected children (Zissis *et al*, 1976; Wenman *et al*, 1979; Grimwood *et al*, 1983; Anderson and Weber, 2004). Wenman *et al* showed child to parent transmission to be a common event, with 36 of 102 adults who had children with rotavirus infection also found to be positive for the virus. Infection has also regularly been documented in medical staff that are in frequent contact with rotavirus-infected infants (Samadi, Huq and Ahmed, 1983; Hjelt *et al*, 1985). As a result the seasonality of adult infection probably reflects that of children (Nakajima *et al*, 2001). However, Cox and Medley tested routine serum samples from hospitalised adults and detected IgM to rotavirus throughout the year thus suggesting a sporadic distribution similar to that in tropical areas (Cox and Medley, 2003).

Other adults at risk of rotavirus infection include travellers (Keswick *et al*, 1982; Vollet *et al*, 1979; Bolivar *et al*, 1979). A recent study of travellers returning from Jamaica with diarrhoea found that rotavirus infection was second only to Enterotoxigenic E-coli (Steffen *et al*, 1999).

Outbreaks of rotavirus have been documented but are rare (MMWR, 2000; Halvorsrud and Orstavik, 1980; Abbas and Denton, 1987; Friedman *et al*, 1988; Foster *et al*, 1980). Closed settings such as hospitals, care homes, and day care centres are at particular risk.

Evidence from molecular epidemiological studies has shown that different G types co-circulate at any location at any given time. A study of UK strains detected between 1995-1999 found a total of 15 different strains circulating during this period. Over 95% were of types G1-4 (Iturriza-Gomara *et al*, 2001). This epidemiological pattern has also been seen in other countries. Santos and Hishino examined 45,571 strains from 124 studies (1989-2004) carried out in 52 countries from 5 continents (Santos and Hishino, 2005). They found that types G1-4 (in conjunction with either P6 or P8 types) accounted for 88% of all circulating types. The newly emerged G9

was the fourth most important rotavirus type (4.1%). Different types were predominant in different geographical areas. For example, G1/P8 type accounted for more than 70% of strains in North America, Europe and Australia. However, this type accounted for only 30% of strains in South America and Asia. Another emerging G type (G5) has recently become common in South America.

Group B rotaviruses show a different epidemiology to that of group A rotaviruses (Sen *et al*, 2001; Kelkar and Zade, 2004). Group B rotaviruses were first discovered as a cause of human illness in 1982 when large outbreaks of severe cholera like-illness were described in adults in China (Yang *et al*, 2002). Since then several outbreaks have been reported in China and more recently in India and Bangladesh (Barman *et al*, 2004; Ahmed *et al*, 2004; Sanekata *et al*, 2003; Kobayashi *et al*, 2001).

Group C rotaviruses cause illness similar to that of group A rotaviruses and can affect children and adults (Kuzuya *et al*, 2003; Nilsson *et al*, 2000; Gabbay *et al*, 1999; Souza *et al*, 1998; Teixeira *et al*, 1998; Otsu, 1998). Studies in numerous countries have shown high sero-positive rates suggesting that infection is common (James *et al*, 1997; Nilsson *et al*, 2000; Steele and James, 1999; Jiang *et al*, 1995; Iturriza-Gomara *et al*, 2004). Targeted studies using sensitive molecular tests have detected group C rotavirus in approximately 1-10% of all cases of infantile gastroenteritis (Sanchez-Fauquier *et al*, 2003; Yan *et al*, 2004; Phan *et al*, 2004; Schnagl *et al*, 2004; Castello *et al*, 2002; Cunliffe *et al*, 2001). These studies have also highlighted that primary group C rotavirus infections may occur in children older than those that suffer group A rotaviruses.

2.3.1.5. Clinical Illness

Infantile rotavirus infection has an incubation period of approximately 1-2 days followed by a sudden onset of watery diarrhoea lasting between 4-7 days. Diarrhoea is often accompanied by vomiting (Sharma *et al*, 2002; Bishop, 1996). The severity of the illness varies (Ruuska and Vesikari, 1990). For example, asymptomatic infection by so called “nursery strains” have been described (Crawley *et al*, 1993). More serious infections have also been described sometimes leading to neurological abnormalities resulting from electrolyte imbalances (Hung *et al*, 2003; Jones and Blikslager, 2002; Lin *et al*, 1996). A recent study by Blutt *et al* detected virus and antigen in the serum of both animals and children raising the possibility that rotavirus may also escape from the gastrointestinal tract and cause disease elsewhere in the

body (Blutt *et al*, 2003; Blatt *et al*, 2007). Studies have demonstrated rotavirus in the Central Nervous System (CNS) of patients with neurological illness such as meningo-encephalitis but whether this is a cause, bystander or contaminant is unclear (Lynch *et al*, 2003; Lynch *et al*, 2001; Pager *et al*, 2000). Rotavirus has also been demonstrated in the kidney, liver and lung (Nuovo *et al*, 2002; Gilger *et al*, 1992). Other reported complications of rotavirus infection include necrotising enterocolitis (Rotbart and Levin, 1983), infantile acute hemorrhagic edema, and possible intussusception (Bines *et al*, 2004; Robeinson *et al*, 2004; Nelson *et al*, 2002; Rennels *et al*, 1998; Mulcahy *et al*, 1982; Konno *et al*, 1978; Sharma *et al*, 2004; Boccia *et al*, 2001; Di Iernia, Lombardi and Lo Scocco, 2004).

In immunocompromised children, chronic and severe rotavirus infections have frequently been documented (Saulsbury, Winkelstein and Yolken, 1980; Troussard *et al*, 1993; van Kraaij *et al*, 2000). In 1980 for example, 2 children with primary immune deficiencies developed chronic diarrhoea that improved after the administration of human breast milk containing high titres of rotavirus antibody (Gilger *et al*, 1999). Gilger *et al* also described 4 children with various immune deficiencies who were suffering from chronic diarrhoea and were found to have rotavirus present in the liver and kidney.

The clinical illness seen in adults has mainly been determined through volunteer studies (Hardy, 1987; Jewkes *et al*, 1981; Kapikian *et al*, 1983; Ward *et al*, 1990; Ward *et al*, 1986). The incubation period seems to be longer than in children (2-6 days) and symptoms of diarrhoea, fever, headache, malaise, nausea and cramping last 1-4 days. Case reports have indicated a similar incubation period and symptoms to those ascertained through volunteer studies, although reports from elderly patients have shown more prolonged symptoms and shedding.

Rotavirus infection has also been described in adult solid organ transplant patients, Bone Marrow Transplant (BMT) patients, patients with cancer and adults who are infected with HIV. Bolivar *et al* investigated 90 adult patients with solid tumours or leukaemia (Bolivar *et al*, 1983). Two patients developed rotavirus infection with subsequent graft versus host disease. Yolken *et al* examined 78 adult BMT patients and found evidence of rotavirus infection in 9 (Yolken *et al*, 1982). Most of those affected suffered abdominal cramps and diarrhoea. Six developed respiratory illness (as proven by chest infiltrates on a chest radiograph) of whom five subsequently died.

Other reports of rotavirus pneumonitis have included a patient undergoing steroid treatment for multiple sclerosis (Thomas, Pollok and Gazzard, 1999). Stelzmueller and colleagues described rotavirus cases in 3 adult solid organ transplant patients. All developed enteritis and one developed toxic megacolon with ulcerative colitis (Stelzmueller *et al*, 2005). In each case the rotavirus infection was associated with secondary infections and prolonged hospitalisation.

Albrecht *et al* followed 66 HIV positive patients from 1987-1991. Thirteen were found to be positive for rotavirus. These patients had evidence of chronic diarrhoea for up to 8 weeks duration (Albrecht *et al*, 1993). Other studies have also described chronic diarrhoea and shedding in HIV positive individuals (Liste *et al*, 2000).

2.3.1.6. Treatment

Treatment of infantile rotavirus is unnecessary in most cases. However, when needed, treatment relies upon oral, intramuscular or intravenous re-hydration (Sachdev, 1996; Mahalanabis *et al*, 1995). The use of probiotics has also been examined with some studies showing reduced duration of diarrhoea (Szajewska and Mrukowicz, 2001). As mentioned above, breast milk containing anti-rotavirus antibodies has also been used (Saulsbury, Winkelstein and Yolken, 1980; Schoub *et al*, 1978). Recent studies using nitrazoxanide, a thiolzolid anti-infective agent, have shown it to reduce the duration of symptoms in paediatric patients (Rossignol and Gohary, 2006; Rossignol *et al*, 2006).

Treatment in adults is similar to that advocated for children. Oral rehydration and (if needed) pain relief utilising codeine, loperamide and diphenoxylate have been used. The use of oral human immunoglobulin has also been found to be of benefit. For example, Guarino *et al* noted a reduction in diarrhoea duration (76 hours vs 131 hours) following this treatment. Such formulations have also been used in the immunocompromised (Guarino *et al*, 2002; Guarino *et al*, 1997; Guarino, 1996; Guarino *et al*, 1996; Guarino *et al*, 1994; Guarino *et al*, 1991).

2.3.1.7. Control And Prevention

Controlling rotavirus infections is difficult due to its low infectious dose, environmental stability and possible respiratory spread (Taylor and Greenough, 1989; Le Baron *et al*, 1990). Most measures are based on universal precautions (frequent

hand washing is important for example) and are similar to those outlined for norovirus infection (Rao, 1995; Sattar *et al*, 1994).

Rotavirus vaccines, however, have shown promise in prevention. The first such vaccine was a bovine strain tested in Finnish children in the 1980s (Vesikari *et al*, 1983). This vaccine was found to be safe and resulted in protection against repeat challenge, and partial cross-protection from other rotavirus types. However, development was halted after further clinical trials in Rwanda and Gambia showed reduced effectiveness (Georges-Courbot *et al*, 1991; Hanlon *et al*, 1987). Similar inconsistent results were found with other animal mono-valent vaccines (Bernstein *et al*, 1995; Snodgrass *et al*, 1984).

Multi-valent vaccines containing animal strains and mono-re-assortments of human outer capsid strains G1-4 were tested in the early 1990s (Kapikian *et al*, 1996). In one trial 1278 children aged between 5-25 weeks were given three doses (at 2, 4, and 6 months) of a multi-valent vaccine. The vaccine resulted in 50% protection from rotavirus infection. In those infected with rotavirus there was a 60-92% reduction in severe gastroenteritis and 100% protection from severe dehydration (Bernstien *et al*, 1995; Rennels *et al*, 1996; Santosham *et al*, 1997; Joesuu *et al*, 1997). Rotashield™, as the vaccine was called, was approved by the FDA in the US in 1998. However, after the administration of more than 1.5 million doses in the first 10 months, an excess of intersusception in vaccinated children was detected by the vaccine adverse event monitoring system (MMWR, 1999; Murphy *et al*, 2001). The risk of intersusception in vaccinated children was estimated to be 1 in 2500 and may have resulted in upwards of 1600 cases. This risk was deemed to be too great in a country where the actual risk of mortality due to rotavirus was 1 in 100,000. The vaccine was immediately removed from the childhood vaccination schedule. However, recent re-appraisal of the data by scientists at the US National Institute of Health found no evidence of an overall increased risk of intersusception (Roberts, 2004). Since then other vaccines have been developed. A mono-valent (G1) vaccine has recently been licensed in Mexico (Perez-Schael *et al*, 2002). Another monovalent and a multi-valent (G1-4 and P1) vaccine has recently been trialled with promising results (Glass and Parashar, 2006). A hexa-valent vaccine (for types G1-4, 8 and 9) is also being trialled (Roberts, 2004; Bonn, 2004).

2.3.2. The Caliciviruses: Norovirus And Sapovirus

Norovirus and sapovirus are two of the four genera in the *Caliciviridae* family (Green *et al*, 2000; Thiel and Konig, 1999). The remaining two genera are vesivirus and lagovirus, which are animal viruses. Norovirus was first discovered in 1968 in the faecal samples of students and teachers from a school outbreak of gastroenteritis in Norwalk, Ohio, US (Kapikian *et al*, 1972). Sapovirus was detected in 1976 by Madeley and Cosgrove and later during a sequence of outbreaks at a home for infants in Sapporo, Japan (Madeley and Cosgrove, 1976; Chiba *et al*, 1977). Caliciviruses are non-enveloped with a diameter of 27-35nm. Under electron microscopy the surface of the viral particles have “cup-like” depressions (Figure 3 and Figure 4) from which these viruses get their name (“Calici” is latin for goblet/cup). Caliciviruses are positive sense, single stranded RNA viruses with a genome of approximately 7.7kb in length.

Figure 3: Norovirus as viewed under electron microscopy.

Figure provided by David Brown, Health Protection Agency, Colindale.

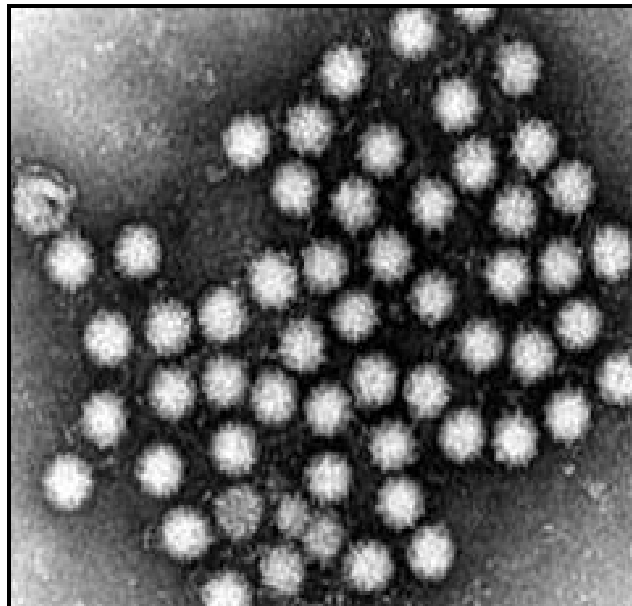
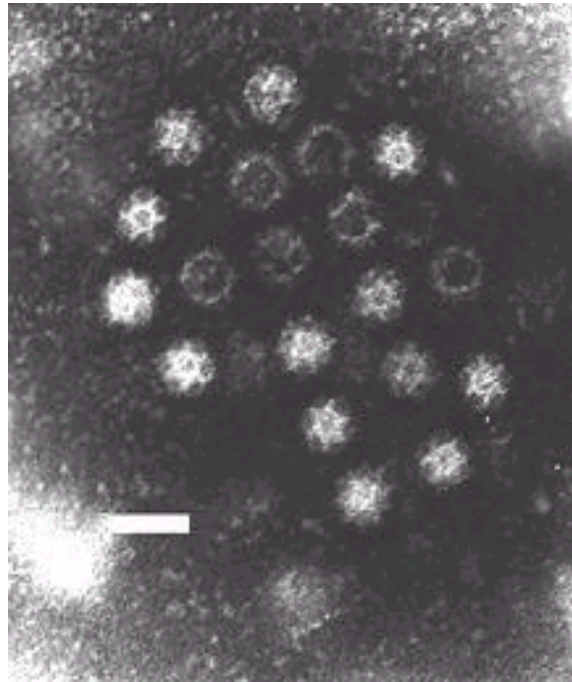


Figure 4: Sapovirus as viewed under electron microscopy.
Figure provided by David Brown, Health Protection Agency, Colindale.

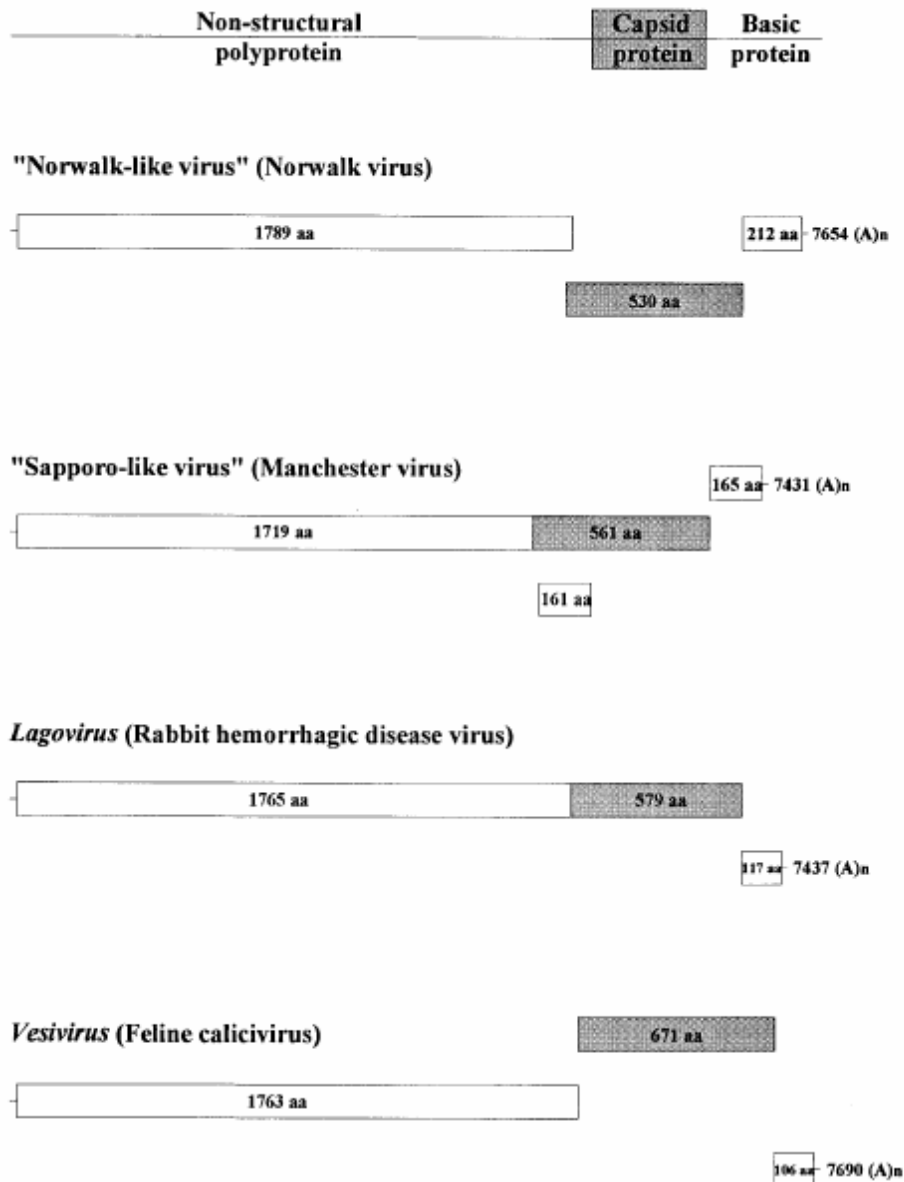


2.3.2.1. Genome Organisation And Function

Norovirus Genome Structure

Most understanding of the norovirus genome organisation and function has come from expression studies with cDNA clones and recombinant expression systems (Hardy, 2005). These studies have shown that noroviruses are genome protein linked at the 5' end and polyadenylated at the 3' end (Jiang *et al*, 1993; Lambden *et al*, 1993). Noroviruses have 3 ORF. ORF1 codes for the non-structural proteins (p48, NTPase, p22, VPg, 3C like protease and the RdRp). ORF2 codes for the major capsid protein VP1 whereas ORF3 codes for the minor structural protein VP2 (Figure 5).

Figure 5: The genomic structure of the caliciviruses.
 Figure taken from: Atmar and Estes, 2001.



Norovirus Non-Structural Proteins

The ORF1 is more conserved than either the ORF2 or ORF3. However, significant variation can still be found. The non-structural protein p48 is located at the N terminal protein of the ORF1 and varies in both length and sequence between different norovirus genogroups (Hardy, 2005). Its role is currently unknown but it is thought to play a role in the regulation of cell proliferation. The p41 NTPase is similar in sequence to the 2C in picornaviruses. Experiments suggest it is an NTPase without

helicase activity with similar activity to the polio 2C (Pfister and Wimmer, 2001). Sequence analysis of different noroviruses suggests that nucleotide homology can be as low as 36% in this region (Atmar and Estes, 2001; Katayama *et al*, 2002). The p22 protein is also of unknown function. It holds a similar position to that of the 3A in the poliovirus genome. As a result it has been suggested that it may have a role in membrane localisation of replication complexes (Belliot *et al*, 2003). Sequence analysis of this region has highlighted areas of significant variation with similarities of approximately 50% (Katayama *et al*, 2002). VPg is a protein of 15kDa in length and is found covalently linked to genomic and subgenomic mRNA (Burroughs and Brown, 1978). Experiments using caliciviruses devoid of VPg have found them to be non-infectious and FCV devoid of VPg has reduced viral protein synthesis (Daughenbaugh *et al*, 2003). Therefore it has been suggested that VPg is involved in ribosome recruitment to viral RNA. 3Clpro is similar in sequence to the picornavirus 3C and is thought to be the viral protease. The RNA dependent RNA polymerase has similar structural and catalytic elements to the RdRp of other RNA viruses (Ng *et al*, 2002; Ng *et al*, 2004). Sequence analysis of the VPg, 3Clpro and the RNA polymerase show that these regions are reasonably conserved amongst norovirus genogroups with similarities of approximately 70% (Katayama *et al*, 2002).

Norovirus Structural Proteins

The structural proteins VP1 and VP2 make up the norovirus capsid. X-ray crystallography has shown the capsid to be composed of 90 dimers of VP1 with a small number of VP2 copies (Prasad *et al*, 1994). These are together in a T=3 icosahedral virion (Prasad *et al*, 1996; Prasad *et al*, 1999).

Studies of VP1 have shown that it folds into 2 domains linked together by a flexible hinge region: S (shell) and P (protrusion). S is essential for the icosahedral virion whereas the P domain forms the arch-like structures extending from the shell. The P domain has 2 sub-domains (P1 and P2). P1 and P2 correspond to the leg and the top of the arch-like capsomere and contain substantial variation structurally. This is of two types: the orientation with respect to the S domain and the size of the P2 region. On an amino acid and nucleotide level, P1 is moderately conserved. However, sequence analysis of the P2 region shows it to be highly variable (Chen *et al*, 2004). Changes in the amino acid sequence are likely to result in structural variations in the size and shape of the determinants of cell binding, antigenicity and host specificity

(Chen *et al*, 2004). This region is also thought to determine the ABO histo susceptibility discussed below (see section 2.3.2.3)(Tan *et al*, 2003). The P2 region is under immune pressure and thus accumulation of amino acid changes occurs rapidly. For example, analysis of the P2 region in an immunocompromised patient with chronic norovirus infection detected up to 32 amino acid changes in one year (Nilsson *et al*, 2003).

The VP2 is a protein of 208-268 amino acids. in length with a molecular weight of 22-29kDa. The nucleotide sequence of the VP2 region varies significantly between strains (Seah *et al*, 1999; Katayama *et al*, 2002). It is a basic protein, which suggests it may bind RNA and be involved in RNA packaging.

Sapovirus Genome Structure

Sapoviruses have two or three open reading frames depending on genotype (Figure 5). Of the five genotypes currently recognised. GI and GIV and GV are predicted to have a third ORF (Schuffenecker *et al*, 2001).

The 2C-like NTPase (NTPase), VPg, 3C-like protease, RNA-dependent RNA polymerase, and capsid protein (VP1) are encoded in the ORF1 polyprotein, which is afterwards cleaved into the nonstructural and structural proteins (Oka *et al*, 2005). The roles and genetic variability of each of the non-structural proteins are likely to be similar to those of the norovirus genome discussed above. The ORF2 codes for minor structural proteins of unknown function (Vinje *et al*, 2000).

Like norovirus, the capsid region of the sapovirus contains most of the nucleotide variation. Analysis of the amino acid sequence of the sapovirus genome shows that the capsid region can be separated into 3-4 regions (Okada *et al*, 2006). Two of these regions contain significant variation: the N terminal variable and the central variable region contain significant variation. The other two regions, known as the N terminal region and the C terminal region show more conservation between genogroups and strains. Chen *et al* suggested that the CVR region is homologous to the P2 region of the noroviruses in that it is located on the viral surface and may confer strain/antigen specificity (Chen *et al*, 2004).

2.3.2.2. Classification

Historically, noroviruses have been classified using cross-challenge studies in volunteers and cross-reactivity by Immune EM. However, this method has poor accuracy and reproducibility. Serotyping with neutralisation is not possible as there is

no cell culture system available (Duizer *et al*, 2004). As a result, nucleotide sequencing has played a major role in norovirus characterisation. Four regions have been used for classification (Ando *et al*, 2000; Vinje *et al*, 2004): region A (the RdRp), region B (the 3' end of the ORF1), region C (short stretch near to the 5' end of ORF2) and region D (located at the 3' end of the ORF2). Nucleotide sequencing has tentatively suggested that there are 5 assigned genogroups, each containing a number of clusters and strains (Fankhauser *et al*, 2002). Zheng *et al* aligned the capsid protein sequences of 164 noroviruses and found genogroups, clusters and strains to differ by 41-61%, 15-45% and 0-14% respectively (Zheng *et al*, 2006). Using sequencing, genogroup 1 and 2 were found to contain the largest number of norovirus clusters. There are at least 8 in genogroup 1 and 17 in genogroup 2. The genogroups 1, 2 and 4 are known to cause human infections. Genogroups 3 and 5 contain animal noroviruses.

Classification of sapoviruses is also an evolving process and because of the issues highlighted with norovirus above has thus far relied upon nucleotide sequencing methods. Various classification schemes have been proposed. Vinje *et al* proposed 3 genogroups based on the sequence analysis of the capsid region (Vinje *et al*, 2000). However, more recent studies have suggested that there are 5 or 6 genogroups, each containing clusters and strains. For example, Gallimore *et al* examined the sapovirus capsid sequence and described 5 genogroups. Genogroups were found to differ by 49-55%, clusters by 19-25% and strains by 1-5%. The sapoviruses known to cause human infections were shown to be members of genogroups 1, 2, 4 and 5, whereas animal strains fell into group 3 (Gallimore *et al*, 2006). Okada *et al*, analysed the capsid region of 15 sapoviruses. In their study these divided into 6 genogroups, 2 of which represented animal sapoviruses (Okada *et al*, 2006). Based on the capsid region the animal strains were shown to differ from the human strains by 40-44%.

The large amount of diversity observed in norovirus and sapovirus strains is a result of the accumulation of point mutations due to error prone RNA replication and recombination events (Matson and Szucs, 2003; Nilsson *et al*, 2003; Dingle, 2004). Nilsson *et al* examined sequential samples taken from an immunocompromised patient with chronic norovirus infection and identified a section in the capsid region (called the P2 domain) where mutations frequently accumulated, eventually leading to the formation of new phenotype (under immune pressure). Jiang *et al* identified the

first norovirus recombinant in 1999 and since this discovery several more have been reported (Jiang *et al*, 1999; Katayama *et al*, 2002; Lochridge and Hardy, 2003; Vinje *et al*, 2000). Evidence from these reports suggests that recombination occurs at the junction between ORF1 and ORF2. Other potential recombination sites include the P2 region (Rohayem *et al*, 2005).

Recently a sapovirus recombinant has also been described (Katayama *et al*, 2004). As with norovirus, the recombination event is thought to have occurred at the RdRp-Capsid junction. Recombination seems to be restricted to intragroup strains since intergroup and intergenus recombination has yet to be described (Katayama *et al*, 2004).

2.3.2.3. Immune Response

The immune response to norovirus is not yet fully understood (Matsui and Greenberg, 2000). Early transmission studies showed that the majority of adults are susceptible to norovirus infection suggesting that childhood norovirus infection fails to elicit long-term protection (Parrino *et al*, 1977; Wyatt *et al*, 1974). Parrino *et al* examined the formation of short and long-term immunity by challenging 12 volunteers with norovirus. Of these, 6 became ill. The 6 volunteers who became ill were then re-challenged with the same virus 27-42 months later. All became ill thus demonstrating an absence of long-term immunity. However, the same 6 patients were then re-challenged 6-14 weeks later and this time all failed to become ill suggesting the formation of short-term immunity.

Long-term immunity has been described in a number of studies and may be a result of repeated exposure (Ewald *et al*, 2000; Black *et al*, 1982; Ryder *et al*, 1985). For example, in 1998 a foodborne outbreak of norovirus occurred in a group of tourists who were staying with an aboriginal community (Matsui and Greenberg, 2000). Although the aborigines ate the same meal as the tourists none became ill. Similar reports have been published in Bangladesh and Panama (Ryder *et al*, 1985).

The relationship between the different norovirus strains is also complicated by some evidence of cross-protection (Wyatt *et al*, 1974). Wyatt *et al* examined the immune relationship between the norovirus strains Norwalk, Hawaii and Montgomery County virus. Volunteers were challenged first with one of the three noroviruses. The results showed that those who became ill were then re-challenged with the same virus or another. Those initially challenged with Norwalk virus became ill when challenged

with Hawaii virus. However if re-challenged with Montgomery County virus some cross protection was observed. Other studies have found similar results (Madore *et al*, 1990; Noel *et al*, 1997).

A confusing relationship exists between the presence of pre-existing antibody to norovirus and susceptibility. Sero-prevalence studies have shown that antibody production to norovirus is lowest in the 0-5 years age group and increases with age, which is consistent with the theory that antibody production aids resistance, since children are more susceptible to norovirus than adults (Jing *et al*, 2000). However, antibody is also often found to be significantly higher in susceptible adults than those able to resist infection.

Recent evidence has pointed towards genetic factors as determinants of norovirus susceptibility (Hutson, Atmar and Estes, 2004; Rockx *et al*, 2005; Hutson *et al*, 2002). Studies utilising Virus Like Particles (VLPs), which are structurally and antigenically similar to norovirus, have shown that noroviruses will only attach to and infect host cells in the gut if they express specific, genetically determined carbohydrates (Lindesmith *et al*, 2003; Hutson *et al*, 2003; White *et al*, 1996). These carbohydrates are thought to include H, Lewis and histo-blood group antigens (Harrington *et al*, 2002; Harrington *et al*, 2004; Marionneau *et al*, 2002; Huang *et al*, 2003; Green *et al*, 1988). These carbohydrates have a wide tissue distribution and are thought to be present on saliva, red blood cells, and gastro duodenal epithelial cells. Evidence from serological and volunteer studies supports this theory. For example, it has been found that convalescent sera and human breast milk block VLPs from binding to the aforementioned carbohydrates (Jiang *et al*, 2004). Individuals unable to produce H antigen do not become infected when challenged with norovirus (Lindesmith *et al*, 2003; Hutson *et al*, 2002; Harrington *et al*, 2002; Meyer *et al*, 2004). Rockx *et al* found that individuals with type B histo-blood group antigen are protected against norovirus G1 strains but not G2 strains thus highlighting that different noroviruses may bind preferentially to different carbohydrates (Rockx *et al*, 2005; Huang *et al*, 2005). This may explain the worldwide predominance of G2.4 norovirus types (see section 2.3.2.4). Unlike some norovirus types, these viruses have been shown to be able to bind to a broad range of carbohydrate targets. As a result they have a large susceptible target population. These findings may also explain the confusing relationship between pre-existing antibody and risk of infection as described above. Pre-existing antibodies to a particular norovirus may be an

indication that the individual is particularly susceptible to that virus and has suffered repeated infections in the past.

These genetic characteristics have also been shown to be important pre-determinates of other gastrointestinal infections. For example, B histo-blood group antigen was shown to be less common in patients with haemolytic uraemic syndrome after an E-coli 0157 outbreak in Sakai, Japan (Hutson, Atmar and Estes, 2004).

The immune response to sapovirus is less well defined. Studies in adults have shown a low incidence of infection thus suggesting that primary infection in childhood results in long-term immunity (Chiba *et al*, 2000).

2.3.2.4. Epidemiology

Since long-term immunity to norovirus is rare, humans of all ages are susceptible to repeat infections throughout life. As a result norovirus is frequently implicated in sporadic cases of IID and is now thought to be the predominant cause of outbreaks of IID, especially in closed settings such as hospitals, residential homes, hotels, schools and cruise ships etc (Matson, 2005; Thornton *et al*, 2005; Isakbaeva *et al*, 2005).

Sapovirus is predominantly a pathogen of children with infections limited to mainly the very young (de Wit, Koopmans and van Duynhoven, 2003). As a result its burden is likely to be less than that of the other viral causes of IID. Outbreaks due to sapovirus have been documented but are rare (Johansson *et al*, 2005).

Limited data exist on the epidemiology of sapoviruses. As a result the remainder of this section mainly focuses on the epidemiology of norovirus infections. Sporadic cases and outbreaks show winter predominance although cases and outbreaks can occur all year round (Zahorsky, 1929; Adler and Zickl, 1969; Mounts *et al*, 2000; Lopman *et al*, 2003; Hale *et al*, 2000). Norovirus has a low infectious dose with fewer than 10 viral copies capable of causing illness in a human adult volunteer (Levett *et al*, 1996; Moe *et al*, 1999). The vast majority of norovirus cases are a result of faecal-oral spread but there is also evidence of airborne spread (via droplets produced during vomiting) and spread by contaminated fomites (Widdowson *et al*, 2005; Fretz *et al*, 2005; Caul, 1994). Environmental studies using surrogate virus feline calicivirus (FeCv) have estimated that (at 20°C) the virus may remain infectious on surfaces for between 21-30 days (Sattar, 2004; Barker, Vipond and Bloomfield,

2004; Steinmann, 2004; Doultree *et al*, 1999; Hota, 2004; Gehrke, Steinmann and Goroncy-Bermes, 2004)

Potential sources of infection include those with asymptomatic infection (Duizer *et al*, 2004; Marshall *et al*, 2001; Vinje *et al*, 1997; de Wit *et al*, 2001). Asymptomatic infections have been detected have been measured in a few studies. For example, in the Netherlands asymptomatic norovirus infections have been detected in between 1-5% of the general population but have been detected in up to 19% of individuals in outbreak settings. Asymptomatic sapovirus infections have also been detected in young children (range 1-13%). Other sources include those with symptomatic infection who have prolonged viral shedding after their symptoms have resolved. There is evidence of norovirus shedding up to 7 weeks or more after resolution of symptoms in immunocompetent individuals (Ball *et al*, 2005; Rockx *et al*, 2002; Cliver, 1997). Longer durations of shedding have also been observed in immunocompromised patients (Nilsson *et al*, 2003). There is also evidence of pre-symptomatic shedding (Lo *et al*, 1994).

The remainder of cases are likely to be a result of contaminated food or water (Hirakata *et al*, 2005; Paez Jimenez *et al*, 2004; Parshionikar *et al*, 2003; Boccia *et al*, 2002). Data suggests that most foodborne infections are caused by norovirus. A recent study in the US estimated that 67% of all foodborne infections were due to norovirus (Mead, 1999). Food can become contaminated in numerous ways. Infected food handlers are a common source (Lo Sv *et al*, 1994; White *et al*, 1986; Gaulin *et al*, 1999). At-risk foods include those with prolonged hands-on preparation such as salads and sandwiches (Holtby *et al*, 2001; Kilgore *et al*, 1996).

A number of large outbreaks have been linked to the consumption of bivalve shellfish such as oysters, which have been shown to filter sewage from sea water and concentrate virus in their flesh (Chalmers and McMillan, 1995; Ang, 1998; Stafford *et al*, 1997; Kohn *et al*, 1995)

Several outbreaks have been linked to the consumption of sewage-contaminated drinking water (van den Berg *et al*, 2005; Kukkula *et al*, 1999; Brown *et al*, 2001; Lodder *et al*, 1999; Hafliger, Hubner and Luthy, 2000). Such water has also been used in the preparation and storage of particular foods.

Noroviruses have been shown to be resistant to freezing and have been implicated in a number of outbreaks relating to frozen foods. Noroviruses have also been detected in bottled mineral water, although the significance of these findings has

been questioned (Sanchez, Joosten and Meyer, 2005; Beuret *et al*, 2002; Beuret, Kohler and Luthi, 2000; Lomothe *et al*, 2003).

Several studies have suggested a possible animal source, since noroviruses have been detected in cattle and other animals (Van der Poel *et al*, 2003; Van der Poel *et al*, 2000; Smiley, 2002; Smiley *et al*, 2003). These strains have yet to be associated with human illness. A recent study by Widdowson *et al* found significant levels of antibodies to bovine norovirus strains in vets and others in frequent contact with cattle. These data suggest that infection with these animal strains is possible and may contribute to the diversity of noroviruses (Widdowson *et al*, 2005).

Recent studies have suggested that norovirus may also be an important travel associated infection. Chapin *et al* found norovirus in 65% of travellers returning to the US from Mexico and Guatemala with travel-associated diarrhoea (Chapin *et al*, 2005).

Molecular epidemiological studies have shown that a number of norovirus strains and serotypes circulate at any one time, with one type predominant worldwide (Gallimore *et al*, 2004; Hale *et al*, 2000; Kageyama *et al*, 2004). Since 1996 type G2.4 strains have dominated worldwide (Lopman *et al*, 2004; Noel *et al*, 1999). Occasionally a new strain may supersede previous strains to become predominant. This was the case in 2002 when a new G2.4 variant emerged worldwide (Lopman *et al*, 2004). This emergence was accompanied by several alterations in the epidemiology of norovirus infection. For example, although there still appeared to be a peak of activity over the winter months particularly in closed settings, there was also an increase in outbreak reports during the summer months (Lopman *et al*, 2003). This new variant norovirus contained a mutation at position 4820 (from AACTTG to AATCTG). It is unclear, however, how this change led to such an increase in activity. It has been suggested that this variant is more stable in the environment or more virulent. However, some researchers have suggested that the sudden increase in norovirus infection may instead be the result of a large, as yet, unidentified introduction of this new variant.

A similar increase in activity was observed in 2005 and again in 2006 (CDR weekly, 2005; HPS weekly report, 2005; Bull *et al*, 2006; Gallimore *et al*, 2007). These have also been associated with the emergence of a new norovirus G2.4 norovirus variant. Recent data also suggests that sapovirus may act in a similar fashion (Gallimore *et al*, 2006). Gallimore and colleagues sequenced the sapoviruses

detected in the UK between 1989 and 2004 and found a change in the predominant strain in 2004 (from G1.1 to G2.1).

2.3.2.5. Clinical Illness

Classically, the clinical symptoms of norovirus and sapovirus were derived from transmission studies (Dolin *et al*, 1971; Gotz *et al*, 2001; Kaplan *et al*, 1982; Graham *et al*, 1994). These studies reported that after an incubation period of 24-48 hours the illness is of sudden onset with nausea, headache, cramps, vomiting and diarrhoea. Symptoms resolve after 24-48 hours. The studies also showed that children with norovirus infection are more likely to have episodes of vomiting whereas adults have a predominance of diarrhoea. However more recent work has suggested that these findings may be inaccurate (Rockx *et al*, 2002). A recent community cohort study in the Netherlands found that vomiting was less common in children under 1 year of age than in older patients. This study also showed that the symptoms of norovirus and sapovirus lasted longer than previously thought (5 days and 6 days respectively).

Clinically, most infections caused by norovirus and sapovirus are thought to be mild. However, it is increasingly recognised that norovirus is responsible for a significant proportion of childhood hospitalisation due to IID (see 2.3.6). Studies of norovirus hospital outbreaks have shown that infection lasts longer in this setting, and can often be severe, with an increased risk of mortality-probably as a result of underlying illness (Lopman *et al*, 2004; Meakins *et al*, 2003, Mattner *et al*, 2006).

Unusual presentations of norovirus have been observed in some individuals, usually as a result of physical stress or immunocompromise. For example, an outbreak of norovirus at a military field hospital in Afghanistan resulted in neck stiffness, confusion and photophobia, and in one case disseminated intravascular coagulation (MMWR, 2002). These symptoms were probably a result of electrolyte imbalance similar to that seen in severe rotavirus infections. Studies of norovirus infection in the elderly have also highlighted the occurrence of post infection complications (Goller *et al*, 2004; Oliver *et al*, 1985). For example, in one elderly care home outbreak a “residual malaise” was present in 42% of those affected 1-4 weeks after onset.

Other studies have highlighted the presence of anorexia, lethargy and vertigo in the weeks after symptoms. In some elderly patients these non-specific symptoms and even the original acute symptoms can recur (Goller *et al*, 2004).

Chronic diarrhoea and shedding have been observed in both solid organ transplant patients and bone marrow transplant patients (Kaufman *et al*, 2003; Gallimore *et al*, 2004). In some cases shedding for up to 2 years has been observed (Nilsson *et al*, 2003). The importance of caliciviruses in HIV positive individuals is uncertain. A recent study by Rodriguez-Guillen *et al* suggested that caliciviruses are not implicated in HIV associated diarrhoea in adults. However, caliciviruses were found to be more frequent in HIV positive children (Rodriguez-Guillen *et al*, 2005).

2.3.2.6. Control

Controlling an outbreak of norovirus is difficult due to its low infectious dose and numerous modes of transmission.

Most of the current guidelines concern norovirus outbreaks in the hospital setting (Chadwick *et al*, 2000)¹. Controlling an outbreak on a ward or other closed setting requires rapid implementation of infection control procedures. Lopman *et al* estimated that control procedures must be implemented within 3 days of an outbreak onset if they are to be successful. However, detecting an outbreak at such an early stage is difficult due to the high frequency of non-infectious gastrointestinal disease such as incontinence and anti-microbial associated diarrhoea (Lopman *et al*, 2003).

Recommended control procedures include the wearing of gloves and aprons when in contact with infected patients or contaminated areas (see Table 1 and Table 2). Hand washing is advocated at all times. Studies using FeCv have shown ethanol based hand rubs with a 70-95% concentration of ethanol to be best (Kampf, Grotheer and Steinmann, 2005). However, other studies have questioned this finding and suggested that these protocols need to be compared to standard hand washing procedures before such conclusions can be drawn (Duizer and Koopmans, 2006). Guidelines suggest that affected staff should be excluded for 48-72 hours after suffering their last symptoms (this should also be applied to infected food handlers). Wards should also be closed to further admissions. Staff and patients from the affected areas should be prevented from entering other unaffected areas. There is also evidence that patient cohorting is effective in containing affected patients and contaminated areas.

¹ <http://www.hps.scot.nhs.uk/enviro/guidelinedetail.aspx?id=18578>

Cleaning norovirus from the environment is essential but difficult, and re-infections have been documented following the re-introduction of individuals into previously contaminated areas. Since there is no culture method for measuring the viability of norovirus or sapovirus many cleaning and disinfection studies have utilised animal caliciviruses known to be related to norovirus in an attempt to establish efficient methods. Studies using FeCv have shown conflicting results (Duizer *et al*, 2004; Sattar, 2004; Barker, Vipond and Bloomfield, 2004; Steinmann, 2004; Doultree *et al*, 1999; Hota, 2004; Gehrke, Steinmann and Goroncy-Bermes, 2004). For example, Steinmann demonstrated that ethanol was effective in cleansing FeCV from the environment whereas Duizer *et al* found that ethanol was not effective. Doultree *et al* showed that glutaraldehyde, iodine and hypochlorite at 1000ppm were effective. Other studies have suggested the use of sodium bicarbonate (Malik and Goyal, 2006). For soft furnishing a steam clean of at least 70°C is thought to be effective (Doultree *et al*, 1999).

Although these procedures have proved successful in containing some ward outbreaks, spread to other wards is a common occurrence. The reasons for this are unclear. It has been suggested that large multi-ward outbreaks are a result of a breakdown in the infection control procedures, which can often be difficult to achieve in practice (Christie, 2002; Cowden, 2002). For example, closing wards is difficult when faced with acute bed shortages due to other winter pressures. The failure to close a ward may prevent extensive cleaning, which is needed to remove virus from the environment. Restricting staff and patient movement is also difficult. Many staff may be asked to work in a number of wards and patients will be frequently moved between different wards. The duration of exclusion (48-72 hours) may also be too short, as many studies have indicated that virus can be excreted for longer periods. This raises the possibility that a staff member may return to work before viral shedding has ceased and spread further infection. Furthermore, agency nursing staff may not be entitled to sick pay and will therefore be tempted to return to work before their illness has resolved. However, it may also be possible that large multi-ward/area outbreaks may be a result of the multiple introductions of different noroviruses.

Table 1: Recommended infection control guidelines within wards (Chadwick *et al*, 2000).

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1. Cohort nurse or isolate symptomatic individuals (Category II)
 2. Wear gloves and apron for contact with an affected patient or environment (Category II)
 3. Wash hands with soap and water after contact with an affected patient or environment, after removing gloves and apron (Category I)
 4. Remove exposed food such as fruit (No Category)
 5. Consider use of antiemetics for patients with vomiting (No Category)
 6. Exclude affected staff from the ward immediately and until 48 h symptom-free (Category II)
 7. Close the ward to prevent the introduction of new susceptibles. Avoid transfer to unaffected wards or departments (unless medically urgent and after consultation with infection control staff). The priority is to stop spread of the virus to other areas (Category II).
 8. Exclude non-essential personnel from the ward (Category II)
 9. Caution visitors and emphasize hand hygiene (Category II)
 10. Clean and disinfect vomit and faeces spillages promptly: Appendix B (Category II)
 11. Increase the frequency of routine ward, bathroom and toilet cleaning (Category II)
 12. Use freshly prepared 0.1% (1000 ppm) hypochlorite to disinfect hard surfaces after cleaning (Category II)
 13. The ward should not be re-opened until 72 h after the last new case and 72 h after uncontained vomiting and diarrhoea (Category II)
 14. Thoroughly clean the ward and change the bed curtains before re-opening (Category II)
 15. Clean carpets and soft furnishings with hot water and detergent, or steam clean. Vacuum cleaning is not recommended (No Category)
-

Table 2: Recommended infection control guidelines between wards (Chadwick *et al*, 2000).

-
1. Staff working in affected areas must not then work in unaffected areas of 48h (includes agency and bank staff) (Category II)
 2. Avoid patient movements to unaffected areas (unless medically urgent and after consultation with infection control staff) and other institutions (Category II)
-

2.3.2.7. Prevention

At present there is no vaccine for norovirus or sapovirus infection. Since the immune response to norovirus infection is not yet understood, developing a vaccine may prove difficult. Most of the vaccine studies pre-date the discovery of the genetic determinants of norovirus immunity (as discussed earlier). Most have utilised VLPs. VLPs are morphologically and antigenically similar to noroviruses, are stable, immunogenic, can be produced in large amounts, and can be stored in lyophilised form (Estes *et al*, 2000). Initial studies in mice have shown them to be highly immunogenic after oral administration and to result in serum and mucosal immunity (Guerrero, Ball and Estes, 1997). Phase 1 trials in humans have shown them to be safe

but the immune response elicited was significantly less than that observed after natural infection (Ball *et al*, 1999). Further trials are to be carried out (Tacket, 2005).

2.3.3. Astrovirus

Astrovirus was first discovered in 1975 by Appleton and Higgens in hospitalised children with gastroenteritis (Appleton and Higgens, 1975). Astroviruses are members of the Astroviridae family. Under electron microscopy the astrovirus capsids are 28-30nm in diameter and have a characteristic 5-6 pointed star appearance (Figure 6).

2.3.3.1. Genome Organisation and Function

Astroviruses are positive sense, ssRNA viruses with a genome of approximately 6.8 kb in length (Figure 7). The genomic organisation is as follows: a 5NCR region followed by three ORF (1a, 1b and 2), a 80 nucleotide NCR and a poly A tail of 30 nucleotides (Belliot *et al*, 1997; Jiang *et al*, 1993; Lewis *et al*, 1994).

The ORF1a is 2700 nucleotides in length whereas ORF1b is 1550 nucleotides in length. ORF1a and 1b code for the non-structural proteins such as the protease and the RNA dependent RNA polymerase. ORF1a also encodes other motifs including transmembrane helices, a nuclear localisation signal and immunogenic epitopes. ORF1a length can also vary amongst strains although the significance of this is at present uncertain.

The ORF2 codes for the viral capsid. The viral capsid precursor is 86-90kDa in size and after protease processing forms 2-5 major proteins (Herring *et al*, 1981; Monroe *et al*, 1991; Belliot *et al*, 1997; Wang *et al*, 2001). ORF2 is less conserved than ORF1a and 1b and varies significantly between astrovirus types (Wang *et al*, 2001). Analysis of the ORF2 regions has detected 4 main areas: the N region, the central upper, central lower regions and C region. The N and C regions were shown to be relatively conserved between different astroviruses. The two regions between these, the central upper and central lower, are the regions that show the most variation. The central upper region can vary in sequence length between serotypes. It is not thought to be present on the virion surface. The central lower region contains several stem loops. These genes are thought to contain the regions that determine immune response, cell attachment and entry. As a result these regions differ between serotypes.

The 3' NCR is highly conserved between the 8 astrovirus types and it is thought it may play a role in the initiation of RNA synthesis (Monceyron *et al*, 1997). In addition, it has been postulated that it may also play a role in RNA stability, translation initiation and intracellular localisation.

2.3.3.2. Classification

Astroviruses are serotyped using neutralisation antibodies. Various methods exist including immunofluorescence, neutralisation assays or IEM. Sequence analysis of a 348bp region of the ORF2 has been shown to correlate with serotype.

Overall there are 8 recognised types of astrovirus (Monroe, Holmes and Belliot, 2001). Types 1-7 were distinguished by 1995 whereas type 8 was recognised more recently (Taylor *et al*, 2001). Types 1-7 cluster into 2 groups. Group A contains types 1-5 whereas group B contains types 6 and 7. Type 8 can cluster with either group depending on the area sequenced (Taylor *et al*, 2001; Walter *et al*, 2001; Belliot, Laveran and Monroe, 1997). This has led some researchers to speculate that type 8 is a recombinant virus. The area most studies suggest as the potential recombination site is the ORF1b/ORF2 junction.

2.3.3.3. Immune Response

At present the immune response to astrovirus is not well understood. The fact that cases are mainly restricted to the young or elderly suggests that primary infection results in long-term protection, which then wanes in old age (Glass *et al*, 1996). Koopmans *et al* showed that immunity was homotypic (Koopmans *et al*, 1998). Infections in adolescence and adulthood are therefore likely to be caused by rarer types of the virus.

Figure 6: Astrovirus as viewed under the electron microscope.
Figure taken from: <http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/05000000.htm>

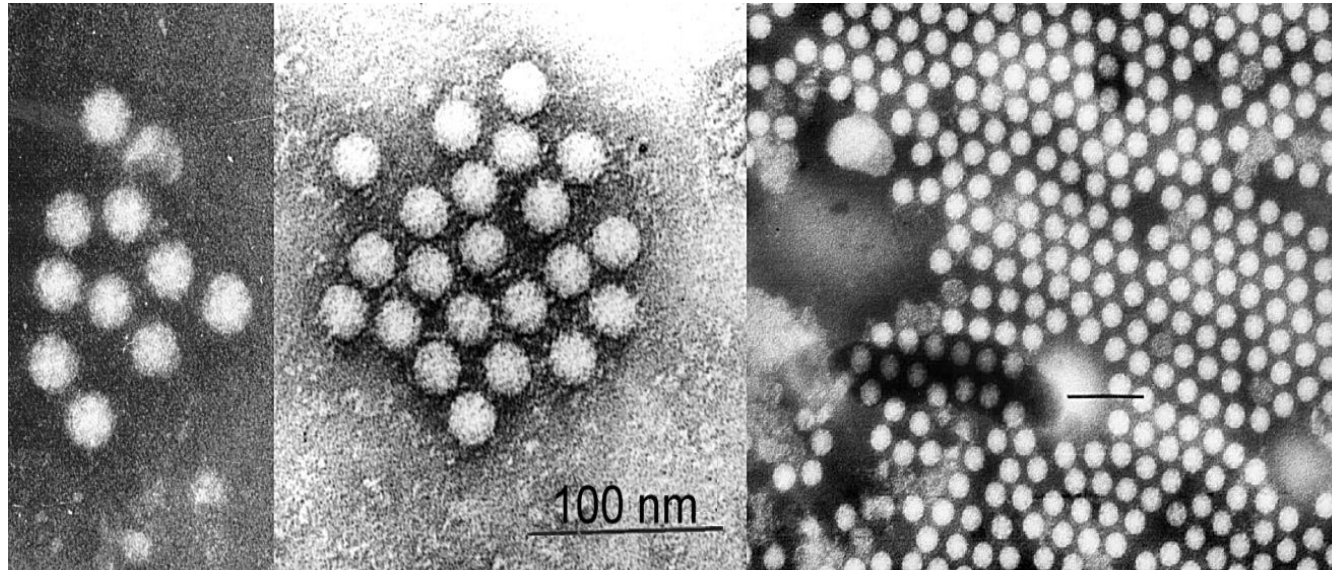
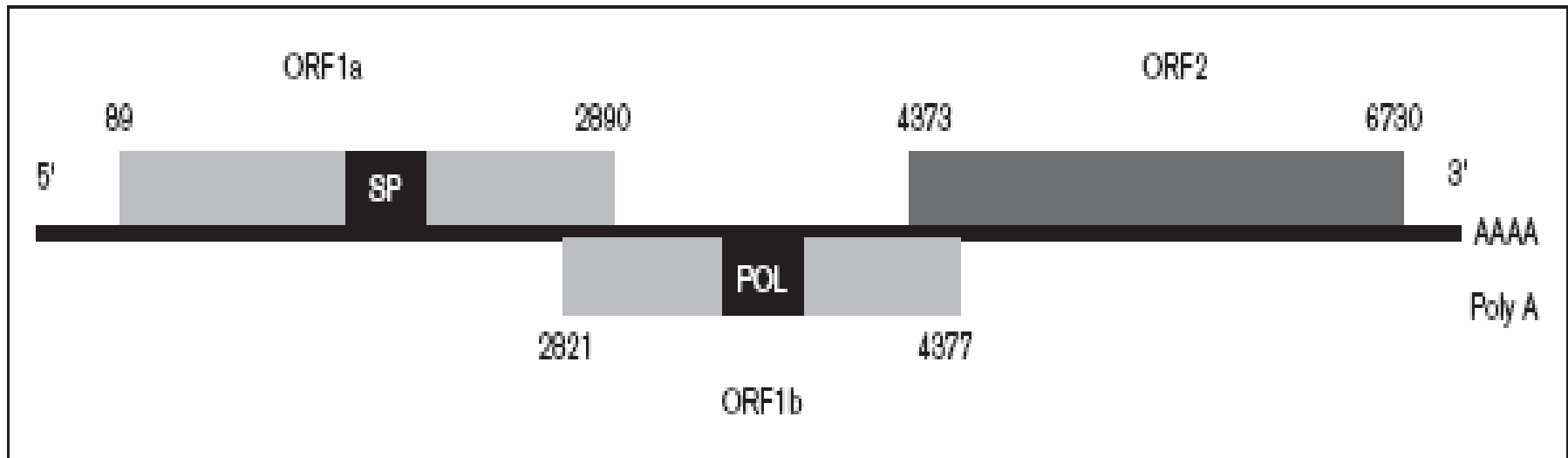


Figure 7: The genomic structure of astrovirus (Walter and Mitchell, 2003).



2.3.3.4. Epidemiology

Astroviruses have a worldwide distribution (Walter and Mitchell, 2003) and are found in humans, lambs, calves, deer, piglets, mice, dogs and ducks. Human infections are mainly seen in infants and the elderly (Noel *et al*, 1996). Reports of the age of initial infection vary in different studies. A hospital study in France showed acquisition to be in those aged less than 7 months (Marie-Cardine *et al*, 2002). However, a community-based study in France and a hospital study in Spain showed the age of acquisition to be between 2-4 years (Chikhi-Brachet *et al*, 2002; Guix *et al*, 2002). Interestingly the age of acquisition may vary according to type, with astrovirus types 1-3 acquired in children under 3 years whereas types 4 and 8 occur at an older age (Guix *et al*, 2002). This is no doubt related to the predominance of the various types (see below).

The seasonality of astrovirus varies in different regions (Walter and Mitchell, 2003). In Egypt, Brazil and Eastern USA a spring/summer peak of activity has been reported. However, in Korea, Argentina and Western USA a winter predominance occurring alongside rotavirus infections has been observed (Giordano *et al*, 2001; Kang *et al*, 2002; Rodriguez-Baez *et al*, 2002).

Astroviruses have been shown to cause endemic illness and outbreaks in closed settings such as hospitals, schools and care settings (Cubitt *et al*, 1999; Mitchell *et al*, 1995; Oishi *et al*, 1994). Transmission is mainly fecal-oral but may also involve contaminated fomites (Abad *et al*, 2001). Other sources include contaminated food (including oysters and mussels) and sewage-contaminated water (Pinto *et al*, 2001; Le Guyader *et al*, 2000; Taylor *et al*, 2001).

Numerous studies have shown that, like other viral causes of IID, multiple serotypes co-circulate at any one time (Lee and Kurtz, 1994; Mustafa, Palombo and Bishop, 2000; Palombo and Bishop, 1996). Overall, type 1 astrovirus is the most common. Types 2-4 are of medium frequency whereas types 5-7 are rare. Type 8 has had an increasing incidence in some countries including Egypt, Spain, France and Australia. This raises the possibility that type 8 is an emerging genotype.

2.3.3.5. Clinical Illness And Treatment

The clinical illness caused by astrovirus is similar to rotavirus but is milder (Dennehy *et al*, 2001; Pang and Vesikari, 1999; Guerrero *et al*, 1998; Kurtz *et al*,

1979). Studies show that after an incubation period of 1-4 days there is a sudden onset of watery diarrhoea. Illness is mainly seen in infants over 6 months of age.

Immunocompetent individuals can shed virus for up to 2 weeks post resolution of symptoms. Some studies have suggested that persistence may be linked to infection with certain astrovirus types. Caballero *et al* found higher levels of persistence and viral shedding in faecal samples showing infection with astrovirus type 3 (Caballero *et al*, 2003).

Severe infections have been observed especially in the immunocompromised, those with malnutrition and those co-infected with another viral enteric pathogen (Walter and Mitchell, 2000; Grohmann *et al*, 1993; Roman *et al*, 2003). There have been several reports of prolonged and recurrent illness in immunocompromised individuals.

However, the illness caused by astrovirus is usually mild and self-limiting and therefore no specific therapy is needed. For severe infection replacement of fluids and electrolytes may be necessary.

2.3.3.6. Control And Prevention

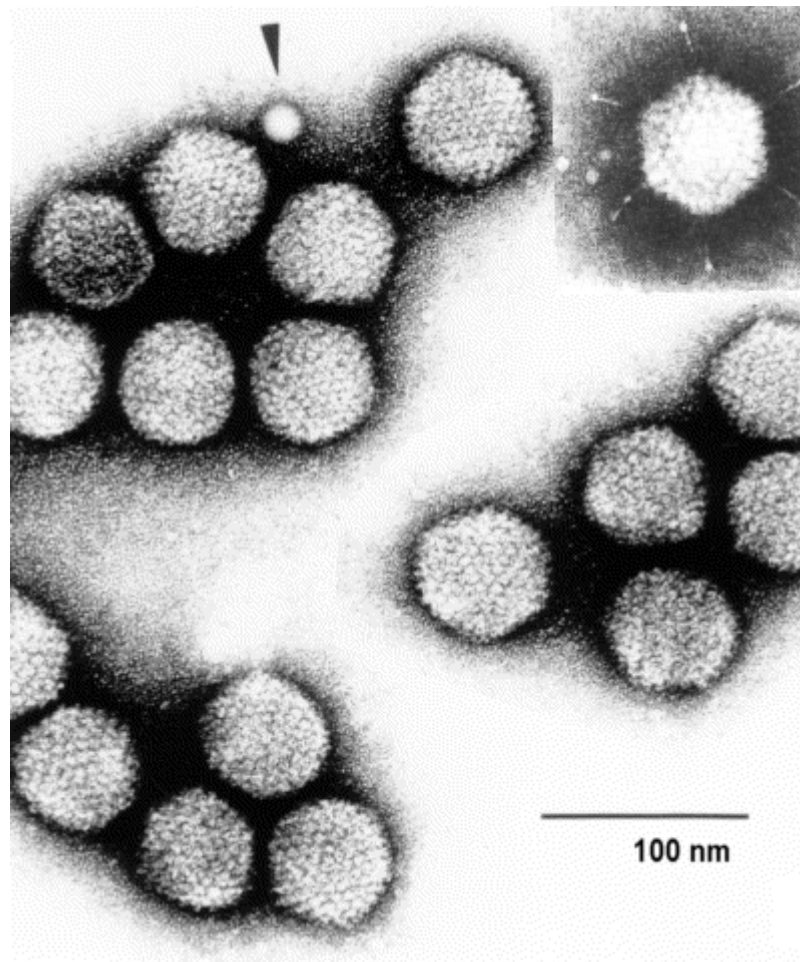
Control of astrovirus infection relies upon the application of universal hygienic and disinfection practices especially in hospitals and other closed settings. Food handlers are advised to follow guidelines excluding them from work until several days after symptoms subside (Table 1 and Table 2). At present there is no vaccine available for the prevention of astrovirus.

2.3.4. Adenovirus

Adenoviruses are non-enveloped icosahedral viruses of 70-80nm in diameter (Figure 8). Adenoviruses were first isolated in 1953 from tissue cultures of human adenoidal tissue (Cocchi, 1960). They belong to *Adenoviridae* family, genus *Mastadenovirus*. Adenoviruses have an icosahedral capsid with icosahedral symmetry. The protein capsid is made up of 252 capsomers: 240 hexons and 12 pentons. Each penton has a protruding fiber.

Figure 8: Adenovirus as viewed under the electron microscope.

Figure taken from: http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/em_adeno.gif



2.3.4.1. Genome Organisation and Function

Adenoviruses are dsDNA viruses with a genome of approximately 335kb in length (Brown *et al*, 1996; Horwitz, 1996). The genome contains several inverted terminal repeats that vary in length between different adenoviruses (Wold and Gooding, 1991). As a result the adenovirus genome differs between genera and species. All adenoviruses produce early (E1-4), intermediate and late RNA (1-5) transcripts and virus-associated RNA gene transcripts.

The Early RNA Transcripts

The E1 is a transcription unit with 2 regions, E1A and 1B (Shenk and Flint, 1991; Shenk, 2001). The E1A is a transcription activator of virus (of other early and late proteins) and some cellular promoters, whereas E1B contributes to evading the immune response by blocking host mRNA transport to the cytoplasm and inhibiting p52-mediated apoptosis (Wold and Gooding, 1991). High levels of sequence identity

in the E1/A region have been observed between members of the same subgroup. For example, Avvakumov *et al*, found four regions which were relatively conserved between adenoviruses (similarity scores of between 50-60%) (Avvakumov *et al*, 2002). However, between these regions significant sequence variation is observed (similarity scores of <30%).

The E2 transcript codes for the viral proteins that are directly involved in adenovirus replication. It contains 2 regions (E2A and 2B), which code for a number of proteins. These include E2A-DBP (a single stranded DNA binding protein), E2B-pTP (a terminal precursor protein functioning as a protein primer for initiation of viral DNA replication) and E2B-ad pol (DNA polymerase).

The E3 transcript has at least 8 recognised ORFs, which vary according to adenovirus serotype. The E3 transcripts are mainly used to counteract host antiviral defences. Modes of action include the inhibition of CTL responses and TNF alpha mediated cell lysis.

The E4 transcript has numerous ORF that, like that of E3, vary according to adenovirus serotype. For example adenovirus type 5 has six recognised ORFs whereas adenovirus 11 has five. The E4 transcript is thought to encode for proteins that regulate viral and cellular gene expression at the transcriptional and post transcriptional level.

The Intermediate Region

The adenovirus intermediate region produces 2 proteins: pIX and pIVa2. The pIX is a structural component of the virus and is a transcriptional activator of the major late promoter (Lutz *et al*, 1997; van Oostrum and Burnett, 1985). The pIVa2 is also a transcriptional activator of the major late promoter, but is also thought to be involved in genome packaging.

Late Proteins.

The late proteins are organised into one primary transcript unit that is processed using different poly A sites to produce distinct mRNAs. These are recognised as late proteins L1-5.

The L1 late protein facilitates capsid assembly and enables genome encapsidation via interaction with pIVa2 (Shenk, 2001).

The L2 late protein contains 3 ORFs coding for the Penton, pVII and pV. The penton, alongside the fiber, plays an important role in host cell binding and

penetration. pVII is the major component of the adenovirus core whereas pV acts as the bridge between the core and the penton base. It should be noted that pV contains a second ORF of unknown function (Shenk, 2001).

The L3 late protein contains 3 ORFs, which code for the pVI, pII (hexon) and L3-23 (Shenk, 2001). The pVI is a capsid component that is associated with the hexon. It possibly acts as a bridge between capsid and core components. The pII (hexon) trimerises with itself to form the major capsid. It contains at least 7 hyper variable regions but there are regions outside these areas that are conserved between adenovirus serotypes (Crawford-Miksza and Schnurr, 1996). These areas are thought to determine the adenovirus serotype. The L3-23K is the adenovirus protease. It cleaves viral precursor proteins during viral maturation.

The L4 has 4 ORFs (Andrade *et al*, 2001). Three proteins have thus far been identified. The L4-100K is a non-structural protein that is required for hexon assembly, late protein synthesis and inhibition of granzyme B mediated lysis. The L4-33k is a protein of unknown function. The pVIII is associated with hexons and is possibly a link between hexon capsomere and core capsids.

The L5 late protein is a single ORF. It codes for the adenovirus fibre. Analysis of the nucleotide sequence shows that the fibre contains areas of significant variation. It forms a structure consisting of a tail, shaft and knob. The knob determines the adenovirus tissue tropism via cell surface receptors.

2.3.4.2. Classification

Adenovirus serotypes are classified according to the ability to induce specific neutralising antibodies. The serotype specific antigens are located on the virion surface (possibly via the hyper variable regions on the hexon region). The results of analysis of the HVR regions of the hexon region match their serotypic classification. The adenovirus genera (A-F) are determined by numerous factors including the haemagglutination of rat and monkey cells or the oncogenicity in hamsters.

2.3.4.3. Epidemiology

Adenovirus IID is mainly endemic but outbreaks have also been reported in closed settings (Richmond *et al*, 1979; Retter *et al*, 1979; Yolken *et al*, 1982; Murphy, 1981; Albert, 1986). Adenovirus IID has no specific seasonal pattern and spread is mainly via the fecal-oral route. Most infections occur in childhood and studies have

shown that by 3 years of age 30-100% of children are seropositive for adenovirus (type 40 and 41).

2.3.4.4. Clinical Illness And Treatment

Clinical illness due to adenovirus is similar to that of other viral causes of illness (Bhan *et al*, 1988; Whitelaw, Davies and Parry, 1977; Richmond *et al*, 1979; Vesikari *et al*, 1981; Johansson *et al*, 1980; Blaskovic, Freitag and McLaughlin, 1982). Symptoms include watery, non-bloody faecal samples and vomiting. The duration of symptoms (3-11 days) is possibly longer than that produced by other viral causes of IID (Dowling and Wynne, 1981) and it is thought that type 41 causes a longer illness than type 40. In general, adenovirus IID results in shedding for between 1-14 days.

Although fatal cases have been described, the illness is usually mild and treatment is not needed (Whitelaw, Davies and Parry, 1977). Disseminated adenovirus type 40 or 41 infection has rarely been described in immunocompromised patients, although a recent case report by Slatter *et al* described a paediatric BMT patient who died of disseminated adenovirus 41 infection (Slatter *et al*, 2005).

2.3.4.5. Control And Prevention

Control of infection relies upon universal precautions and strict decontamination procedures as described for norovirus (Table 1 and Table 2). No vaccine is available for adenoviruses that cause IID.

2.3.5. Other Possible Viral Causes Of IID

There are many other possible causes of viral IID mentioned in the literature. In most cases a causative role has yet to be proven.

It has been suggested, for example, that the enteroviruses (members of the *Picornaviridae* family) are a rare cause of gastroenteritis in humans. Echoviruses (4, 11, 14, 18, 19 and 22) and coxsackie A1 have been implicated in outbreaks of diarrhoea in immunocompetent and immunocompromised humans (Townsend *et al*, 1982; Patel *et al*, 1985; Melnick, 1996). Another member of the *Picornaviridae* family, aichivirus, has been associated with both sporadic and outbreaks of IID in Japan and south East Asia (Yamashita *et al*, 1993; Yamashita *et al*, 1995). However, a role for this virus in IID in other countries has yet to be determined.

Reoviruses (members of the *Reoviridae* family) have regularly been found in faecal samples and most children have antibodies to all 3 serotypes (Tyler and Fields, 1996). However, although a role in childhood IID has been suggested, firm evidence has yet to be established (Giordano *et al*, 2005).

Toroviruses and coronaviruses are members of the virus family *Coronaviridae*. Toroviruses have been established as a cause of IID in cattle and horses, and recent studies have suggested a role in sporadic IID in children (Koopmans *et al*, 1997; Waters *et al*, 2000). Coronaviruses are recognised as causes of the common cold in humans. Like toroviruses, they have been shown to be a cause of IID in animals. Coronavirus particles have been found in the faecal samples of cases of IID but have also been found in healthy volunteers (Holmes, 2001).

Like the *coronaviridae* family, parvovirus is associated with IID in animals. In addition, parvovirus-like particles have frequently been found by EM in faecal samples. However, a causative role has yet to be established. Picobirnaviruses are members of the *Birnaviridae* family and have been found to be significantly associated with IID in Aids patients (Grohmann *et al*, 1993). They have also been found to be associated with IID in various animals (Leong *et al*, 2000).

2.3.6. The Contribution of Viral Pathogens To The Burden Of IID

The significant morbidity and economic burden of IID in the developed world has already been outlined (section 2.2). But what proportion of the total number of cases is due to viruses?

The contribution of each of the five main viral pathogens to overall IID in children, adults and outbreaks has only recently been ascertained using sensitive detection techniques such as PCR (see section 2.4.3.3) and is discussed in the following section.

2.3.6.1. Burden Of Viral IID In Children

Recent studies using sensitive techniques have established that viral pathogens are the major cause of sporadic IID in children, both those requiring hospitalisation and those presenting to GPs. For example, Simpson *et al* detected viral pathogens in 60.3% of children hospitalised for IID in Addenbrookes hospital, England (Simpson *et al*, 2003). de Wit *et al* detected viral pathogens in over 44% of community managed cases of IID in children presenting to GPs in the Netherlands (de Wit *et al*, 2001).

Pang *et al* investigated 832 samples from community cases of IID in children (aged under 2 years) in Finland and found a viral pathogen in approximately 60% of cases (Pang *et al*, 2000). Similar detection rates (48-75%) have been shown in other countries (Phan *et al*, 2005; Bon *et al*, 1999; Bereciartu, Bok and Gomez, 2002; Qiao *et al*, 1999; Oh, Gaedicke and Schreier, 2003).

Rotavirus is the predominant cause of childhood IID in both the community and hospital setting (Brandt *et al*, 1983; McIver *et al*, 2001; Fruhwirth *et al*, 2001). For example, Barnes *et al* examined 4,637 samples from children admitted to hospital with IID between 1980-93 and found rotavirus in 39.6% of cases (Barnes *et al*, 1998). Bon *et al* examined 414 children who consulted their GP for IID in France between 1995-99 (Bon *et al*, 1999). Overall, 61% of all cases were attributed to rotavirus. Similar findings have been found in numerous other studies (Giordano *et al*, 2001; Fischer, 2001; Donelli *et al*, 1993; Sethi, Khuffash and al-Nakib, 1989; Waters *et al*, 2000; Boga *et al*, 2004; Oh, Gaedicke and Schreier, 2003; Subekti *et al*, 2002; Qiao *et al*, 1999; de Wit *et al*, 2001; Pang *et al*, 2000; Froggatt *et al*, 2004; Chikhi-Brachet *et al*, 2002).

An increasing number of studies have shown that norovirus is second only to rotavirus as a cause of community or hospital managed IID in children. For example, Oh *et al* examined the burden of norovirus in children hospitalised in Germany (Oh, Gaedicke and Schreier, 2003). Between February 2001 and January 2002, 20.7% of cases were attributed to norovirus. Chikhi-Brachet and colleagues examined 161 patients with community managed IID in France in 1998-99. Norovirus was detected in 19% of cases (Chikhi-Brachet *et al*, 2002). Similar findings (7.6-29.9%) have been reported in numerous other studies (Kirkwood and Bishop, 2001; Simpson *et al*, 2003; McIver *et al*, 2001; Subekti *et al*, 2002; Qiao *et al*, 1999; Phan *et al*, 2004; Bereciartu, Bok and Gomez, 2002; Sakai *et al*, 2001; Boga *et al*, 2004; Rodriguez-Baez *et al*, 2002; Phan *et al*, 2005; Buesa *et al*, 2002; de Wit *et al*, 2001; Bonn *et al*, 1999; Farkas *et al*, 2000; Pang *et al*, 2000; Froggatt *et al*, 2004; Chikhi-Brachet *et al*, 2002; Simpson *et al*, 2003; Fretz *et al*, 2005).

The burden of adenovirus in children with community and hospital managed IID is less than that of rotavirus and norovirus. Studies have demonstrated detection rates between 2-7.9% of children hospitalised with IID or managed in the community (Wood *et al*, 1988; Barnes *et al*, 1998; Giordano *et al*, 2001; Donelli *et al*, 1993; Sethi, Khuffash and al-Nakib, 1989; Simpson *et al*, 2003; Bereciartu, Bok and

Gomez, 2002; Waters *et al*, 2000; de Wit *et al*, 2001; Pang *et al*, 2000; Froggatt *et al*, 2004; Chikhi-Brachet *et al*, 2002; Simpson *et al*, 2003; Basu *et al*, 2003).

The burden of astrovirus in hospital or community managed IID is similar to the proportion attributable to adenovirus with detection rates ranging between 0-16.2% (Phan *et al*, 2004; Qiao *et al*, 1999; McIver *et al*, 2001; Liu *et al*, 2004; Dennehy *et al*, 2001; de Wit *et al*, 2001; Bonn *et al*, 1999; Pang *et al*, 2000; Froggatt *et al*, 2004; Chikhi-Brachet *et al*, 2002; Simpson *et al*, 2003; De Grazia *et al*, 2004; Espul *et al*, 2004).

Sapovirus is predominantly a pathogen of very young children. As a result, sapovirus is the least common of the viral causes of IID. Studies have demonstrated detection rates of 0-9.2% (Froggatt *et al*, 2004; Phan *et al*, 2005; Pang *et al*, 2000; Pang, 2001; Farkas *et al*, 2000; Simpson *et al*, 2003). In general, studies reporting higher rates of sapovirus infections have usually investigated only younger study populations (those under 2 years).

2.3.6.2. Burden Of Viral IID In Adults

Viral pathogens contribute significantly to IID in adults. de Wit *et al* examined the incidence and cause of IID in two studies; one of patients attending GPs between 1996-99 and the second a general population-based cohort study in the Netherlands (de Wit *et al*, 2001 (i); de Wit *et al*, 2001 (ii)). The GP study (which examined 34 sites) covered all age groups and took place between 1996-99. Faecal samples were taken from 857 patients and tested for viral and other microbiological pathogens. Overall, 37.5% of cases of IID tested positive for at least one pathogen (15.4% of cases were positive for a virus, 16.5% were positive for bacteria, and 8.3% were positive for parasites). The burden of viral pathogens was greatest in the 0-4 years age group (44.7%). It was less in the older age groups (15.4% in the 5-14 years age group, 14.1% in those aged 15-29 years, 7.3% in 30-59 year olds, and 7.8% in those aged over 60 years). In comparison the bacterial pathogens, in particular *Campylobacter* spp, were restricted mainly to the older age groups (2.6% in the 0-4 age group, 15.6% in the 5-14 age group, 14.7% in those aged 15-29 years, 10.5% in 30-59 year olds and 7.8% in patients aged over 60 years). From this study it can be seen that although viruses are not the most common cause of IID in adults presenting to GPs the disease burden is still substantial. In the population-based cohort viruses were detected in 33.5% of community managed cases of IID (bacteria were detected in 2.4% of cases

and parasites 6.8%). Viral pathogens were found to be responsible for most cases of IID in all age groups with the majority of viral diagnoses attributable to norovirus. The norovirus burden was thus higher in the community setting, possibly because it results in milder infections that do not necessarily require GP or hospital attention.

2.3.6.3. Burden Of Viral IID In Outbreaks

It has recently been established that the majority of IID outbreaks are caused by norovirus. Fankhauser *et al* examined 284 outbreaks submitted to the Centre for Disease Control (CDC) from 1992-2000 (Fankhauser *et al*, 2002). Of these, 93% were found to be caused by norovirus. Similar findings have been reported elsewhere (Chatterjee *et al*, 2004; Inouye *et al*, 2000; van Duynhoven *et al*, 2005). For example, Lopman *et al* reviewed the causes of outbreaks reported in a number of European studies occurring between 1995-2000 (Lopman *et al*, 2003). Noroviruses were shown to cause most cases of outbreaks in England and Wales (96%), Germany (100%), Sweden (97%), the Netherlands (84%), Slovenia (43%) and Spain (57%). The other viruses discussed are also known to cause outbreaks of IID, however outbreaks of this type are much less common than those caused by norovirus.

2.4. Laboratory Methods For The Diagnosis Of Viral Causes Of IID

Laboratory diagnosis of viral IID is important, as the aetiological causes of IID (bacteria, parasites or viruses) cannot be differentiated based on clinical or epidemiological data alone. There are many tests available for the detection of viral causes of IID. Some are predominantly used in the research setting whereas others are commonly used in routine diagnostic services.

The requirements of a diagnostic test are quite different to an assay that is to be used in the research setting. Both settings require tests that have high sensitivity and specificity, however in the diagnostic setting speed, throughput and ease of use are also essential. Ideally, for a diagnostic test to be clinically useful it should be sensitive, specific, rapid and high throughput. A test with these characteristics will bring several advantages. First, the high sensitivity and specificity will increase the likelihood of the clinician receiving an accurate etiological diagnosis. Providing an etiological cause rather than a “syndromic” diagnosis allows clinicians and public health officials to make informed management decisions regarding patient care based on knowledge of the prognosis, length of infectiousness and possible transmission

routes. A rapid diagnosis may also prevent unnecessary tests for other potential causes or empirical treatment. Although not generally recommended for treating IID, a recent study in Ireland found that approximately 7.5% of patients who consulted a GP for symptoms of IID received antibiotics, which represented 15% of all treatments prescribed (Acute Gastroenteritis in Ireland, North and South-A Telephone Study, 2003). A rapid and accurate diagnosis may also help reduce unnecessary or prolonged hospitalisations whilst other tests are carried out. Taken together, all these factors will also help reduce health care costs. Rapidity is particularly important in the outbreak setting as it may influence decisions on infection control measures such as patient isolation or cohort nursing. A high throughput test will contribute to rapidity and will increase the robustness of the assay when faced with seasonal peaks in sample submissions and sudden increases resulting from the emergence of a new virus type. From the laboratory standpoint, an ideal test should not be expensive to implement and maintain. In addition, it should also be easy to use and robust since the tests may be operated by less specialised staff.

Accurate and rapid diagnosis will also inform public health surveillance systems. Rapidity ensures surveillance data is timely whereas the ability to provide an accurate result allows the morbidity, mortality and economic cost of particular infections to be measured and prevention measures to be planned or evaluated.

Since the initial discovery of viral causes of IID by EM in 1972 several techniques for the detection of viral causes of IID have been developed. These include the direct detection techniques culture and EM, antigen detection assays and the nucleic acid detection technologies such as PAGE, hybridisation and PCR. The advantages and disadvantages of each of are described in the following sections

2.4.1. Direct Detection Of Viral IID.

2.4.1.1. Culture Methods

One of the shared characteristics of the different viral causes of IID is their fastidious nature. There have been few reports of the successful use of culture systems to grow these viruses. Those that have been successful have often used specialist cell lines that are not commonly used in laboratories. For example, a permissive cell line for rotavirus is the monkey kidney epithelial cell line MA104 (Brydon *et al*, 1977; Purdham *et al*, 1975; Babiuk *et al*, 1977; Urasawa, Urasawa and Taniguchi, 1981).

Group B rotavirus has also been successfully cultured and is distinguished from group A rotavirus by forming multinucleated syncytia. Norovirus and sapovirus have proved impossible, thus far, to culture in either cell lines or organ cultures. Duizer *et al* attempted to culture norovirus in 24 different cell lines with and without various supplements (Duizer *et al*, 2004). No successful growth was reported. Wobus *et al* successfully cultured a murine norovirus in dendritic and macrophage cell lines (Wobus *et al*, 2005). However, whether or not this cell line can be used in human norovirus strains remains to be seen. Astrovirus has been frequently isolated using the cell line Ca-Co-2 (Grimm *et al*, 2004; Brinker *et al*, 2000; Pinto, Diez and Bosch, 1994). Rohayem *et al* compared Ca-Co-2 to Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for the diagnosis of astrovirus. In 287 samples Ca-Co-2 detected 8 as positive whereas 19 were detected by RT-PCR. Other cell lines that can be used for astrovirus include MA104 and T84. Adenovirus types 40 and 41 have been successfully cultured in the cell line Graham 293 (Szucs and Uj, 1998).

In general most of the methods described above are prolonged and laborious and not suited for high throughput work. As a result viral culture is rarely used for diagnosis, as other methods are more sensitive, rapid and are easier to use. However, a culture system is still desirable in order to carry out viability studies to assess detergents and future antivirals.

2.4.1.2. Electron Microscopy

EM was instrumental in the discovery of the viral causes of IID and as a result has been the diagnostic gold standard for many years. It is an open technique allowing the detection of a number of pathogens if present in the sample (Biel and Madeley, 2001). This characteristic is also useful in the search for new pathogens (Curry, 2003). Of all the viral causes of IID rotavirus is probably the most easily identified using this method as the virus can be excreted in large quantities for prolonged periods (over 10^{10} viral particles/ml has been described). Furthermore, rotavirus can be readily identified by its “wheel like” appearance under EM. However, other viral pathogens are more difficult to detect using EM. The “star like” appearance of astrovirus is often only present in around 10% of the viral particles. The “cup like” depressions of norovirus are often indistinct although they tend to be more distinct in sapovirus. As a result astrovirus and norovirus pathogens can often be mistaken for each other.

The overall detection limit of EM is approximately 10^6 viral particles/ml of faecal sample. However, viruses causing IID are often shed at levels below this detection limit, especially a few days after the onset of symptoms. As a result EM is reliant on collecting samples early on in the illness, when viral shedding is at its greatest. Furthermore, if samples are not refrigerated before examination the viral particles can break down reducing the sensitivity of the assay still further (McCaughey *et al*, 2000).

Samples that contain no virus or levels of virus below the detection limit can take time to examine, since the whole sample has to be scanned in detail to check for the presence of the virus, thus prolonging turn-around-times and lowering throughput. The use of Immune Electron Microscopy (IEM) can increase the sensitivity, specificity and rapidity of EM. IEM utilises convalescent serum or immune serum and mixes this with faecal sample. The viral particles, if present, should then form large antibody-antigen complexes, which are easier to detect by EM. The adaptation of this method to include a grid coated in antibody (called Solid Phase IEM or SPIEM) is also useful (Kjeldsberg, 1994; Wood *et al*, 1989; Svensson, Grandien and Pettersson, 1983; Svensson and von Bonsdorff, 1982). Both methods have also been used to serotype viral pathogens and measure immune responses.

Overall, however, EM is laborious since each sample requires detailed manual examination, which predisposes it to low throughput. It is also insensitive in comparison to other techniques (Buesa *et al*, 1996; Pang *et al*, 1999; Simpson *et al*, 2003). It requires highly trained personnel and expensive equipment. Automated systems have been described which reduce the need for specialised personnel (Utagawa *et al*, 2002), however these are often very expensive. The large initial cost of EM (in terms of both equipment and building costs) and continued maintenance costs has resulted in EM being restricted to specialised virology testing centres.

2.4.2. Antigen Detection Methods

Since the 1980s an increasing number of in-house and commercial antigen detection methods have been described for the detection of viral IID. These methods have many advantages over EM. They are easy to use, high throughput and have a rapid turn-around-time (less than 4 hours from sample arrival to result). They are also less expensive than EM as they do not need specialised equipment or large laboratory space. As a result these assays tend to be used by a wider range of laboratories for the

detection of viral causes of IID. Commercial assays have proved particularly useful, as the reagents are already provided, they have good standardisation, Quality Control (QC) and technical support. These assays are available in numerous formats including Enzyme ImmunoAssay (EIA), Latex Agglutinations assays (LA), and ImmunoChromatoGraphy methods (ICG).

Most of the commercial antigen detection assays available are for the detection of rotavirus and adenovirus. Methods are also available for the simultaneous detection of both viruses (Giordano *et al*, 2005; Rabenau *et al*, 1998).

For rotavirus, most assays utilise monoclonal or polyclonal based assays that target the conserved VP6 antigen (Anand *et al*, 2001; Eing *et al*, 2001; Kelkar *et al*, 2004). Monoclonal-based EIA assays (developed in the 1990s) are more sensitive than polyclonal based assays, whilst both tend to be more sensitive than LA methods (Dennehy, Gauntlett and Tente, 1988; Thomas *et al*, 1988; Altindis *et al*, 2004). However LA is more rapid than EIA (Thomas *et al*, 1994; Kohno *et al*, 2000). ICGs are now regarded to be as sensitive as EIA and as rapid as LA for rotavirus diagnosis (Wilhelmi *et al*, 2001; Bon *et al*, 2005).

All of the above antigen detection methods are considered at least as sensitive for rotavirus, if not more so, than EM. Detection limits tend to be between 10^4 - 10^7 viral particles/ml. However, their reduced specificity can result in false positive results. This is especially true during non-peak times of the year (Moore, Steele and Alexander, 2000; Banyai *et al*, 2003; Sanekata *et al*, 1990). Other problems include their reduced sensitivity in adult patients and in others who shed low levels of rotavirus (Anderson and Weber, 2004). They are also, at present, unable to detect non group A rotavirus strains (James *et al*, 1998).

There are a number of commercial antigen detection methods available for adenovirus including EIA, LA and ICG (Moore, Steele and Alexander, 2000; Banyai *et al*, 2003; Sanekata *et al*, 1990). Most of the assays target the hexon antigen and are either in monoclonal or polyclonal format (Vizzi *et al*, 1996; Wood *et al*, 1989; Wood *et al*, 1989; Nishio *et al*, 1990). Monoclonal-based EIAs tend to be superior to LA and ICG. In comparison to EM, the assays show comparable or slightly greater sensitivity. As with rotavirus however, antigen based methods for adenovirus detection have reduced specificity and can result in false positive reactions (Moore, Steele and Alexander, 2000; Banyai *et al*, 2003; Sanekata *et al*, 1990).

The development of an antigen detection assay for noroviruses has been hampered by their variability and complicated immune reactions (described earlier). Early antigen detection methods were in-house and targeted viral capsid proteins using hyper immune sera from mice, guinea pigs and rabbits, targeting viral capsid proteins. These assays often failed to detect all of the different types of norovirus and thus were restricted to research laboratories only (Nakata, Estes and Chiba, 1988). Some in-house assays were specifically devised to detect the predominant strains, allowing other methods to be used on a smaller subset of samples (Vipond *et al*, 2000; Matson *et al*, 1989). The ability to express norovirus capsid proteins in a baculoexpression system allowed the generation of VLPs. VLPs have been used to immunise animals, producing both monoclonal and polyclonal antibodies. This led to the discovery of genotype and genogroup specific antibodies, which in turn led to the development of a small number of commercial EIAs for the detection of norovirus. Studies have shown these assays to be more sensitive than EM. Richards *et al* compared a commercial polyclonal EIA (IDEIA, Dako Cytomation) with EM on a panel of outbreak specimens (Richards *et al*, 2003). Overall the sensitivity of the EIA was greater than that of EM (55.5% vs 23.9%). It was concluded that this EIA would prove useful in laboratories without access to EM or molecular methods such as PCR. However, this assay was later withdrawn and replaced with a monoclonal-based EIA. Burton-Macleod *et al* compared the monoclonal EIA (IDEIA, Dako Cytomation, Ely, UK) to another commercial EIA called the Denka EIA (Seiken Co. LTD, Tokyo, Japan) on 103 samples containing G1 and G2 norovirus, and representatives of other enteric viral pathogens (Burton-Macleod *et al*, 2004). Although the Denka kit was more sensitive than the Dako assay (70% vs 30%) the specificity was significantly reduced (69% vs 100%). The Denka assay also detected sapovirus. However, neither EIA was recommended for routine diagnosis. Recent assessments of other commercial EIAs for norovirus have also proved disappointing (Dimitradis and Marshall, 2006).

There are relatively few antigen detection methods available for the detection of astrovirus. A number of in-house EIAs have been described in the literature with varying levels of sensitivity (Herrmann *et al*, 1990; Komoriya *et al*, 2003; Kohno *et al*, 2000). Recently commercial assays have become available. Putzker *et al* compared a commercial EIA assay (IDEIA, Dako Diagnostika, Hamburg, Germany) with EM on 213 faecal samples (Putzker *et al*, 2000). Overall the EIA was able to detect all of the sample positives as EM from all 8 astrovirus groups. A recent study by Liu *et al*

compared the same commercial EIA with RT-PCR (using primers mon 269 and 270). They found that the EIA had comparable sensitivity to the RT-PCR assay (Liu *et al*, 2005).

At present there is no commercial assay available for the detection of sapovirus. Several in-house assays have been described. Most use polyclonal antibodies but these have tended to be insensitive (Hansman *et al*, 2005; Hansman *et al*, 2006). The use of VLPs for sapovirus may in future lead to the development of commercial assays for its detection.

Overall, antigen detection methods are a useful laboratory diagnostic method. Studies have shown them to be at least as sensitive as EM with the advantage that they are rapid, easy to use, and high throughput. They are also inexpensive to implement in a laboratory, as they require little extra equipment or specialised training. However, despite these advantages, question marks remain over their specificity, especially for norovirus detection. They also have stringent requirements concerning the time from sample collection to testing taking place, which must be between 3-7 days depending on the pathogen under test. Furthermore, no commercial assay is currently available for sapovirus.

2.4.3. Nucleic Acid Detection Assays

2.4.3.1. Poly-Acrylamide Gel Electrophoresis (PAGE)

An inexpensive and effective alternative to either EM or antigen detection methods for the diagnosis of rotavirus is polyacrylamide gel electrophoresis (PAGE). To carry out this technique the segmented dsRNA is extracted directly from faecal samples, electrophoretically separated on polyacrylamide gels and then detected by silver staining (Steele *et al*, 1993; Johnson and McCrae, 1988). The sensitivity is as good as EIA and can be improved using nucleic acid extraction techniques. This method detects all rotavirus types and allows group A rotavirus to be differentiated from other rotaviruses as it has a distinctive banding pattern to that of group B and C. However, the steps described above mean that this method is laborious and therefore not routinely used in laboratories.

2.4.3.2. Hybridisation

A number of hybridisation assays have been described that use complementary labelled probes such as radio labelled dioxigenin (Willcocks *et al*, 1991; Moe *et al*,

1991). Some of these assays have been shown to be as sensitive and specific as EIAs for rotavirus (Ali *et al*, 1993; Flores *et al*, 1983). However, hybridisation assays for variable RNA viruses such as norovirus and sapovirus have shown reduced sensitivity, mainly because of the difficulty in targeting conserved areas. As a result these assays quickly became redundant, as laboratories preferred the increased sensitivity of other molecular techniques such as PCR (described in the following section).

2.4.3.3. Polymerase Chain Reaction (PCR) Assay

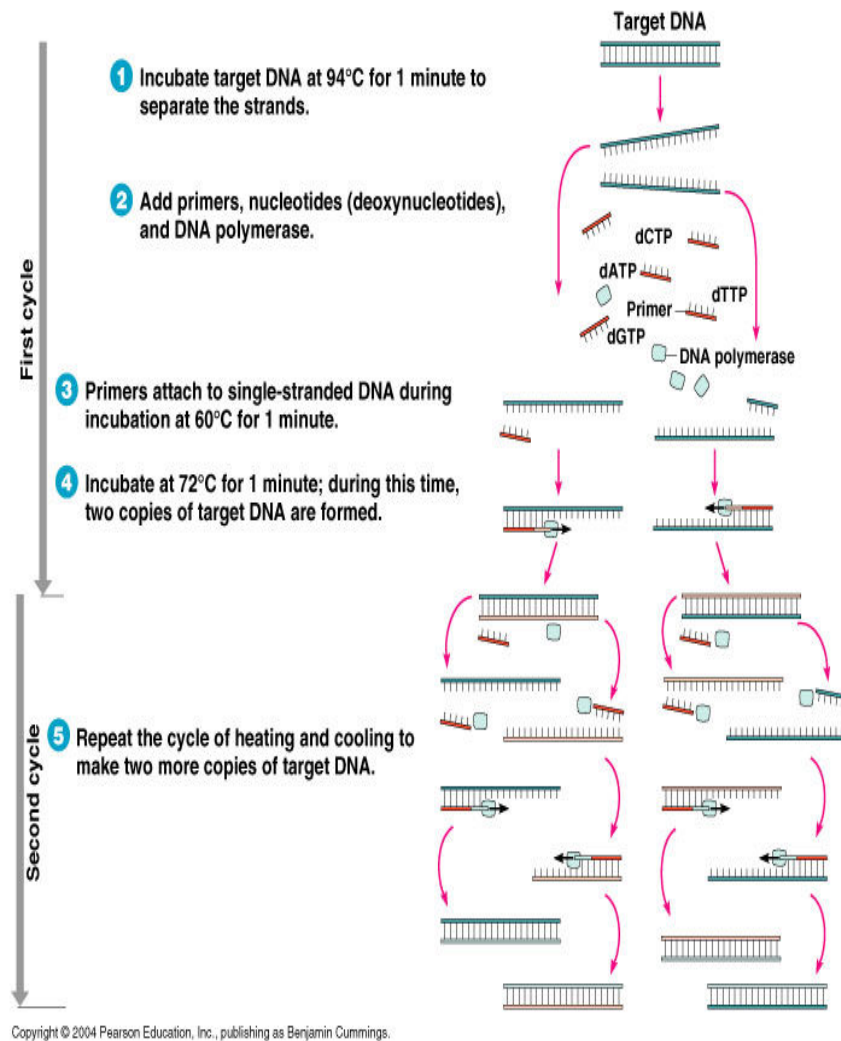
PCR (shown in Figure 9) is a very sensitive technique allowing the detection of minute quantities of virus (Mullis and Faloona, 1987; Holland *et al*, 1991). PCR utilises a pair (or more) of synthetic oligonucleotides called primers, which are specific to a conserved region (of defined length) of the genome of the virus to be detected. These primers are then added to a mixture containing dNTPs (nucleotides), various buffers, and an enzyme called Taq DNA polymerase. To this mixture the nucleic acid from a clinical sample is added. This PCR mixture then undergoes a cycle of temperature changes consisting of: a denaturation step that causes the DNA strands to separate; an annealing step during which the primers bind to the target sequence; and an extension step where the Taq DNA polymerase uses the primers as a substrate to create a complimentary strand, by adding dNTPs to the primers. At the end of one PCR cycle the number of starting target sequences doubles. After 30-50 cycles millions of copies of the original template can be made.

Although principally a DNA detection technique the detection of RNA can be achieved by the addition of a Reverse Transcriptase (RT) step, which converts RNA to cDNA that can then be used in PCR reactions.

Successful PCR relies upon numerous factors. Efficient nucleic acid extraction is needed to purify the nucleic acid from the clinical sample and remove substances that may inhibit the PCR (Rasool, Monroe and Glass, 2002). There are many different methods available for this (Hale, Green and Brown, 1996; Kok *et al*, 2000; Santos and Gouvea, 1994). Many are in a commercial kit format thus improving the reliability and ease of use of the extraction process. Many of these kits also work using an automated robot format allowing multiple samples to be extracted reliably and within a short time period, which is useful in a high throughput (i.e. diagnostic) laboratory and helps improve the rapidity of PCR.

Another important factor for successful PCR is the choice of primers. Primers should be designed to detect and amplify conserved regions of the target virus. Targeting variable regions may prevent primers from binding and can result in false negative PCR results.

Figure 9: The Polymerase Chain Reaction assay.



The use of multiple primers (known as multiplex PCR) allows the detection of multiple pathogens within a single PCR reaction (O’Neil *et al*, 2002). Multiplexing a number of PCR methods into a single tube can improve test rapidity by allowing a number of tests to be carried out in one step and can also reduce tests costs by using less reagent.

Carrying out a nested PCR can increase the sensitivity of a PCR test. Nested PCR consists of a second PCR reaction that uses the PCR product as a template and primers that target an area within the first PCR target region. However, although this

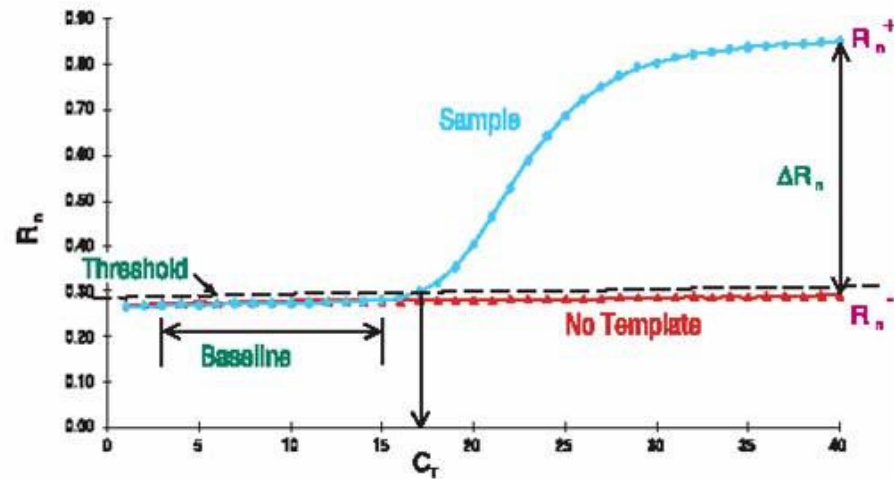
increases the sensitivity of the PCR, it also raises the risk of false positive results due to contamination.

Currently, two forms of PCR assay exist - conventional methods and the more recent real-time PCR techniques. Both are based on the above chemical reaction and differ only in how the result is analysed. In conventional PCR, which was the first kind to be developed, the product from the PCR reaction is detected by adding a volume of the PCR mixture to an agarose gel once the reaction is complete. After electrophoresis the gel is examined under ultraviolet light. If the target was present in the original sample a band of defined length (corresponding to the target sequence) is observed. Other methods of confirmation include micro-titre plate hybridisation and southern hybridisation (van der Vliet, Hermans and Klatser, 1993; Keller *et al*, 1990). This form of PCR therefore provides end point analysis, which means that the result is only available at the end of the test.

Real-time PCR, in contrast, allows visualisation of the PCR process as it occurs. This technique first began to appear in the late 1990s. Instead of a band on a gel a positive reaction is shown by an increase in fluorescence above background levels. The PCR cycle point at which the sample becomes positive is called the cycle threshold (Ct). The Ct is directly related to the starting target copy number. The lower the Ct (i.e. the nearer it is to zero) the greater the starting target copy number (Figure 10), which means that real-time PCR is a semi-quantitative assay and with the use of known standards can be made fully quantitative (Kang *et al*, 2004).

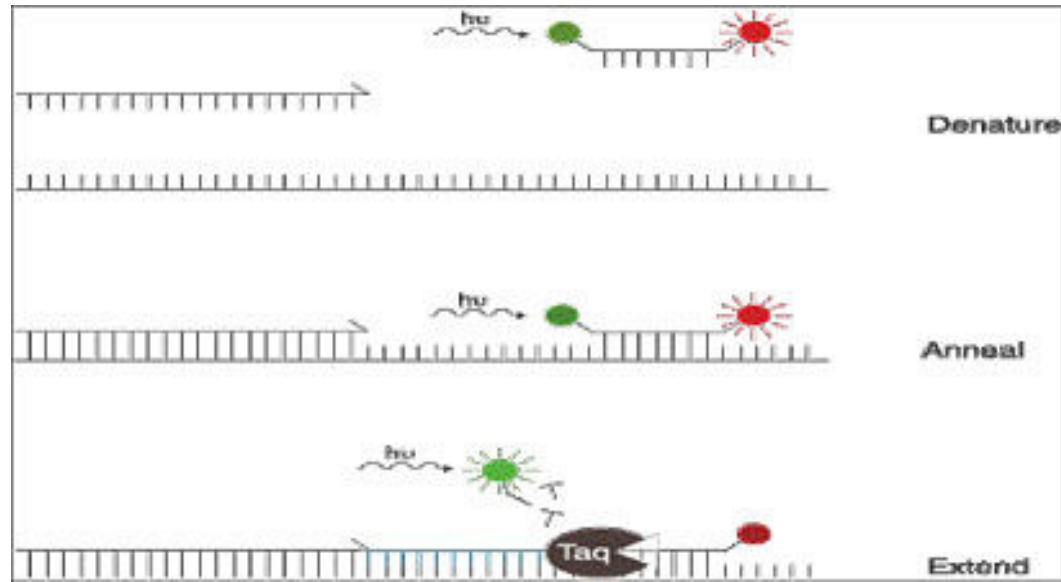
There are a number of chemistries available for real-time PCR including dual-labelled probes (formally known as TaqMan™ probes), molecular beacons, Minor Groove Binding probes (MGB), fluorescent-labelled primers and DNA chelating dyes (Figures 11-14). The advantages and disadvantages of these chemistries are discussed in Table 3. Most published assays use dual-labelled probes or molecular beacons. This is probably due to the fact that the addition of a probe or beacon improves specificity, since this and the primers have to bind to the PCR to provide a positive result.

Figure 10: Real-time PCR fluorescent curve.



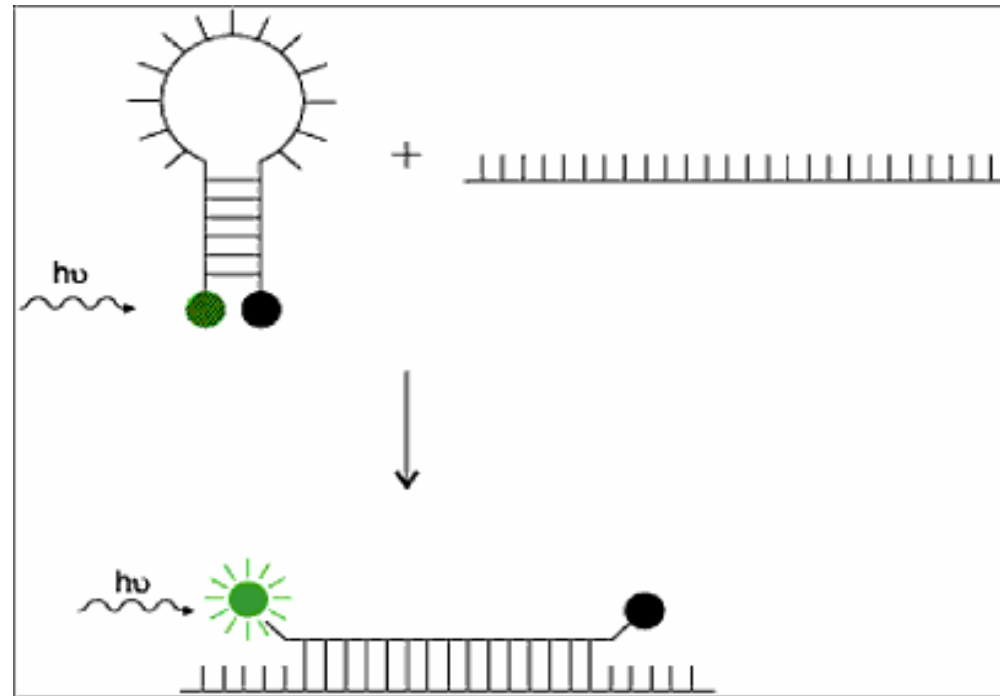
The figure shows a representative amplification plot (fluorescence signal versus cycle number) for real time PCR. In the initial cycles of PCR, there is little change in fluorescence signal (This is the baseline for the amplification plot). An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. The parameter C_t (threshold cycle) is defined as the cycle number at which the fluorescence passes the fixed threshold. So the higher the initial amount of the target in the sample, the sooner accumulated product is detected in the PCR process as a significant increase in fluorescence, and the lower the C_t value.

Figure 11: Dual-Labelled Probes.



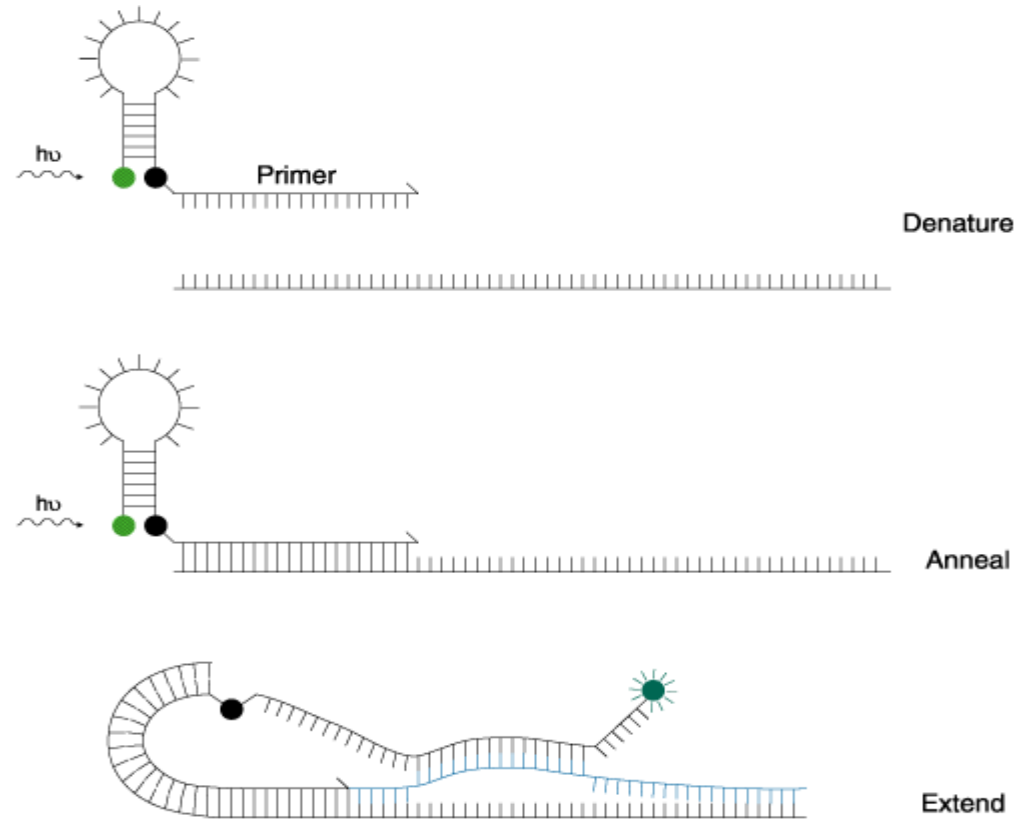
Dual-labelled probes (also known as Taqman™ probes) are oligonucleotides that contain a fluorescent dye on the 5' base, and a quenching dye located on the 3' base. When excited the fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing, resulting in a nonfluorescent probe. Dual labelled probes are designed to hybridise to an internal region of a PCR product. During PCR, when the polymerase replicates a template on which the probe is bound, the 5' exonuclease activity of the polymerase cleaves the probe. This separates the fluorescent and quenching dyes, allowing detection of the signal from the reporter dye. Fluorescence increases in each cycle, proportional to the rate of probe cleavage (<http://probes.invitrogen.com>).

Figure 12: Molecular Beacons.



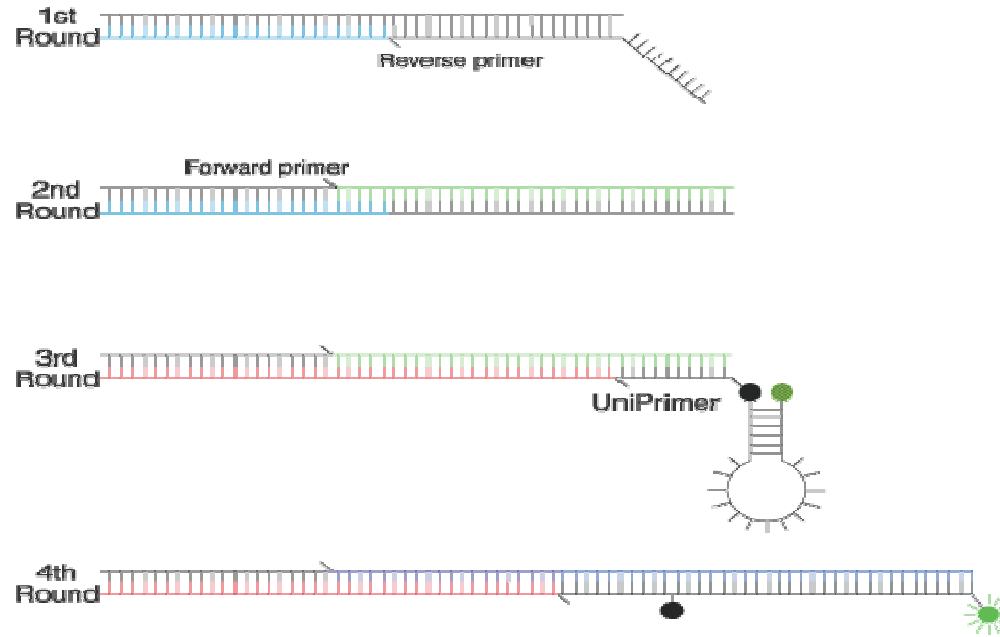
Here the primer binding site is within the loop. Molecular beacons contain fluorescent and quenching dyes and are designed to adopt a hairpin structure while free in solution, bringing the fluorescent dye and quencher into close proximity. When a molecular beacon hybridises to a target, the fluorescent dye and quencher are separated and the fluorescent dye emits light upon irradiation. Unlike dual labelled probes, molecular beacons are designed to remain intact during the amplification reaction. (<http://probes.invitrogen.com>)

Figure 13: Scorpion primers.



Schematic representation of real-time PCR with Scorpion primers. In the hairpin loop structure, the quencher (black circle) forms a nonfluorescent complex with the fluorophore (green circle). Upon extension of the amplicon, the Scorpion probe hybridises to the newly formed complementary sequence, separating the fluorophore from the quencher and restoring the fluorescence (<http://probes.invitrogen.com>).

Figure 14: Uniprimers.



Schematic representation of real-time PCR with UniPrimers. In the first round of amplification, the reverse primer, containing a special sequence tag, primes synthesis along the template. In the second round, the forward primer primes synthesis that extends through the special sequence tag, forming a complementary sequence to the tag. In the third round, the UniPrimer hybridizes to this complementary sequence via the special sequence tag. The hairpin structure of the UniPrimer ensures that the quencher (black circle) suppresses the fluorescence of the fluorophore (green circle). Finally, in the fourth round, synthesis extends through the hairpin loop, relieving the quenching of the fluorophore (<http://probes.invitrogen.com>).

Table 3: Comparisons of the various technologies available for real-time PCR.

Chemistry	Advantages	Disadvantages
Molecular beacons	Specific Sensitive	Susceptible to probe mis-match Expensive Low fluorescence Less available
Dual-labelled probes	Specific Many publications available Increased fluorescence Less susceptible to probe mis-match Many manufacturers	False negative results can still occur
Minor groove binders	Specific Increased fluorescence produced Can be used in small conserved areas	Susceptible to probe mis-match Few suppliers Expensive
Labelled primers (e.g. sunrise, scorpion, and lux)	Cheap (no probe needed) As sensitive as probe-based technology Less homology needed (no probe region)	Primer-dimer formation Strict design criteria
Chelating dyes	Cheap Used on large regions	Primer-dimer formation Less specific than other methods

Since both conventional and real-time PCR methods detect nucleic acid rather than viral particles or antigen, these tests involve less stringent requirements than either EM or EIA concerning the time taken between sample collection and testing. Both methods have also been shown to offer significantly improved sensitivity in comparison to other diagnostic methods (for a full review of each of the PCR methods available see Chapter 3), although more data is available on the performance of conventional PCR due to the relatively recent nature of real-time technology. For example, for rotavirus detection, Buesa *et al* carried out a comparison of conventional RT-PCR, PAGE and EM on 200 samples. The PCR assay detected 66 positive samples, whereas PAGE and EM detected 59 and 56 positive samples respectively (Buesa *et al*, 1996). Similar results were found by Pang *et al*, who compared a different gel-based RT-PCR with a commercial EIA (Rotaclone) on a total of 2398 infants. In Pang *et al*'s study, 256 cases of rotavirus-associated gastroenteritis were detected by EIA whereas with RT-PCR, 84 (33%) more cases of rotavirus gastroenteritis were diagnosed (Pang *et al*, 1999). Similarly, Simpson *et al* compared a real-time RT-PCR assay with EM on 305 samples collected from children under five years of age with acute gastroenteritis (Simpson *et al*, 2003). The RT-PCR method detected 86 rotavirus-positive samples, compared to 70 using EM (an increase in detection rate of 22.9%). Similar increases in detection have been observed when comparing PCR to EM for adenovirus. For example, Simpson *et al* detected 40

adenovirus-positive samples using real-time PCR compared to 12 detected by EM (an increase of 233%).

Similar, if not greater, increases in detection rate have been observed when using PCR for the detection of norovirus, astrovirus and sapovirus. Early studies using EM and EIA often failed to detect these viruses. For example, Waters and colleagues used EM and EIA to examine 1386 samples from children admitted to hospital in Canada with IID. Although the detection rate for rotavirus and adenovirus was in line with other studies, the detection rates for norovirus, astrovirus and sapovirus were found to be 2%, less than 1% and less than 1% respectively (Waters *et al*, 2000). An Italian study examining 417 children hospitalised with IID found, using EM, astrovirus in less than 1% of cases. No cases of norovirus or sapovirus were reported (Donelli *et al*, 1993). The use of PCR has significantly increased the detection rate of these pathogens. For example, Froggatt *et al* examined 3,172 samples from children (aged under 7 years) in Bristol, UK (Froggatt *et al*, 2004). Using EM, 0.9% of cases tested positive for norovirus. The use of EIA and a gel-based RT-PCR increased the detection rate to 4.2% and 10.3% respectively. In the study carried out by Simpson *et al* norovirus was detected using real-time RT-PCR in 46 samples compared to 6 using EM. This study also showed an increase of astrovirus positives (7 vs 3) and sapoviruses (8 vs 1) using the real-time PCR assay.

This increased sensitivity of PCR in comparison to other techniques has in recent years helped establish that viral pathogens are the major cause of sporadic IID in children both requiring hospitalisation and presenting to GPs, and that these pathogens also contribute significantly to sporadic IID in adults (Simpson *et al*, 2003; de Wit *et al*, 2001 (i); de Wit *et al*, 2001 (ii))

The greatest impact, however, has been in the diagnosis and investigation of outbreaks of IID. Before the application of RT-PCR in the late 1990s the aetiology of most outbreaks of IID was undetermined. This was highlighted by Bean and Griffin who examined 7500 outbreaks of IID reported to the CDC between 1973-87 (Bean and Griffin, 1990). Of these, only 38% had a diagnosed aetiological cause (1.8% were attributed to viruses). In contrast, the utilisation of PCR methods has now established that the majority of IID outbreaks are caused by norovirus. Fanklauser *et al* examined 284 outbreaks submitted to the CDC from 1992-2000 using a conventional gel-based PCR (Fankhauser *et al*, 2002). Of these, 93% were found to be caused by norovirus. Similar findings have been reported elsewhere based on the use of PCR techniques

(Chatterjee *et al*, 2004; Inouye *et al*, 2000; van Duynhoven *et al*, 2005). For example, Lopman *et al* reviewed the causes of outbreaks reported in a number of European studies occurring between 1995-2000 (Lopman *et al*, 2003). Noroviruses were shown to cause most cases of outbreaks in England and Wales (96%), Germany (100%), Sweden (97%), the Netherlands (84%), Slovenia (43%) and Spain (57%). The other four viruses discussed are also known to cause outbreaks of IID, however outbreaks of this type are much less common than those caused by norovirus.

There is some concern that PCR techniques may in fact be over-sensitive, detecting low levels of virus that may be due either to prolonged shedding after the resolution of symptoms or to asymptomatic infection in patients with illness due to other causes.

Prolonged viral shedding after the resolution of symptoms has been detected in a number of cases (Richardson *et al*, 1998; Ball *et al*, 2005; Rockx *et al*, 2002; Cliver, 1997). Moreover, the fact that in such cases the more sensitive PCR techniques have detected virus for the longest periods suggests that it is shed at low levels. Richardson *et al*, for example, followed up 37 children with acute rotavirus infection. Using EIA rotavirus was detected up to 21 days (with a mean of 7 days) after infection whereas RT-PCR detected rotavirus up to 57 days post-infection. Here, the mean was 10 days (Richardson *et al*, 1998). The extended duration of shedding could not be linked to disease severity or age. Norovirus shedding, also, has been detected up to 7 weeks or more post-infection (Ball *et al*, 2005; Rockx *et al*, 2002) whereas sapovirus and astrovirus have been detected up to 2 weeks post-infection using RT-PCR (Rockx *et al*, 2002; Cliver, 1997). However, the percentage of infections that result in prolonged shedding has yet to be established and may be in the minority (Rockx *et al*, 2002; de Wit *et al*, 2001).

True asymptomatic infections have also been observed in a small number of studies and are likely to be the result of a complicated relationship between various viral and host factors (Graham *et al*, 1994). For example, Graham *et al* inoculated 50 volunteers with norovirus and of these 32% showed seroconversion and viral shedding in the absence of clinical symptoms. Recent studies have attempted to demonstrate that a link exists between the severity of the illness and the level of virus shed, with the suggestion that less severe or asymptomatic infections result in low levels of shedding. Kang *et al*, for example, used a real-time PCR to quantitate rotavirus shedding and compared it to Vesikari severity scores in 66 children

presenting to hospital with IID (Kang *et al*, 2004). Those with mild to asymptomatic infection were found to have very low viral loads (i.e. Ct values close to 40 cycles) compared to those with more severe infection. Similarly, Pang *et al* compared RT-PCR to EIA on children with IID (Pang *et al*, 1999). The RT-PCR detected more cases of rotavirus in comparison to the EIA, but these were more often from those with mild/asymptomatic infection. These findings have not been restricted to rotavirus. Richards *et al* used real-time PCR to quantitate norovirus shedding in volunteers with symptomatic and asymptomatic infection (Richards *et al*, 2004). Two of the 3 volunteers with asymptomatic infection (note a very small sample size) were found to have a low level of viral shedding. Gallimore *et al* tested asymptomatic staff and patients after an outbreak of norovirus in a hospital ward (Gallimore *et al*, 2004). In total, 87 asymptomatic staff were tested and of these 26% were positive for norovirus. Since these were detected by a nested method but not by a less sensitive single round PCR it was suggested that the virus in the asymptomatic cases was being shed at low levels.

However, others studies have failed to find a link between viral shedding and disease severity. In the study mentioned above by Richards *et al* one of the asymptomatic patients was found to have a high viral load in the stool. Conversely, low levels were also detected in a number of symptomatic volunteers with more severe infection. Zhang *et al* also used real-time PCR to correlate astrovirus viral load and severity in 54 patients presenting at hospital with gastroenteritis (Zhang *et al*, 2006). Here, no link between viral load and severity was observed. Consequently, the link between low level viral shedding and asymptomatic infection has yet to be confirmed.

Furthermore, the rate of asymptomatic infection in different patient groups and the population at large has also yet to be determined. Much of what is known about the rate of asymptomatic IID infections has come from a small number of case-control studies. Some of these studies have suggested that asymptomatic shedding is very common, whereas other studies have found it to be rare or have failed to detect such cases at all. Many of these differences are likely to be a result of the tests used, the cohort examined and the country the study was carried out in. For example, Tompkins *et al* examined the detection rate of various pathogens in cases of IID and controls in both a general population and a GP cohort (Tompkins *et al*, 1999). This study used EM and EIA to test for viral pathogens. Detection rates were found to be low (1.1%-

1.2%) in both control groups, with rotavirus and norovirus most common in both populations. Recently, the same samples were re-tested using more sensitive PCR methods for norovirus, rotavirus and sapovirus (Amar *et al*, 2007). The detection rate for norovirus, rotavirus and sapovirus in the control groups increased significantly from the previous study to means of 16% (range 5-31%), 14% (range 4-29%) and 2% (range 0-13%) respectively. Examining the detection rate by age group showed that the highest values were detected in the young. It is important to note, however, that children under 5 yrs of age may have been over-represented in the study resulting in an inflated mean detection rate. Moreover, information on whether the control cases had had a recent gastrointestinal illness was not known, which is a serious flaw as the detections may not have been due to true asymptomatic cases but examples of prolonged shedding following infection. Other case control studies have used molecular methods for the detection of viral pathogens. For example, de Wit *et al* used RT-PCR to test for norovirus and sapovirus and EIA methods to test for adenovirus, astrovirus and rotavirus. They detected viruses in approximately 9.1% of controls taking part in a community cohort study (de Wit *et al*, 2001). Of this total, norovirus was detected in 5.2% of the control samples. Again, however, this value may have been an over-estimate as children were over-represented in the study (Marshall *et al*, 2004). However, similar rates for norovirus asymptomatic infection have been found in studies in Japan and Mexico (Garcia *et al*, 2006; Akihara *et al*, 2005). High levels of asymptomatic infection have also been found in the outbreak setting (Vinje *et al*, 1997) and in countries with low socioeconomic conditions (Garcia *et al*, 2006). On the other hand other studies have failed to detect norovirus at all in asymptomatic control groups (Marshall *et al*, 2004). Marshall *et al* tested samples from 399 asymptomatic individuals with a nested RT-PCR and failed to detect a single norovirus case. Meanwhile, few studies have applied molecular techniques to investigate the detection rate of adenovirus and astrovirus in asymptomatic patients.

In summary, therefore, the concern that PCR techniques may be over-sensitive, detecting asymptomatic cases of IID or cases following resolution of the symptoms, is based on several assumptions that have yet to be fully proven. Although there is evidence that prolonged shedding post-infection does take place, the proportion of infections that this applies to has yet to be established and may be in the minority. Meanwhile, data on whether asymptomatic infection is associated with viral shedding (at low levels) is more contradictory, as is the evidence on the rate of such

infections in patient groups and the population at large. While it is possible, therefore, that the use of PCR may result in some cases of unnecessary detection of IID these are likely to be in the minority.

Whilst similar levels of sensitivity are achievable using both types of PCR, in practical terms there are some differences between them that mean real-time techniques are more likely to be of use in a routine diagnostic setting. Despite conventional gel-based PCR having been available for some time, its use is still mainly restricted to specialist testing centres and research facilities. This is a result of many factors. Firstly, in order to reduce the risk of contamination the laboratory needs to be set up with a particular structure or flow through to prevent amplified product being reintroduced into subsequent PCR reactions. Conventional PCR is also technically demanding and requires specially trained staff in order to develop, implement and “trouble shoot” assays. The need for a post-amplification step such as agarose gel electrophoresis reduces the likelihood of automation and thus the potential for high throughput. As a result, although sensitive, the assay is not rapid or capable of processing a large number of samples at one time. The combination of these factors means that little use of conventional PCR is made in non-specialist and routine diagnosis laboratories. Despite this adaptations can be used to improve conventional PCR and increase its usefulness in the routine setting. For example, multiplexing more than one PCR test into a single test can reduce test costs and improve rapidity.

Real-time PCR offers the potential to overcome many of the problems encountered with traditional gel-based PCR (Mackay, Arden and Nitsche, 2002; Lomeli *et al*, 1989; Heid *et al*, 1996; Pfaffl, 2001). In contrast to traditional gel-based PCR, for example, real-time PCR is a closed technique, which means all of the PCR steps take place in one tube (reverse transcriptase, PCR and detection). This characteristic is likely to improve reproducibility and robustness, and reduces the risk of contamination thus favouring automation and high throughput. As a result, real-time PCR has the potential to be more rapid. The closed system also reduces the need for a strict laboratory set-up and as a result reduces implementation costs. The cost of reagents and equipment are also starting to reduce, as companies compete to provide to an increasing numbers of users. As a result, of all the methods discussed in this section, this thesis takes the view that it is real-time PCR that is most near to the ideal test outlined in the introduction.

2.5. The WOSSVC Diagnostic Service For Viral IID.

The West of Scotland Specialist Virology Centre (WOSSVC) is based at Gartnavel General Hospital (GGH) in Glasgow. It is one of only two specialist virology centres in Scotland and provides a virology service for the whole of Glasgow. In addition, a significant proportion of its workload (over 40%) is from health boards outside Glasgow. The users include hospital-based users, users in primary care and general practice and private industry. Each year over 200,000 investigations are performed. Since the late 1990s, as part of an ongoing programme of innovative service development the number of in-house molecular tests has increased significantly and these are now used in routine services for detecting viral and bacterial pathogens in Cerebrospinal Fluid (CSF) samples, respiratory samples, blood, urine, vesicle fluids and genital swabs.

At the start of the research documented in this thesis, although the process of implementing molecular methods had begun in other areas, the WOSSVC diagnostic service for viral gastroenteritis was based solely upon electron microscopy. The laboratory offered this service for samples from outbreaks of IID, and samples from sporadic cases of IID in children less than 10 years of age and immunocompromised patients. However, as made clear in previous sections, EM is laborious and not suited to testing large numbers of samples. Furthermore recent research has clearly highlighted the insensitivity of EM in comparison to emerging techniques, particularly PCR. In the interests of continuing to improve the diagnostic service offered therefore, a need was identified to replace EM in the laboratory with an improved test for the detection of viral IID. Based on the literature PCR was identified as the most suitable candidate. However, although highly sensitive and able to test a number of samples at any one time, PCR has been found to be technically demanding to implement, maintain and troubleshoot. To date these factors have resulted in its use being largely restricted to a research setting. However, the view taken in this thesis is that such tests have the potential to be adapted for use in the routine setting, particularly real-time versions of this technique.

Consequently, the overall aim of this work was to improve the routine diagnostic service for IID offered at the WOSSVC, by replacing EM as the frontline test with PCR methods adapted from the literature. This was broken down into several smaller objectives. The initial objective was to select and adapt appropriate PCR

techniques from the literature. The second was to compare these techniques to possible alternatives such as EIA and EM through a series of laboratory experiments to ensure their suitability. The final aim was to implement the new techniques in the routine service and examine their impact on the quality of the service offered.

2.6. Summary

This chapter has provided the background to the research presented in this thesis and through this the motivation for its existence. The chapter began with an overview of the public health importance of IID in developing and developed countries. Particular focus was then given to the cost of IID in developed countries in order to show that IID is a significant cause of morbidity in such countries and although mortality is low, the economic cost is substantial.

This section was followed by a description of the five main viral causes of IID. For each pathogen a number of characteristics were described including: their virology, classification, elicited immune response, epidemiology, the clinical illness in which they result, methods of preventing this illness, infection control and treatment. This section was designed to show that these genetically complex pathogens are a cause of world wide sporadic illness and outbreaks in all age groups, and can cause severe and chronic illness in numerous patient groups. The multiple types and subtypes and the complex immune responses were highlighted to show that repeat infections can occur with each virus. The virology of each virus was also described in order to highlight the variable nature of some parts of the viral genomes. This is important when considering the design of molecular assays to test for these pathogens, which is a major part of the next chapter. Their numerous sources and transmission routes were also described in order to highlight how difficult these pathogens are to control and prevent.

Following this, a review of the different laboratory methods available for the detection of viral IID was presented. This began with a description of the characteristics of an ideal diagnostic test in order to highlight that a routine diagnostic test has different requirements to an assay that is to be used as a research tool. The advantages and disadvantages of each diagnostic method were then described in this context. This section outlined the increased sensitivity offered by PCR techniques, which have helped to establish that viral pathogens are the most common cause of IID

in children and outbreaks of IID and contribute significantly to such illness in adults. As such, services for their diagnosis are particularly important. It was then highlighted that conventional and in particular real-time PCR methods have the potential to be adapted for use in the routine diagnostic laboratory and the aims of the research were described.

Chapter 3

Selection And Development Of The PCR Protocols For Investigation

3.1. Overview

This chapter describes in detail the selection and development of the PCR tests to be investigated as a potential replacement for the existing EM-based diagnostic service for viral gastroenteritis. The chapter begins by describing key decisions taken with regard to the structure of the proposed new service, based on a review of the literature (described in Chapter 2). These include the decision to split the service in two, with samples from outbreaks treated separately to those from sporadic cases of IID, and the decision on which pathogens to test for in each case. This is followed by a summary of the steps involved in a generic PCR assay. For each key stage (i.e. the sample preparation followed by the PCR method itself) the available protocols are then reviewed, and the factors affecting the eventual choices made for each of the outbreak and sporadic services are discussed. The methods selected in each case are described in detail, together with any adaptations necessary for use in the routine laboratory setting. The chapter ends with a full technical description of the sample preparation and PCR methods assessed in the research described in Chapter 4.

3.2. Introduction

It was established in Chapter 2 that norovirus is the principle cause of outbreaks of gastroenteritis, whilst for sporadic cases of IID there are five main viral causes: adenovirus, astrovirus, norovirus, rotavirus and sapovirus. Based on this information, the decision was taken to divide the new diagnostic service into two parts: a separate service for samples from outbreaks of IID and one for those from sporadic cases, with the distinction between the two types made via the clinical details provided on the specimen request form. This division of the service allows the tests in each case to be tailored to best match the known characteristics of the two types of viral IID. For the outbreak service, for example, the decision was taken to adopt a norovirus assay as the frontline test for sample investigation, based on an increasing number of reports showing norovirus to be the principle cause of outbreaks of IID (see Chapter 2). For the sporadic service, the decision was taken to employ this same norovirus assay together with PCR tests for adenovirus, astrovirus, rotavirus and

sapovirus to investigate sporadic cases of gastroenteritis in children under 10 years of age and immunocompromised patients (the patient groups already tested using the EM-based service). As shown in Chapter 2, these viral pathogens contribute significantly to IID in children and can cause severe and chronic infection in immunocompromised individuals of all age groups. In the case of the outbreak service, moreover, it was decided that samples which tested negative for norovirus should be examined further using the tests for the other non-norovirus pathogens. Testing for other possible causes of IID was rejected (in both services) since this would mean additional cost when the clinical significance of many of these pathogens is unclear.

A PCR test for viral IID is made up of numerous individual stages including the sample preparation steps (stool extraction followed by nucleic acid extraction), and the PCR-related steps such as reverse transcription (if the target is RNA), the PCR step itself (or two PCR steps if using a nested protocol) and, if using a conventional PCR method, agarose gel analysis for the interpretation of results. Numerous methods are available for each of these steps that each differ in their sensitivity, specificity, ease of use, cost and rapidity. Furthermore, some of these steps can be bought from commercial companies whereas others must be carried out using in-house methods. As a result each method must be chosen carefully in order to develop a PCR test that is both effective and applicable for use in the routine diagnostic setting.

This chapter motivates the choice of laboratory protocols for use in the proposed new molecular service for IID and describes each of the methods in detail. It begins by describing the methods chosen for stool and nucleic acid extraction. A review of the published PCR methods for the detection of the five main viral pathogens is then given. This discusses the advantages and disadvantages of each method and outlines relevant evaluation data. The factors that affected which of these assays were selected for further investigation in this research are then described, together with details of the eventual methods chosen and the adaptation of these to the routine setting.

3.3. Laboratory Methods

3.3.1. Sample Preparation Methods

Sample preparation in the form of stool extraction followed by nucleic acid extraction is a core part of all PCR based tests carried out on stool. Stool extraction is carried out in order to remove any sample debris that may inhibit nucleic acid extraction and other laboratory equipment. The nucleic acid extraction method is carried out in order to remove PCR inhibitors and, in some cases, to concentrate nucleic acid (Atmar and Estes, 2001). Concentrating the nucleic acid in a sample aids the sensitivity of the PCR test, as more nucleic acid can be added to a PCR reaction. A number of methods are available for each of the stool and nucleic acid extraction steps, the choice of which is independent of the PCR method chosen.

3.3.1.1. Stool Extraction Protocol

Faecal extraction consists of three main steps. First the stool is added to some form of liquid medium to make a 10-20% solution, which is then centrifuged in order to pellet cell debris. The supernatant is then removed and can be used for nucleic acid extraction. Various solutions and centrifugation speeds have been described in the literature (Schaffer *et al*, 1985; Logan *et al*, 2006; Lennette, Balows, Hausler and Sahdow, 1985 (Fourth Edition)). Solutions described include distilled water, phosphate buffered saline (PBS), viral transport medium (VTM), Hanks medium and specially designed medium from commercial companies, although no studies exist that focus on comparing the utility of these methods. The method implemented in this research uses PBS to make the 10-20% solution, which is then centrifuged at 3,000 rpm for 30 minutes. Examples of its use can be found in the literature (Lennette *et al*, 1985) and its effectiveness was already established in the laboratory as part of the existing EM protocol. Details of the exact implementation are outlined in section 3.4.

3.3.1.2. Nucleic Acid Extraction Method

Nucleic acid extraction is necessary to purify and concentrate nucleic acid (DNA and RNA) from stool samples and to remove any factors that may inhibit PCR. There are numerous in-house and commercial methods available for nucleic acid extraction.

An in-house method commonly used for the extraction of nucleic acid from stool samples is the “Boom” method (Boom *et al*, 1990). This method uses guanidium

thiocyanate to lyse the stool samples. Acid phenol-chloroform-isoamyl alcohol is then used to extract nucleic acid from the lysed faecal samples and the nucleic acid is then purified by silica binding. This protocol has been shown to successfully extract ssRNA, dsRNA and DNA from stool samples (Rasool *et al*, 2002). Other published in-house methods used for the extraction of nucleic acids from stool samples include (Atmar and Estes, 2001); exclusion chromatography (using spin columns containing sephadex G200); heat release (heating stool samples for 5 minutes at 95-99°C); and antibody capture (virus-specific antibodies, bound to a 96-well plate, bind virus from clinical samples and viral RNA and/or DNA is released from the capsid after washing and heating). These methods differ in their ability to purify nucleic acids and are variously suited to particular sample types, viruses and nucleic acid types.

In general, in-house extraction procedures are inexpensive and are therefore suited to use in the research setting. Their primary aim is to remove inhibitors from the sample and then efficiently extract nucleic acid. Of less importance are their ease of use and the number of samples that can be extracted at any one time. As a result, these methods tend not to be high throughput, can be difficult to standardise and require prolonged hands-on time, which increases the risk of error and contamination. These factors combined mean that such methods were not considered for use in the research described in this thesis.

Numerous commercial companies offer methods for nucleic acid extraction (Fiebelkorn *et al*, 2002; Loens *et al*, 2007; Jongerius *et al*, 2000; Chiu *et al*, 2006). Most of these are based on either silica-gel-membrane or magnetic-particle extraction technology. Purification using silica-gel-membrane technology uses the “Boom” method described above and numerous kits are available that can extract RNA or DNA, or can perform total nucleic acid extraction from numerous sample types. Magnetic particle systems can also be used to extract both RNA and DNA. Samples are first lysed and then the target RNA or DNA (or both) is bound to the magnetic particles. The nucleic acid is then washed before elution.

Although more expensive than in-house techniques, commercial methods are easy to use, standardised, and as a result offer improved Quality Control (QC). All of the necessary components are delivered in a single kit, including reagents that have been pre-optimised for the process. In comparison to in-house methods such systems can result in fewer errors, are more consistent, and reduce the risk of contamination. They are often available in a manual format that is suited to processing small numbers

of samples. However, a number of companies also offer automated extraction platforms that allow rapid, reliable and high throughput extraction (between 8-96 samples in 1-4 hrs). As a result these are ideal for use in the routine diagnostic setting and were considered suitable for use in this research.

At the time of the research the Qiagen Biorobot 9604 with the Qiagen Blood kit was already in use for the nucleic acid extraction of samples for other molecular tests at the laboratory. Since this system was also found to meet the requirements of the proposed new service for viral IID it was also used in this research. Implementing an alternative additional system was not considered as this could be expensive and may not be of use for the other molecular assays that test for alternative sample or virus types. The Qiagen system utilises silica-gel-membrane extraction technology (based on the Boom method) and can process up to 96 samples and controls in approximately 2hrs. Following initial set-up the process is fully automated, which aids ease of use. This kit has been shown to offer sensitive extraction of both RNA and DNA from numerous sample types including faecal samples, blood, urine, vesicle fluid, genital swabs, respiratory samples (throat swabs, nasopharyngeal aspirates etc), CSF, tissue and blood spots (Qiagen, 2001). Moreover, PCR methods that use this extraction system have been shown to be sensitive via numerous External Quality Control (EQC) schemes including those provided by Quality Control for Molecular Diagnostics (QCMD), the National External Quality Assurance Scheme (NEQAS) and the United Kingdom Clinical Virology Network (UKCVN).

As an indication of the Qiagen platform's ability to sensitively extract RNA and DNA the results of three EQC panels tested at the WOSSVC are presented here (Table 4, Table 5 and Table 6). Results are shown for PCR tests on CSF, RSV, Human metapneumovirus (hMpV) and HSV positive samples (since no EQA panel is currently available for viral causes of IID). Although the panels are concerned with the PCR systems as a whole, the sensitivity of such systems is dependent on effective nucleic acid extraction and in each case was found to be high. This, together with its ability to extract both DNA and RNA from stool samples, and its high throughput, rapidity and ease of use made this automated extraction system highly suitable for the proposed molecular diagnostic service for viral gastroenteritis. Details of the exact extraction protocol are outlined in section 3.4.

Table 4: Results of a NEQAS EQA panel for molecular detection in CSF (2004).

Sample Number	Expected Result	WOSSVC Result	% Of NEQAS Participants With Correct Result (N=123)
1	Enterovirus	Enterovirus	98%
2	HSV-1	HSV-1	100%
3	HSV-2	HSV-2	97%
4	VZV	VZV	98%
5	HSV-1	HSV-1	90%
6	Negative	Negative	100%

Table 5: Results of a QCMD EQA panel for molecular detection in respiratory samples (2005).

Sample Number	Target Concentration (Genome Equi/MI)	Expected Result	WOSSVC Result	% Of QCMD Participants With Correct Result (N=16)
1	10E-3	RSV	RSV	87%
2	10E-5	RSV	RSV	69%
3	10E-6	RSV	RSV	56%
4	-	Negative	Negative	100%
5	10E-3	hMPV	hMPV	93%
6	10E-4	hMPV	hMPV	86%
7	10E-2	hMPV	hMPV	93%

Table 6: Results of a QCMD EQA panel for HSV-1 and 2 samples (2004).

Sample Number	Target Concentration (Genome Equi/MI)	Expected Results	WOSSVC Results	% Of QCMD Participants With Correct Result (N=143)
1	-	Negative	Negative	95%
2	7.5×10^6	HSV-2	HSV-2	98%
3	2×10^3	HSV-1	HSV-1	62%
4	2×10^3	HSV-1	HSV-1	64%
5	2×10^7	HSV-1	HSV-1	97%
6	-	Negative	Negative	95%
7	1.2×10^6	VZV	VZV	93%
8	2.5×10^3	HSV-2	HSV-2	92%
9	7.5×10^2	HSV-2	HSV-2	77%
10	2×10^4	HSV-1	HSV-1	93%
11	7.5×10^3	HSV-2	HSV-2	93%
12	6.7×10^3	HSV-1	HSV-1	83%

3.3.2. PCR Methods

There are numerous PCR methods available in the literature that can be used for the detection of adenovirus, astrovirus, norovirus, rotavirus and sapovirus. The majority of these are conventional single round and nested gel-based PCR methods,

although the number of real-time PCR methods using SYBR green or dual-labelled probe-based technology has increased in recent years. The following section provides a review of the published PCR protocols for each viral pathogen that were considered for use in the new outbreak and sporadic service. For each pathogen the areas of the genome targeted by the PCR assays are outlined, alongside data on the variability of each target region. In addition, the findings from relevant studies comparing the PCR methods to other diagnostic methods (molecular and non-molecular) are given. Following the review, the methods chosen to be adapted for use in each of the proposed services are described, alongside the reasons for each choice and details of any adaptations necessary for use in the routine diagnostic laboratory setting.

3.3.2.1. PCR Methods Considered For The Outbreak Service

RT-PCR Assays For The Detection Of Norovirus

There have been numerous RT-PCR assays published for norovirus detection and a number of targets have been described. Early versions were gel-based and tended to target the RNA dependent RNA polymerase (RdRp) part of the genome, as this region was initially thought to be the most functionally conserved region between noroviruses (Kojima *et al*, 2002). PCR assays that target this region have remained popular. However, significant variation can still be found in this region, which can hamper the detection of a number of norovirus strains and genotypes (Katayama *et al*, 2002). On the other hand, the advantage of this variability is that the RT-PCR product from this region can further analysed in order to identify types and subtypes.

In some of the earliest work in this area, Ando *et al* developed two broadly reactive RT-PCR assays that targeted the RdRp region, were shown to be specific and could detect and differentiate between norovirus strains from genogroup 1 and 2 (Ando *et al*, 1995). Green *et al* developed a RT-PCR that used primers Ni and E3 to amplify a 113bp region of the norovirus RdRp (Green *et al*, 1995). This RT-PCR was compared to EM on 132 samples from outbreaks and sporadic cases of gastroenteritis that had occurred in the UK between 1992-1994. Of the 101 samples that tested positive for norovirus using EM, the PCR detected 93 as positive. Of the 31 samples that tested negative for norovirus using EM, the PCR detected 5 as positive. Based on the results of this study the authors judged that this PCR method offered a sensitive alternative to EM. The assay was also shown to be specific by cross testing with other pathogens including sapovirus. In the years following it became commonly used by

testing centres throughout Europe and was used as the frontline test by the Health Protection Agency (HPA) in London (Vinje *et al*, 2003).

Numerous other methods that target the RdRp region have been published for the detection of norovirus in clinical samples, shellfish, water and other environmental material (Green *et al*, 1995; Ando *et al*, 1995; Green *et al*, 1995; Guyader *et al*, 1996; Hafliger *et al*, 1997; Lees *et al*, 1995; Wyn Jones *et al*, 2000; Myrmel *et al*, 1999; Green *et al*, 1998). Nested and hemi-nested PCR formats have also been described, sometimes offering up to 1000 times more sensitivity than single round PCR assays (Green *et al*, 1998; Hafliger *et al*, 1997).

However, despite the large number of tests available that target the RdRp region, few studies have been published that directly compare these methods. In 2003, Vinje *et al* carried out an assessment of a number of conventional RT-PCR methods that targeted this region. The aim was to determine which were commonly used in European laboratories, and if possible, determine which of them offered the best sensitivity. This study showed that the most commonly used methods were single round RT-PCR assays that utilised either the primers Ni and E3, JV12 and JV13, or mon 431-434, together with a nested assay that used the primers 32 and 33 for the first round RT-PCR, and primers 35 and 36 for the second nested step. Each assay was tested using a panel of 82 samples that tested positive for norovirus using EM and contained representatives from genogroups 1, 2 and 4. The end point detection limit of each test was determined using a number of dilution series, of multiple norovirus genotypes. Overall, no one assay was found to be significantly superior to another. Looking at the data in more detail showed that the RT-PCR test that used primers Ni and E3 detected approximately 85% of the G1 types examined and 58% of the G2 types. The assay that used JV12 and JV13 detected 100% of the G1 types examined and 75% of the G2 types. The nested method utilising the primer sets 32-33 and 35-36 detected 85% of the G1 types examined and 85% of the G2 types, whereas the single round RT-PCR method using the primers mon 431-434 detected 85% of the G1 types examined and 69% of the G2 types. When these assays were compared on dilution series a wide variation in their end point detection limits was found. This study highlights that even tests targeting the same region can vary significantly in their sensitivity. Of the methods compared, the authors concluded that the primers JV12 and JV13 offered more universal detection and as a result recommended these primers to laboratories that had yet to implement RT-PCR. Subsequently, these primers have

also been used successfully in a nested format (Medici *et al*, 2005) and have been used in a multiplex format in tests for adenovirus and astrovirus (Rohayem *et al*, 2004).

A number of Nucleic Acid Sequence-Based Amplification (NASBA) assays have also been described that target the RdRp region. Comparisons with established RT-PCR methods have shown that these have comparable sensitivity (Moore *et al*, 2004; Houde *et al*, 2004; Rutjes *et al*, 2006; Greene *et al*, 2003; Patterson *et al*, 2006). This technology can also be used to quantitate the amount of virus in a stool sample via the amplification of known standards.

Real-time PCR is a relatively new technique and therefore few methods of this type have been described that target the RdRp region. Pang *et al* described a SYBR green method which, for G2 detection only, targeted the RdRp area (the G1 assay targeted the capsid region)(Pang *et al*, 2004). Compared to conventional RT-PCR using the primers JV12 and JV13, the real-time PCR was found to be more sensitive for G2 types. However, specificity was reduced as cross-reactions were observed between the G1 and G2 assay reducing its value as a typing assay. In another study Simpson *et al* used the primers JV12 and JV13 in a SYBR green format. Their method was shown to be significantly more sensitive than EM (Simpson *et al*, 2004). Richards *et al* also used previously published primers (mon 431-434) in a SYBR green real-time format (Richards *et al*, 2004). Here, although the assay was not directly compared to any others it was shown to be sensitive.

As an alternative approach, a number of norovirus RT-PCR assays have been published that target the capsid area. Yamazaki *et al*, for example, described an assay that targeted the capsid region (Yamazaki *et al*, 1996). This assay was compared to EM and was shown to be more sensitive. Vinje *et al* described a broadly reactive RT-PCR that targeted the capsid VP1 region. This was used for both diagnosis and epidemiological study but was not compared to an alternative method (Vinje *et al*, 2004). However, compared to the RdRp region, the capsid contains significantly more variation as it includes the highly variable regions that determine antigenic determinants, cell attachment and entry (Katayama *et al*, 2002). These areas come under immune pressure, which in turn promotes the emergence of new norovirus phenotypes (Nilsson *et al*, 2003). The variable nature of the capsid region thus increases the risk of false negative reactions due to nucleotide changes in the primer regions. Careful design is needed to ensure detection of a wide range of types and

continued test monitoring is needed to maintain test sensitivity. The variable nature of the capsid region has also hampered the development of real-time probe-based RT-PCR methods, as these methods require a greater area of nucleotide conservation than conventional PCR methods. Some examples do exist, however. Pang *et al* described a real-time RT-PCR method that targeted the capsid region in order to detect G1 noroviruses (Pang *et al*, 2004). However, although it could detect all of the G1 types tested, the detection limit was found to be approximately 3 logs less sensitive than conventional RT-PCR using primers JV12 and JV13. As described above, cross reactions between G1 and G2 types were also seen.

In 2002, Katayama *et al* published a similarity study of 18 norovirus sequences that showed for the first time that the most conserved region of the norovirus genome is the ORF1-ORF2 junction (Katayama *et al*, 2002). As a result, most of the recently published tests have targeted this region rather than the RdRp or capsid region, with the aim of detecting a wide range of norovirus types and subtypes. This finding, moreover, has significantly aided the development of real-time PCR methods since these require greater regions of conservation to allow probe as well as primer binding.

One of the first assays to be published that targeted the ORF1-ORF2 junction was described by Kojima *et al* (Kojima *et al*, 2002). This assay was a gel-based multiplex assay that utilised two primer sets for the detection and differentiation of genogroup 1 and genogroup 2 noroviruses. It was compared to two other RT-PCR methods that targeted the RdRp and the capsid regions respectively, using 35 samples that tested positive for norovirus using EM. The new assay developed by Kojima *et al* was shown to be specific and detected all 35 samples as positive whereas the other assays detected 28 and 27 as positive. The new test was also shown to detect a wide range of norovirus strains, and the PCR product could be used for epidemiological studies using sequence analysis.

Kageyama *et al* described the first real-time RT-PCR assay to target this region (Kageyama *et al*, 2003). Aligning the sequence data provided by Katayama's original study with a further 70 partial ORF1-ORF2 sequences led to the development of two probe-based real-time RT-PCR methods for the separate detection of norovirus types G1 and G2. The assays were compared to two conventional RT-PCR methods, one targeting the capsid region and one the RdRp. Of the 81 samples that tested positive for norovirus using EM, the real-time PCR methods together detected 80

whereas the two conventional RT-PCR methods detected 62 and 67, respectively. In addition, the real-time RT-PCR method was shown to be specific and detected a further 20 positive samples in a panel of 28 samples that had tested negative using EM.

Houde *et al* compared this real-time PCR assay to both a NASBA test and a conventional RT-PCR that used the primers described by Ando *et al* (Ando *et al*, 1995; Houde *et al*, 2006). All of the assays detected the same number of norovirus-positive clinical samples from a panel of G2 types. However, the real-time RT-PCR method was found to be more sensitive by at least 1-2 logs than either of the others when the endpoint detection limits of each assay were assessed. Jolkihumar *et al* further improved the sensitivity of this real-time RT-PCR method by up to 100 fold by removing the degeneracies from the forward G2 primer (Jolkihumar *et al*, 2005). A new probe-based G1 RT-PCR was also developed that targeted the ORF1-ORF2 junction and which was shown to be more sensitive than the G1 real-time assay developed by Kageyama and described above. Other studies have also published real-time RT-PCR methods (including dual-labelled probe-based and ones based on MGB chemistries) that target the ORF1-ORF2 region (Pang *et al*, 2004; Schmid *et al*, 2004; Hoehne *et al*, 2006). In each case the assays were compared to conventional RT-PCR methods and were found to be more sensitive.

3.3.2.2. Selection And Development Of The PCR Methods For Use In The Proposed Outbreak Service

As described in Chapter 2, norovirus is highly variable “genomically” and, although one strain tends to dominate at any one time, there exists a wide range of types and subtypes (Katayama *et al*, 2002; Fankhauser *et al*, 2002; Green *et al*, 1995; Koopmans, 2000; Gallimore *et al*, 2004; Hale *et al*, 2000; Kageyama *et al*, 2004). Moreover, individuals with norovirus infection have been shown to shed virus at levels below the detection limits of current tests, such as EM, soon after disease onset (Waters *et al*, 2000; Donelli *et al*, 1993).

In terms of a diagnostic test, therefore, the priority is to select one that can detect a wide range of norovirus types and that has a low limit of detection. It is important, moreover, that the sensitivity remains stable over long periods of use with a minimum level of monitoring, which means that tests that target the most conserved area of the genome are preferred. Although, as shown, it is possible to develop

sensitive tests for norovirus that target other areas, the variable nature of these regions increases the risk of sensitivity drift over time and/or sudden loss of sensitivity due to the emergence of a new virus type or subtype.

The first norovirus RT-PCR for use in the outbreak service was developed during 2000. At this time the RdRp region was widely believed to be most functionally conserved area amongst noroviruses. As a result tests that targeted this region were considered most appropriate for use in the proposed diagnostic service, since assays that targeted the ORF-1 and ORF-2 junction had yet to be published.

Various single round, nested and hemi-nested RT-PCR methods have been published that target the RdRp region. Many of these have been shown to be specific and more sensitive than EM (Froggatt *et al*, 2004; Simpson *et al*, 2003; Ando *et al*, 1995; Green *et al*, 1995 (i); Green *et al*, 1995 (ii)). However, although nested and hemi-nested RT-PCRs have been shown in some cases to offer high sensitivity the trade off is that the additional steps involved reduces test rapidity and ease of use (Green *et al*, 1998; Hafliger *et al*, 1997). The larger number of steps also results in more potential for error. Consequently, for the purposes of this work, single round RT-PCR assays were preferred as these are made up of fewer steps and as a result are likely to be more rapid and easier to use. Numerous single round RT-PCR were published at the time this research began and some had already been shown to offer improved sensitivity in comparison to EM (Ando *et al*, 1995; Green *et al*, 1995; Green *et al*, 1995). Of these, the RT-PCR described by Green *et al* was chosen to be adapted for the detection of norovirus in outbreak samples (Green *et al*, 1995). This is a conventional single round RT-PCR assay that had been shown to be specific and able to detect a broad range of norovirus types, and had been compared to EM on outbreak samples and found to be sensitive. A major contributing factor that led to the selection of this assay over the other candidates outlined above was that a gel-based version of the assay was used as the frontline test by the HPA in England (Vinje *et al*, 2003). During its use there it had been shown to be able to detect the predominant strains circulating in England and it was hypothesised that similar types may be encountered in Scotland.

Once this assay was selected the aim was to adapt this conventional gel-based RT-PCR into a real-time format, in order to increase its potential for rapid turn-around-times and, by association, high throughput. At the time no real-time RT-PCR assays for the detection of norovirus had been published (highlighting the novelty of

this work). Rapidity is particularly important in a norovirus test that is to be used in the outbreak setting as rapid results may inform decisions on the infection control procedures to be used.

In order to convert the chosen assay into a real-time format SYBR green technology was employed, which was the predominant real-time technology available at the time. This is an established means of converting conventional PCR tests into a real-time PCR format. SYBR green is a DNA chelating dye that is incorporated into DNA, fluoresces under ultra violet excitation and is measured at the end of each PCR cycle. This negates the need for post amplification analysis (such as gel-based analysis) and can therefore reduce turn-around-times, improve ease of use and reduce the risk of contamination. However, SYBR green is non-specific and will bind to any DNA being produced during the PCR reaction including non-specific products and primer-dimers. With careful primer design and reagent optimisation such pitfalls can be limited but, to increase specificity, it is good practice to confirm positive results using melt curve analysis. The PCR product melting point is directly related to its sequence composition, length and concentration (Mackie *et al*, 2002). A small PCR product, for example, tends to result in a melting point that is consistently within a narrow temperature range, thus aiding interpretation of the results. As such, the PCR product produced by the chosen assay developed by Green *et al* (which was 113 bp in length) was highly suited to this technique.

The real-time version of the assay was implemented on the Lightcycler™ RT-PCR platform, which was one of only two PCR platforms available in the laboratory at the time of this initial development phase, the other being a conventional PCR block (the ABI 9700). The Lightcycler™ platform was a leading edge commercial platform at the time, as evidenced by its widespread use in the published real-time PCR assays (Schalasta *et al*, 2000; Ohyashiki *et al*, 2000; Kamihari *et al*, 2000; Espy *et al*, 2000; Kessler *et al*, 2000), although the number and choice of real-time platforms available has since increased.

The Lightcycler™ platform allows the testing of 32 reactions per run, compared to a typical 48-96 in conventional PCR platforms. It is designed for small PCR reaction volumes (<10ul), which are placed in glass capillaries that can be rapidly heated and cooled. As a result, the PCR parameters are much shorter than in conventional PCR methods. Using this system, 40 cycles of PCR can be carried out in less than 40 minutes (whereas conventional PCR parameters take between 3-5 hours).

It therefore has the potential for both high throughput and rapid testing, making it highly suitable for the proposed norovirus outbreak service.

Other adaptations of the assay described by Green *et al* included the replacement of the stated RT step with a previously published in-house method (Ellis *et al*, 1997). This method utilises random hexamers and is therefore a universal RT-step, converting all RNA in the extracted sample to cDNA. This means that the cDNA produced is not specific to a particular test and may be used for all PCR assays. This has significant value in a routine diagnostic setting since, for example, samples of different types can be processed together and samples that are to be tested for multiple pathogens do not have to be repeatedly processed for each individual test (increasing the efficiency and reducing costs in both cases). Another advantage of this approach is that it reduces the potential for error since the RT step is standardised in the laboratory for all RT-PCR assays, which in turn aids quality control.

For the PCR step itself, the primers (Ni and E3) were used as described in Green *et al*. Reagents that were optimised for use on the Lightcycler™ platform were employed in the form of a commercial mastermix. The SYBR green was added to this mastermix before use, as was Taq start antibody, which helps prevent non-specific Taq polymerase activity. Details of the exact protocol used in implementing the assay are provided in section 3.4.

From 2003, several other real-time PCR platforms were obtained by the laboratory, including the Rotorgene (Corbitt research™), Icyler (Biorad™) and ABI 7500 (Applied Biosystems™). Each of these systems supports a number of different real-time PCR chemistries including SYBR green, dual-labelled probes, MGB, molecular beacons and labelled primers. Compared to the Lightcycler™ platform these offer improved throughput as they can process 72-96 samples in approximately 1-2 hours.

At the same time, it had recently been shown that the most conserved region of norovirus was in fact the ORF1-ORF2 junction, rather than the RdRp region (Katayama *et al*, 2002) and assays that target this region had begun to appear (Kojima *et al*, 2002; Kageyama *et al*, 2003). One was a real-time RT-PCR method (Kageyama *et al*, 2003) and had been shown to detect both G1 and G2 noroviruses, which together are responsible for the majority of human cases of norovirus IID. The real-time RT-PCR assay used dual-labelled probe-based technology and was shown to be more sensitive than two conventional RT-PCR assays. Dual-labelled probes (also

known as Taqman™ probes) are oligonucleotides that contain a fluorescent dye on the 5' base, and a quenching dye located on the 3' base. They are designed to hybridise to an internal region of a PCR product. During PCR, when the polymerase replicates a template on which the probe is bound, the 5' exonuclease activity of the polymerase cleaves the probe. This separates the fluorescent and quenching dyes, allowing detection of the signal from the reporter dye. Fluorescence increases in each cycle, proportional to the rate of probe cleavage. Compared to SYBR green based assays, the advantage of this technique is that the incorporation of a probe means it can be more specific, and therefore a melt curve step is not required. From a diagnostic standpoint, the removal of the need for a melt curve step reduces the duration of the test and further reduces the potential for human error.

The assay described by Kageyama *et al* was therefore chosen for investigation as a further possible development of the routine norovirus outbreak service in 2003. The primers, probes and PCR parameters (i.e. the concentration and annealing temperatures) were used as described. However, the published method used two separate dual-labelled probe-based real-time assays to detect G1 and G2 noroviruses, whereas here the two assays were multiplexed with the aim of reducing turn-around-times and test costs. The multiplexed test was implemented on the ABI 7500 platform as this offered the maximum capacity of 96 sample reactions. Reagents that were optimised for use on the platform were employed in the form of a commercial mastermix. With this kit the RT step, PCR and detection all take place in a single tube. This was a further improvement on the previous test, reducing the number of steps involved. Details of the exact protocol used in implementing the assay are provided in section 3.4.

3.3.2.3. PCR Methods Considered For The Sporadic Service

PCR Assays For The Detection Of Adenovirus

Numerous adenovirus PCR tests are available in the literature. The vast majority target the hexon region of the adenovirus genome. The hexon is a product of L3 and is the major component of the adenovirus capsid. Although there are also 7 hyper variable regions that determine the subgenera and serotypes, it also contains regions that are conserved throughout most adenovirus serotypes (Crawford-Mikszta and Schnurr, 1996). The combination of conserved and variable regions means that, with careful PCR design, the hexon region can be used both for diagnosis and can be

used for epidemiological purposes. Conventional single round and nested PCR assays have been described that target this region (Allard *et al*, 1990; Morris *et al*, 1996; Echavarria *et al*, 1999; Na *et al*, 2002; Lee *et al*, 2005; Formiga-Cruz *et al*, 2005). Furthermore, the conserved regions have also proven to be useful targets for dual-labelled probe-based real-time PCR methods (Heim *et al*, 2003; Claas *et al*, 2005).

Allard *et al* provided an example of a single round PCR method that, using a low annealing temperature, could detect all known adenovirus serotypes (Allard *et al*, 1990). This assay was shown to be more sensitive than a latex agglutination method and a group F specific PCR. However, later studies found this assay to be insensitive for the detection of group B adenoviruses and serotype 11 (Morris *et al*, 1996).

A number of other single round PCR methods exist that target the hexon region and detect all adenovirus serotypes (Echavarria *et al*, 1999; Na *et al*, 2002; Lee *et al*, 2005). For example, Hierholzer *et al* developed a single round PCR that detected all adenovirus serotypes by targeting a 162bp region of the P2 domain within the 3' region of the hexon (Hierholzer *et al*, 1993). Freymuth *et al* compared this assay to immunofluorescence on 277 nasopharyngeal aspirates (NPA) samples and showed a significant increase in detection of adenovirus (in 30 samples vs 4) (Freymuth *et al*, 1997). Cooper *et al* described a single round PCR method that targeted a 140bp region of the hexon region using primers ADJC1 and ADJC2 (Cooper *et al*, 1999). This PCR was compared to tissue culture on 59 eye swabs. The PCR detected adenovirus in 51 eye swabs whereas using culture it was detected in 40. A later study multiplexed this assay with in-house PCR methods for HSV and *Chlamydia trachomatis* (Elnifro *et al*, 2000 (i)). Compared to tissue culture the PCR detected adenovirus in 48 of 49 eye swabs whereas tissue culture detected 26 samples as positive. In a subsequent study this assay was also shown to provide typing information after restriction enzyme digestion (Elnifro *et al*, 2000 (ii)).

Single round multiplex PCR assays have also been described that allow detection of all adenovirus serotypes. Pring-Akerblom *et al* provided an example of such an assay where each adenovirus subgroup could be determined by its PCR product size (Pring-Akerblom *et al*, 1999). A number of PCR methods have also been published that are in a nested format (Takeuchi *et al*, 1999; Avellon *et al*, 2001; Inagara *et al*, 1996) or detect a particular serotype, serotypes or subgroup (Cooper *et al*, 1996; Houg *et al*, 2002; Fujimoto *et al*, 2000; Echavarria *et al*, 1999). The nested

assays were found to offer high sensitivity and have been used to detect adenovirus in water sources (Lee *et al*, 2005; Formiga-Cruz *et al*, 2005).

In recent years a number of dual-labelled probe-based real-time PCR methods have also been described that target the hexon region. A number of these have been designed to target only a particular sub-genera or serotype of adenovirus. For example, Logan *et al* described a dual-labelled probe-based assay that detected group F adenoviruses (Logan *et al*, 2006; Jiang *et al*, 2005). Other such assays include one for the detection of group C adenoviruses (Garnell *et al*, 2002), one for type 4 adenovirus (Houng *et al*, 2002) and one for all non group F adenoviruses (Faux *et al*, 2004). Others have been described that detect all adenovirus serotypes via the hexon region. Ebner *et al* described a 2 reaction adenovirus real-time PCR that utilised 5 probes and 8 primers and was designed to detect and differentiate between adenovirus subgenera A, C, F and B, D, E (Ebner *et al*, 2005). However, it should be noted that the group F adenoviruses were detected by both assays. Heim *et al* also developed a real-time dual-labelled probe-based PCR assay that targeted the hexon region and was able to detect all adenovirus serotypes (Heim *et al*, 2003). This, unlike the assay published by Ebner, utilised a single set of primers and one probe. The new assay was compared to the nested PCR described by Allard *et al* (Allard *et al*, 1992) and was shown to be more sensitive. Claas *et al*, meanwhile, described a MGB assay that targeted the hexon region and could detect all adenovirus serotypes (Claas *et al*, 2005).

Although the hexon region is targeted in most of the PCR assays for the detection of adenovirus, alternative targets have also been used. The E1 region is one example. As described in Chapter 2, the E1a/b region of adenoviruses is a transcription unit that encodes two proteins which play a major role in the adenovirus infection cycle (Shenk and Flint, 1991; Shenk, 2001). However, although moderately high levels of E1 sequence identity have been observed between members of the same subgroup, with similarity scores of approximately 50-60%, significant variation has been found between adenoviruses from different subgroups with similarity scores of less than 30% (Avvakumov *et al*, 2002). As a result it can be difficult to design assays that detect all adenovirus subgroups and serotypes based on targeting this region. Allard *et al* described a group F specific PCR that targeted this region (Allard *et al*, 1990). The PCR products for the group F PCR were 2187 bp and 838 bp for adenovirus 40 and 41 respectively. This assay was compared to a universal PCR that

targeted the hexon region of the adenovirus genome (308 bp) and to a latex agglutination assay on 60 stool samples taken from children with gastroenteritis. Although both PCR assays were found to be more sensitive than the latex agglutination assay, the group F specific assay also detected a number of non group F adenovirus types indicating low specificity. This assay was also found to be 10 times less sensitive than the PCR assay that targeted the hexon region. In 1992, the group F PCR assay was adapted to a two-step nested assay, which resulted in an improvement in sensitivity that made it comparable to the PCR assay which targeted the hexon region (Allard *et al*, 1992). Roussel *et al* also developed a group F specific PCR that targeted the E1b region. This assay successfully detected adenovirus type 41 in samples that had previously tested positive using EM. However, it was not compared in detail to any other diagnostic method (Roussel *et al*, 1993).

A few published assays have targeted the adenovirus pIX gene. The pIX gene is encoded by the adenovirus intermediate region. The pIX gene is the minor structural component of the capsid and serves to cement the hexon in place (Akalu *et al*, 1998). Members of different adenovirus genera show wide variation in the sequence of the pIX region, especially in the C terminal part. As a result careful design of the PCR parameters (e.g. primers, annealing temperatures etc) is warranted if all adenovirus serotypes are to be detected. Akalu *et al* developed a PCR test that targeted this region. However, assessment showed that the PCR failed to detect adenoviruses from groups A, D and E.

Finally, there are some examples of PCR assays that target the fiber gene. The adenovirus fiber is coded for by the late protein L5. The fiber is present on the capsid surface and determines tissue tropism (Stone *et al*, 2003). The fiber has a role in haemagglutination and the formation of neutralisation antibody. As a result considerable fiber sequence variation can be found between serotypes. Consequently, it has proven difficult to design primers that detect all adenovirus serotypes, with the published assays described using separate PCR tests to detect each adenovirus type (Xu *et al*, 2000) or detecting groups B and C only (Adhikary *et al*, 2001; Bruzzone *et al*, 2000).

RT-PCR Assays For The Detection Of Astrovirus

There are numerous astrovirus RT-PCR assays published in the literature. Single round and nested conventional RT-PCR assays and real-time RT-PCR assays

exist for the detection of astrovirus (Cubitt *et al*, 1999, Akhter *et al*, 1995; Major *et al*, 1992; Jonassen *et al*, 1993; Jonassen *et al*, 1995; Noel *et al*, 1995; Zhang *et al*, 2006; Le Cann *et al*, 2004; Royeula *et al*, 2006). In general, these assays target either the 3' terminus NCR region or the capsid region.

Sequence alignments of all known astrovirus serotypes (1-8) have shown the 88 bp 3' end of the astrovirus genome to be highly conserved (Monceyron *et al*, 1997; Belliot *et al*, 1997; Wang *et al*, 2002). This makes it a highly effective target for diagnostic PCR assays but less useful for epidemiological study. Numerous diagnostic RT-PCR methods have been developed that target this small but conserved region (Cubitt *et al*, 1999, Akhter *et al*, 1995; Major *et al*, 1992). Jonassen *et al*, for example, developed an RT-PCR assay targeting this region, which produced a PCR product of 77 bp (Jonassen *et al*, 1993; Jonassen *et al*, 1995). This assay was assessed on 38 samples that tested positive for astrovirus using EM and was shown to detect all but 2 of the panel. However, it was also shown to be non specific. Cubitt *et al* used a similar RT-PCR method to investigate an outbreak of astrovirus in a BMT ward. Compared to EM and EIA, the RT-PCR was shown to be more sensitive, detecting positive cases earlier than the other methods and showing that those infected were shedding virus for longer periods.

In recent years, the 3'NCR region has also proved to be a good target for real-time PCR (Zhang *et al*, 2006; Le Cann *et al*, 2004; Royeula *et al*, 2006). Le Cann *et al* described a dual-labelled probe-based assay that was used to detect astrovirus in sewage. Although not assessed on clinical samples or samples of known subtypes it was shown theoretically to detect all 8 serotypes. Zhang *et al*, meanwhile, used a SYBR green based assay to quantify the level of astrovirus in several patient groups in order to examine whether there was a correlation between viral load and disease severity (no relationship could be found). Royeula *et al* also developed a real-time PCR based on using SYBR green that was shown to be more sensitive than both an EIA and a conventional PCR.

An alternative PCR target for the detection of astrovirus is the capsid region of the genome. However, as with other RNA viruses the capsid region contains areas of high variability (Belliot *et al*, 1997; Wang *et al*, 2001). Wang *et al* described 4 sub-regions in the capsid, each with differing levels of variability. The terminal N and the C regions were relatively conserved (56-86%) between astrovirus serotypes making them suitable targets for diagnostic purposes, whereas the two regions in-between

contained significant levels of variation. These in-between areas are thought to contain the regions that determine antigenicity and cell attachment and entry and are therefore useful for epidemiological study. Researchers have taken advantage of these characteristics to design RT-PCR assays that can detect all astrovirus serotypes, and can also provide a PCR product that can be sequenced for epidemiological study (Yan *et al*, 2003; Royahem *et al*, 2004). Noel *et al* described two RT-PCR methods that targeted the capsid region, one using primers mon 244-245 and one using mon 269-270 (Noel *et al*, 1995). Both offered universal detection, and typing of the product by sequence analysis. Of the two sets of primers, mon 269-270 was found to result in the most sensitive test. These primers have been used in numerous other studies since (Mustafa *et al*, 1998; McIver *et al*, 2000; Simpson *et al*, 2004; Medina *et al*, 2000). McIver *et al* compared this RT-PCR to EM on clinical samples and found it to detect astrovirus in the same number of cases as EM. Medina *et al* showed that it was more sensitive than EIA on 251 samples from children with acute gastroenteritis, detecting an extra 12 positive samples. These primers have also been used to target the capsid region in a SYBR green format and the resulting assay was shown to be more sensitive than EM (Simpson *et al*, 2004). As of yet, however, no probe-based real-time PCR assay has been published that targets this region. This is likely to be due to the variable nature of the region.

RT-PCR Assays For The Detection Of Rotavirus

Most of the published RT-PCR assays for the detection of rotavirus target the following parts of the genome: the VP7, VP4, NSP3, VP6 and NSP2 regions.

One of the first RT-PCR assays described for the detection of rotavirus targeted the VP7 gene (Gouvea *et al*, 1990). The product VP7 is the major constituent of the rotavirus outer capsid and is the target of type-specific antibodies (Jayaram *et al*, 2004). The advantage of targeting this region with PCR is that it contains at least 6 discrete regions that determine the rotavirus subgenera. Therefore, with careful primer design, it can be used for the simultaneous detection and subtyping of rotavirus from clinical samples. However, careful monitoring is needed to maintain the sensitivity of such an assay to ensure that changes in the primer binding sites which result in reduced sensitivity do not occur.

In the assay described by Gouvea *et al* the primers Beg9 and End9 were used as a first round PCR to detect the 5' and 3' end of gene segment 9, giving a RT-PCR

product of 1062 bp. Following this, a nested step was used that employed primers specific for the main rotavirus G types.

Several studies have since utilised this nested method for diagnosis and epidemiology (Pang *et al*, 1999; Ushijima *et al*, 1994). Pang *et al*, for example, found it to offer increased diagnostic sensitivity when compared to an EIA test on samples from children with IID (Pang *et al*, 1999). Ushijima *et al* used this method to show the presence of rotavirus in various clinical specimens (such as CSF, serum and throat swabs) in patients with acute rotavirus infection.

Other methods that target the VP7 region have also been described. Xu *et al* described a single round RT-PCR that amplified the full length of gene segment 9. This assay was found to be significantly more sensitive than a hybridisation assay and a standard electropherotype method when compared on stool samples (Xu *et al*, 1990).

The rotavirus VP4 protein has also been used as a target for PCR. This is a viral haemagglutinin and plays a major role in cell attachment, entry and penetration (Estes, 2001; Gorziglia *et al*, 1990). It is a product of gene segment 4 and contains the variable regions that are used to determine the rotavirus P type. Like the VP7 region, with careful primer design, assays that target this region can be used for both diagnosis and epidemiological study. Numerous methods have been described that target this region for the detection of rotavirus. Gentsch *et al* described a nested assay that could be used to detect and type rotaviruses (Gentsch *et al*, 1992). Masendycz *et al* also described a nested RT-PCR that could be used for the same purpose (Masendycz *et al*, 1997). This was compared to an EIA method and a cDNA hybridisation probe on 102 clinical samples. Overall, the nested RT-PCR assay was found to be the most sensitive, with the hybridisation probe second followed by the EIA. Rasool *et al* adapted the assay described by Gentsch *et al* to a single round RT-PCR that was shown to detect all human rotaviruses (Gentsch *et al*, 1992; Rasool *et al*, 2002). Following this, Min *et al* described a SYBR green based real-time RT-PCR method that utilised the primers reported by Rasool *et al* for the same purpose, although here again this was not compared to any other method (Min *et al*, 2006).

An alternative genome target for the detection of rotavirus is the VP6 region. The VP6 is the major constituent of the intermediate layer of the rotavirus. It has an important structural and immunological role (Greenberg *et al*, 1983; Pothier *et al*, 1987). Sequence analysis has shown that the VP6 is relatively conserved between

human rotaviruses. As a result it is a potentially good target for the detection of a wide range of rotaviruses, although in practice few assays of this type have been developed to date. Kang *et al* described a real-time SYBR green based assay that targeted a 379 bp region of VP6 (Kang *et al*, 2004). This quantitative assay was not compared to any other diagnostic methods but was used to investigate the relationship between the severity of the rotavirus illness and the amount of rotavirus detected in the stool (they found that low viral loads were often found in less severe and asymptomatic infections).

Other areas of the rotavirus genome have also been considered for PCR targeting including the NSP2 and the NSP3 regions. The role of NSP2 is currently unclear although it has been suggested to have a role in RNA replication and packaging (Gombold *et al*, 1985). Sequence analysis has shown that the first 75 nucleotides of the 5' end and the last 28 nucleotides of the 3' end are conserved in all rotaviruses (Patton *et al*, 1993). Xu *et al* described a one step RT-PCR that targeted the 3' and 5' end of the NSP2. The assay detected all group A rotaviruses (Xu *et al*, 1990). It was compared to PAGE and a hybridisation assay and was shown to be significantly more sensitive than either method (between 5000-100,000 times). The large PCR product also contained restriction enzyme sites, which favours cloning and sequencing.

Little is also known about the role of NSP3, although some have suggested a role in protein synthesis or viral replication (Poncet *et al*, 1996). The NSP3 protein is coded for by rotavirus gene segment 7. It contains areas of sequence variability that can be used to divide rotaviruses into 3 evolutionary groups (SaII, Wa and S2). However, there is also a region of approximately 80 nucleotides in the 3' UTR that is highly conserved in all rotavirus strains (Rao *et al*, 1995). Pang *et al* described a probe-based real-time RT-PCR assay that targeted this highly conserved area (Pang *et al*, 2004). This assay was compared to both EM and the nested RT-PCR that targets the VP7 (Goveau *et al*, 1990) on 623 samples from children with gastroenteritis. The assays were also compared on a dilution series of a sample that tested positive for rotavirus using EM. Overall, the real-time RT-PCR detected 123 samples as positive, whereas the conventional nested RT-PCR detected 113 and EM detected 79. On the dilution series the real-time RT-PCR was shown to have a detection limit that was 2 logs greater than the nested assay.

RT- PCR Assays For The Detection Of Sapovirus

In comparison to the number of PCR methods published for the other viral causes of IID there are considerably fewer available for the detection of sapovirus. Amongst those published, most target the RdRp or capsid regions or the polymerase-capsid junction.

Early examples in particular targeted the RdRp region, since early studies had shown that this area was more conserved than the capsid region (Berke *et al*, 1997; Liu *et al*, 1995; Matson *et al*, 1995). One of the first RT-PCR assays described for the detection of sapoviruses was described by Jiang *et al* (Jiang *et al*, 1999). This RT-PCR detected both norovirus and sapovirus using the primers p289-290. Sapovirus was distinguished from norovirus based on the PCR product size (the norovirus PCR product was 312 bp whereas sapovirus was 331 bp). Later studies highlighted that these primers also detected rotavirus via a cross reaction with gene segment 1 (Lindert *et al*, 2004). Robinson *et al* also published a single round RT-PCR assay that targeted this region. It was used to examine the epidemiology of sapoviruses in South West England but was not compared to any other method (Robinson *et al*, 2002). Vinje *et al* compared a single round assay that targeted the RdRp (using primers JV33 and SR80) with two other RT-PCR assays that also targeted the RdRp (Ando *et al*, 1995; Noel *et al*, 1997). The assay was designed using a sequence alignment of the known sapovirus sequences, which represented the three recognised genogroups (Vinje *et al*, 2000). The three assays were then compared using 31 stool samples that had tested positive for sapovirus using EM. The assay using the primers JV33-SR80 detected considerably more samples as positive than the other assays. Since then these primers have also been used in a real-time SYBR green based assay by Simpson *et al* (Simpson *et al*, 2004). This method was shown to be more sensitive than EM on samples from children with IID, both hospitalised and managed in the community. However, it should be noted that significant variation can still be found within the RdRp (Oka *et al*, 2006). As a result designing an assay that can detect all sapovirus types and subtypes remains difficult and requires careful primer design.

The capsid has been targeted by a small number of sapovirus RT-PCR assays (Okada *et al*, 2006; Okada *et al*, 2002; Vinje *et al*, 2000). Like noroviruses, the capsid region of the sapovirus contains some areas of high genetic variability, which are likely to be the determinants of antigenicity, cell attachment and entry. Thus, although

the capsid region can be used for diagnosis, the variable nature of the capsid region tends to make it more suitable for epidemiological studies (Gallimore *et al*, 2006). For example, Okada *et al* described a nested RT-PCR multiplex assay that targeted the capsid and gave genotype data without the need for sequencing (Okada *et al*, 2002).

In recent years, RT-PCR assays have been described that target the RdRp-Capsid junction. This work is based on growing awareness that this is the most conserved region of the sapovirus genome, rather than the RdRp region. This was highlighted by the similarity study carried out by Oka *et al* described earlier in Chapter 2 (Oka *et al*, 2006). Okada *et al* developed a nested RT-PCR that could be used to detect and type all sapovirus genogroups (Okada *et al*, 2006). This assay consisted of universal primers and primer sets for each sapovirus genogroup but was not compared to any other assay. A small number of dual-labelled probe-based RT-PCR assays have also been developed which also target this region. Oka *et al* carried out a similarity study of a number of sapovirus types from all genogroups to develop a real-time probe-based RT-PCR able to detect all 5 sapovirus genogroups using 4 primers and 2 MGB probes (Oka *et al*, 2006). This assay was compared to another real-time RT-PCR assay developed by Chen *et al* (Chen *et al*, 2006), which also targeted the RdRp-Capsid junction. Both assays were shown to offer similar sensitivity (an improvement over conventional RT-PCR), with the exception that the assay described by Chen *et al* was unable to detect the newly discovered genogroup G5.

3.3.2.4. Selection And Adaptation Of The PCR Assays For Use In The Proposed Service For Sporadic Cases Of IID

As with the norovirus outbreak service described above, the research relating to the sporadic service took place in two stages. The first development phase took place between 2000 and 2002.

As outlined earlier, at this time there were two PCR platforms available in the laboratory; the LightcyclerTM, which is real-time; and the ABI 9700, which is a conventional PCR block. However, based on the fact that norovirus had been found to be the cause of most outbreaks of IID, together with the high level of disruption caused by outbreaks of gastroenteritis in closed settings such as hospitals, the decision was taken to prioritise use of the LightcyclerTM for the proposed norovirus outbreak service. This meant that, initially, the development of new tests for the sporadic

service focused on conventional PCR methods that could be implemented on the ABI 9700. This platform allows the processing of 96 samples and controls per run. On it, the PCR step of an assay takes approximately 3-5hrs to run, depending on the exact nature of the reaction. Post amplification analysis is then required to complete the test, which is carried out within the laboratory using agarose gel analysis (an established means of observing PCR results).

As with the outbreak service, the selection of sensitive assays able to detect all or the majority of the known subtypes of each pathogen was again a priority, as was the use of single round assays in order to minimise the number of steps involved. Here, moreover, in order to further reduce the number of steps in the overall testing process (and thus the associated costs) the ambition was to develop a multiplex assay that could test for all four pathogens simultaneously. Since an RT step is, in theory, only required to test for three out of the four pathogens (not adenovirus, which is a DNA virus) this would mean applying an unnecessary RT step in the case of adenovirus, as it is not possible to add both cDNA and DNA to a multiplex PCR simultaneously. However, there are several examples in the literature of tests that employ this approach (Yan *et al*, 2004; O'Neill *et al*, 2002), which is possible since DNA may still be present in the reverse transcribed template. Moreover, in comparison to four separate tests a multiplex assay was felt to have significant benefits in terms of the lower material and staffing costs, and reduced turn-around-times (the latter was also important in providing an improvement on the existing EM service). It did, however, mean that in order to aid result interpretation it was important to choose assays for each pathogen with different PCR product lengths.

With these factors in mind the first adenovirus PCR selected for investigation was the single round PCR described by Cooper *et al* (Cooper *et al*, 1999; Elnifro *et al*, 2000; Elnifro *et al*, 2000 (ii)). This PCR assay targeted the adenovirus hexon region and was selected since it was shown in more than one study to be both sensitive and able to detect all adenovirus serotypes. Also, its PCR product was small (140bp) which favoured multiplexing with one or more of the other non-norovirus assays.

For astrovirus, the RT-PCR described by Noel *et al* using primers mon 269-270 (Noel *et al*, 1995) was chosen. This assay targeted the capsid region of the astrovirus genome, detected all known astrovirus serotypes, and was chosen because it had been tested in several research studies and shown to be more sensitive than both EM and EIA without the need for a nested step (Mustafa *et al*, 1998; McIver *et al*,

2000; Simpson *et al*, 2004; Medina *et al*, 2000). In addition, the size of the PCR product was favourable to multiplexing as it was different to that of the assay chosen for adenovirus (449bp vs 140bp). This was crucial since although the 3'NCR region of astrovirus is generally held to be less variable than the capsid region the RT-PCR product of assays that target the 3'NCR are similar in length to that of the adenovirus PCR (<100bp).

The RT-PCR assessed for rotavirus was a single round version of the assay that used the primers con2 and con3 to target the VP4 region of the rotavirus genome (Gentsch *et al*, 1992; Rasool *et al*, 2002). This was chosen because it was a single round assay and was shown to detect all rotavirus types. Many of the other assays available were nested RT-PCR assays that may have offered increased sensitivity but involved a greater number of steps. Of the assays that were single round RT-PCR assays, moreover, a further benefit of this one was that the PCR product was smaller than many of the other published assays (220 bp), which makes the PCR reaction more efficient. The PCR product length was also different to that of the tests chosen for the other pathogens.

The initial test selected for sapovirus was the assay described by Vinje *et al* (Vinje *et al*, 2000). This assay targeted the RdRp region of the sapovirus genome, which was thought to be the most conserved region at the time of the initial developments. The assay was chosen since it was a single round RT-PCR and had been shown to be sensitive and capable of detecting all of the recognised genogroups of sapovirus at the time. The size of the predicted RT-PCR product was 349 bp, which was different to that of the other PCR assays.

For each of the selected assays the primers were used as described in the original paper, which also specified the annealing temperature used. This was different in each case. However, for a multiplex assay a single temperature is required. It was therefore decided to employ the median temperature of all four tests in the first iteration of the new multiplex assay. A reverse transcriptase step was also applied as part of the sample preparation in all cases to samples for all four pathogens, despite the fact that adenovirus is a DNA virus. In theory a reverse transcriptase step was required in tests for only three of the four pathogens (i.e. all except adenovirus). The step was carried out using the laboratory-standard method described earlier for norovirus detection. Details of the exact protocols used in implementing the assay are provided in section 3.4.

In 2003, when the other real-time platforms became available in the laboratory the possibility of implementing real-time PCR for all tests in the sporadic service was introduced, although in fact the developments did not take place until 2004 (for reasons described in Chapter 4). Here the decision was taken to move straight to dual-labelled probe-based technology, rather than SYBR green based assays, in line with the ongoing developments in tests for the outbreak service and for the reasons described earlier. Due to the relatively recent nature of this technology, however, the choice of available assays was limited.

For adenovirus, the real-time dual-labelled probe-based assay described by Heim *et al* was examined (Heim *et al*, 2003). This targeted the hexon region of the virus and was chosen since it could detect all adenovirus serotypes and used just one set of primers and probes. Other published assays used multiple primers and probes, which would increase test costs and complexity with little potential gain in sensitivity. For astrovirus the real-time RT-PCR chosen was that published by Le Cann *et al* (Le Cann *et al*, 2004). This assay targeted the 3'NCR region of the genome and had been shown theoretically to detect all astrovirus serotypes. It was also the only dual-labelled probe-based assay published for astrovirus at this point. For rotavirus detection the dual-labelled probe-based assay published by Pang *et al* was examined (Pang *et al*, 2004). This targeted the NSP3 region and was again the only assay of this type published at the time. It had been shown to detect all rotavirus types and in tests was more sensitive than a nested RT-PCR (Gouvea *et al*, 1990) and EM.

At the time of development, no dual-labelled probe-based real-time PCR assay was available for the detection of sapovirus in the literature (the first assay was published in 2006). As a result an in-house assay was developed. Analysis of available sapovirus sequences detailed in the BLAST (Basic Local Alignment Search Tool) database identified small conserved regions of the capsid and RdRp areas (300-500 bp). Attempts using the software package Beacon Designer (Premier Biosoft) to design a dual-labelled probe-based assay that targeted the submitted sequence from the RdRp were unsuccessful, as no test could be designed to meet the recommended guidelines (Qiagen, 2004). However, a suitable test that targeted the capsid region was created.

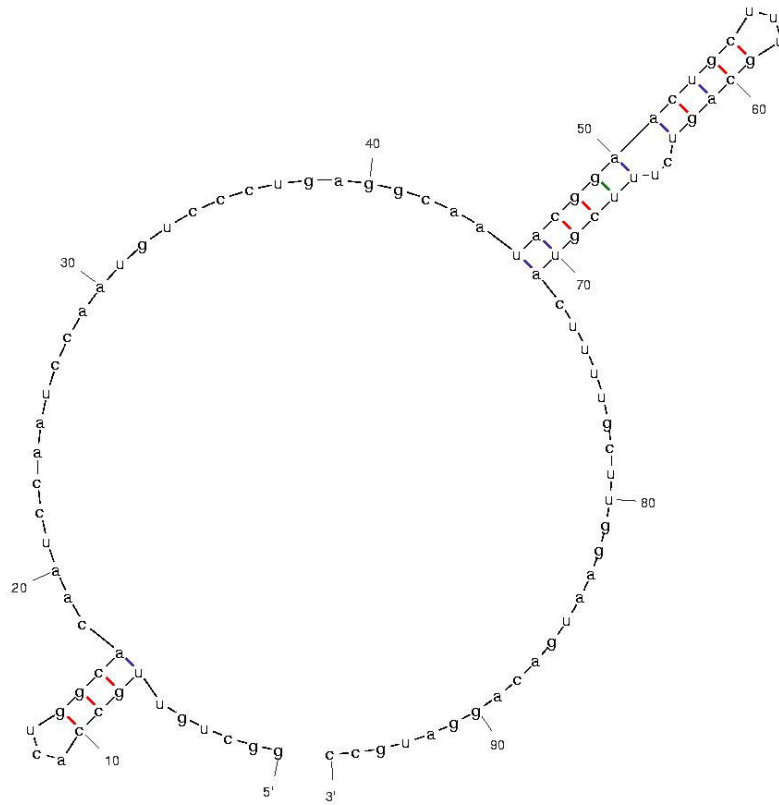
The real-time PCR amplicon was then tested for secondary structure, and for specificity. Secondary structure prediction was carried out using Michael Zukers' m-fold server². The predicted secondary structure for the sapovirus amplicon is shown in Figure 11. The software predicted that there was little secondary structure of note (as measured by the low dG), which suggests it would have little effect on test sensitivity. The amplicon was then resubmitted to BLAST to determine which sapovirus genotypes and types it would theoretically be able to detect. This process showed that the assay was only complimentary to sapovirus G1 types (Table 7). However, no cross-reactions to other viruses or organisms were predicted suggesting the test would be specific. Data from the HPA and other studies suggested that sapovirus G1 types were the most predominant strains pre-2004 (Okada *et al*, 2002; Gallimore *et al*, 2006). Thus, although this test would be unlikely to detect non G1 types, it was decided to assess it in comparison to the gel-based RT-PCR described above with the intention of replacing it either when a new assay was published or improved software design programs were available in the laboratory to allow the development of a more sensitive assay capable of detecting more types.

Where assays were selected from the literature the primers, probes and annealing temperatures were used exactly as described. The in-house sapovirus real-time PCR test used standard dual-labelled probe-based PCR conditions. All RNA real-time PCR assays utilised a commercial one step real-time RT-PCR kit from Invitrogen. This kit, also used in the norovirus dual-labelled probe-based RT-PCR allows the RT step, PCR and detection all to take place in a single tube, removing the need for a separate RT step. This again increases the efficiency of the process and reduces the potential for error. Such kits, however, only became economically viable around this time. The adenovirus assay utilises a commercial DNA mastermix (Invitrogen). Details of the exact protocol are described in section 3.4.

² (<http://www.bioinfo.rpi.edu/~zukerm/rna/>)

Figure 11: Predicted structure of the sapovirus real-time PCR amplicon.

Output of `slr_graph` (6)
by D. Stewart and M. Zuker



$dG = -0.76$ 07May08-12-20-16

Table 7: Alignment of primers and probe in real-time PCR developed for sapovirus.

Prototype Strain	Genogroup	Forward Primer	Probe	Reverse Primer
Real-time assay		GCTGTTSCYACTGGTGCA	CCAATCSAATGTCCCTGAGGCAATACGSAA	TGCTTGAARGACAGGATGCC
Leeds/00/UK (DQ158098)	1.4	GCTGTTGCCACTGGTGCA	CCAATCCAATGTCCCTGAGGCAATACGCAA	TGCTTGAACGACAGGATGCC
Sapporo/82/JP (U65427)	1.1	GCTGTTGCCACTGGTGCA	CCAATCCAATGTCCCTGAGGCAATACGCAA	TGCTTGAATGACAGGATGCC
Stockholm/97/SE (AF104182)	1.3	GCTGTCGCTACTGGGTCA	CCAATCAAATGTCCCTGATGCAATACGCAA	TGCTTGAACGATAGAATGCC
Houston/90/US (U95644)	1.2	GCTGTTGCTACTGGTTCC	CCAATCAAATGTCCCTGAAGCGGTACCAGA	TGCTTGAATGACAGAATGCC
Mex340/90 (AF435812)	2.2	GCTATGGCGACAGGAGCA	CACGAGCAACGTACCAAATTGCATCCGGGA	ACCCTGGACTACACGGCAAGC
Lyon598/97/FR (AJ271056)	2.1	GCAATGGCCACAGGGGCA	AACCAGCAATGTGCCCAACTGCATCCGGGA	ACCATGGACCACCCGGCAAGC
Bristol/98/UK (AJ249939)	2.1	GCAATGGCCACAGGGGCA	GACCAGCAATGTGCCCAACTGCATCCGGGA	ACCATGGACCACCCGGCAAGC

**Primers could not find a match for representatives of sapovirus genogroups G3 (AF182760), G4 (AF435814) and G5 (AY289803).*

3.4. Technical Description Of Laboratory Methods

3.4.1. Sample Preparation Methods

3.4.1.1. Faecal Extraction

Faecal samples were processed by making a 10% solution in Phosphate Buffered Saline (PBS). Samples were then centrifuged at 3,000 rpm for 30 minutes. Sample supernatants were then transferred to labelled specimen tubes before nucleic acid extraction.

3.4.1.2. Nucleic Acid Extraction

The nucleic acid extraction procedure was carried out on the Qiagen 96 DNA blood biorobot 9604 (Qiagen, Crawley, West Sussex, UK). Briefly, 40ul of protease (140mg/ml), 220ul of specimen and 240ul of buffer AL (Guanidium chloride) was added to each well and incubated at 56°C for 10 minutes. 250 µl of ethanol (96-100%) was then added to each well. 670ul of the sample lysate was then added to the vacuum manifold and 310ul of buffer AW1 (Wash Buffer) was added. AW1 was removed by vacuuming for 7 minutes. 1100ul of AW2 (Wash Buffer) were then added to each specimen and removed by vacuuming for 2 minutes. This step was then repeated. The extraction plate was then placed on the S block and centrifuged for 10 min at 6,000 rpm to dry the membrane. 200ul Buffer AE (10mM Tris-Cl; 0.5mM EDTA; pH9) was then added to each well incubation at room temperature for 1 minute. The extraction plate was then placed upon a collection tube plate and centrifuged at 6,000 rpm for 3 min to elute the nucleic acid.

3.4.2. PCR Methods

3.4.2.1. Reverse Transcriptase Step (Gel-Based RT-PCR)

Where the PCR assay involved an RNA target, after nucleic acid extraction the following reverse transcription step was employed for all conventional and SYBR green based real-time PCR (Ellis *et al*, 1997). Briefly, 22.2ul of RNA was added to a reaction mixture (17.8ul) containing 20mM Tris (pH 8.4), 50mM KCl (Invitrogen, Paisley, UK), 7.5mM MgCl₂ (Invitrogen, Paisley, UK), 1.5mM dNTPs (Roche, Lewes, UK), 20ng of pd(N)₆, 1.6U RNA guard (Amersham Pharmacia Biotech, US) and 200U of Malony murine leukaemia virus reverse transcriptase (Invitrogen,

Paisley, UK). The RT step was carried out under the following cycling conditions: 10 minutes at 25°C, 45 minutes at 37°C, 5 mins at 99°C. Samples were then centrifuged to ensure that the sample was thoroughly mixed.

3.4.2.2. PCR Methods For The Outbreak Service

Real-Time SYBR Green Based Assay

In the first PCR method investigated in 2000 amplification was performed in a 10ul mixture, containing 5ul of 5mM Mg²⁺ universal LightCycler™ mastermix (BioGene, Cambs, United Kingdom), 5pM of each primer Ni and E3 (Table 8), Sybergreen and 2.5ul of cDNA template. The PCR parameters were used as in the original publication (Green *et al*, 1995). First a pre-amplification step of 94°C for 45 seconds was carried out. This was followed by 40 cycles of: 94°C for 1 sec, 43°C for 5 seconds and 74°C for 1 sec. The amplification product was then melted by raising the temperature from 70 to 94°C.

Dual-Labelled Probe-Based Real-Time PCR For Norovirus

In the new assay developed for norovirus in 2003, amplification of norovirus was carried out in a 50ul reaction volume using the Invitrogen superscript III one step q-RT-PCR system (Invitrogen, Paisley, UK) containing 10ul of extracted nucleic acid, 0.05uM of each probe PROBE RING 1a, PROBE RING 1b and PROBE RING 2, and 0.5uM of each primer COG1F, COG 1R, COG 2F, COG 2R (Table 9). Reverse transcription was performed for 30 min at 50°C. Using an ABI 7500 SDS platform, platinum taq polymerase was activated at 95°C for 2 min followed by 40 cycles of: 95°C for 30 seconds and 56°C for 60 seconds.

3.4.2.3. PCR Methods For The Sporadic Service (Adenovirus, Astrovirus, Rotavirus And Sapovirus)

The following section details the implementation of the gel-based multiplex assay developed and assessed in 2000, and the real-time assays subsequently investigated in 2004. Note that in the case of the real-time assays for astrovirus, rotavirus and sapovirus the PCR conditions (i.e. cycling times etc.) were the same in each case, with the result that these tests could all be run on the same plate.

Gel-Based Multiplex Assay For Adenovirus, Astrovirus, Rotavirus And Sapovirus

In the test assessed in 2000 10ul of cDNA was added to a 50ul reaction containing 10xPCR buffer (100mM tris-HCl, 15mM MgCl₂, 500mM KCL), 0.2mM of each dNTP, and 0.2mM of each primer (Table 8). A mixture of 2U of Taq DNA polymerase, 0.028uM TaqStart antibody (Clontech Laboratories, Paolo Alto, USA), and 4xTaqStart antibody dilution buffer (Clontech Laboratories, Paolo Alto, USA) was incubated at room temperature for 10 minutes before being added to the final reaction mixture. PCR conditions were as follows: 94°C for 3 mins; 40 cycles of 94°C for 30 seconds, 47°C for 40 seconds, 72°C for 30 seconds; with a final extension step of 72°C for 7 min. Products were then subjected to agarose gel electrophoresis. Positive bands were expected at 140bp for adenovirus, 220bp for rotavirus, 449bp for astrovirus and 320bp for sapovirus.

Dual-Labelled Probe-Based Real-Time PCR For Adenovirus

Amplification of adenovirus was carried out in a 50ul reaction volume using Invitrogen platinum Taq DNA (Invitrogen, Paisley, UK) containing 10ul of extracted sample, 0.05uM of ADENO probe and 0.5uM of each primer ADENO 1 and ADENO 2 (Table 9). Using an ABI 7500 SDS platform, platinum taq polymerase was activated at 95°C for 2 min, followed by 40 cycles of: 95°C for 30 seconds and 60°C for 60 seconds.

Dual-Labelled Probe-Based Real-Time PCR For Astrovirus

Amplification of astrovirus was carried out in a 50ul reaction volume using the Invitrogen superscript III one step q-RT-PCR system (Invitrogen, Paisley, UK) containing 10ul of extracted nucleic acid, 0.05uM of ASTRO probe, and 0.5uM of each primer ASTRO 1 and ASTRO 2 (Table 9). Reverse transcription was performed for 30 min at 50°C. Using an ABI 7500 SDS platform, platinum taq DNA polymerase was activated at 95°C for 2 min, followed by 40 cycles of: 95°C for 30 seconds and 60°C for 60 seconds.

Dual-Labelled Probe-Based Real-Time PCR For Rotavirus

Amplification of rotavirus was carried out in a 50ul reaction volume using the Invitrogen superscript III one step q-RT-PCR system (Invitrogen, Paisley, UK) containing 10ul of extracted nucleic acid, 0.05uM of ROTA probe, and 0.5uM of each primer ROTA 1 and ROTA 2 (

Table 4). Reverse transcription was performed for 30 min at 50°C. Using an ABI 7500 SDS platform, platinum taq polymerase was activated at 95°C for 2 min, followed by 40 cycles of: 95°C for 30 seconds and 60°C for 60 seconds.

Dual-Labelled Probe-Based Real-Time PCR For Sapovirus

Amplification of sapovirus was carried out in a 50ul reaction volume using the Invitrogen superscript III one step q-RT-PCR system (Invitrogen, Paisley, UK) containing 10ul of extracted nucleic acid, 0.05uM of probe SAPO probe, and 0.5uM of each primer SAPO 1 and SAPO 2 (Table 9). Reverse transcription was performed for 30 min at 50°C. Using an ABI 7500 SDS platform, platinum taq polymerase was activated at 95°C for 2 min, followed by 40 cycles of: 95°C for 30 seconds and 60°C for 60 seconds.

Table 8: Oligonucleotide primers for PCR tests in the outbreak and sporadic service (2000-2003).

Virus	Service	Primers	Sequence	Amplicon size	Nucleotide position
Norovirus (SYBR green assay)	Outbreak and sporadic service	Ni	GAA TTC CAT CGC CCA CTG GCT	113bp	4495-4608 bp ^a
		E3	ATC TCA TCA TCA CCA TA		
Rotavirus	Sporadic service and outbreak samples that test negative for norovirus	Con1 Con2	TTGCCACCAATTCAAATAC ATTTCCGGACCATTTATAACC	220bp	676-896 bp ^b
Astrovirus		Mon269 Mon270	TCAGATGCATTGTCATTGGT CAACTCAGGAAACAGGGTGT	449bp	212-676 bp ^c
Adenovirus		ADRJC1 ADRJC2	GACATGACTTTCGAGGTCGATCCCATGGA CCGGCTGAGAAGGGTGTGCGCAGGTA	140bp	2840-2980 bp ^d
Sapovirus		Jv66 Sr80	GTGTANATCCARTCATCACC TGGGCATTCTACACAAAACCC	320bp	XX

^a Based on alignment with AB220921 (Norovirus Hu/ Chiba/complete genome)

^b Based on alignment with AB222786 (Human rotavirus VP4 complete sequence)

^c Based on alignment with AB000288 (Human astrovirus serotype 1 capsid gene)

^d Based on alignment with X51783 (adenovirus type 41 hexon region)

Table 9: Oligonucleotide primers and probes for real-time PCR tests in the outbreak and sporadic service (2004-2006).

Virus	Service	Primers	Probe	Nucleotide position
Norovirus	Outbreak and sporadic service	<i>COG1F</i> - CGYTGGATGCGNTTYCATGA <i>COG1R</i> - CTTAGACGCCATCATCATTYAC <i>COG2F</i> - CARGARBCNATGTTYAGRTGGATGAG <i>COG2R</i> - TCGACGCCATCTTCATTCACA	PROBE RING 1A- VIC-AGATYGCGATCYCCTGTCCA-BHQ-1 PROBE RING 1B- CY5-AGATCGCGGTCTCCTGTCCA-BHQ-1 PROBE RING 2- FAM-TGGGAGGGCGATCGCAATCT-BHQ-1	For G1 (5291-5375) ^a For G2 (5003-5100) ^b
Rotavirus	Sporadic/ service and outbreak samples that test negative for norovirus	<i>ROTA 1</i> -ACCATCTACACATGACCCTC <i>ROTA 2</i> -GGTCACATAACGCCCC	ROTA PROBE FAM-ATGAGCACAATAGTTAAAAGCTAACACTGTCAA-BHQ-1	987-1083 ^c
Astrovirus		<i>ASTRO 1</i> -CCGAGTAGGATCGAGGGT <i>ASTRO 2</i> - GCTTCTGATTAATCAATTTTAA	ASTRO PROBE FAM-CTTTTCTGTCTCTGTTTAGATTATTTTAAATCACC-BHQ-1	236-312 ^d
Adenovirus		<i>ADF</i> -GCCACGGTGGGGTTTCTAAACTT <i>ADR</i> -GCCCCAGTGGTCTTACATGCACATC	ADPRO-FAM TGCACCAGACCCGGGCTCAGGACTCCGA-BHQ1	17363-17495 ^e
Sapovirus		<i>SAPO 1</i> -GCTGTTSCYACTGGTGCA <i>SAPO 2</i> -GGCATCCTGTCRTTCCAAGCA	SAPO PROBE FAM-CCAATCSAATGTCCCTGAGGCAATACGSAA-BHQ-1	139-233 ^f

^a Based on the alignment of M87661

^b Based on the alignment of AF145896

^c Based on the alignment of DQ490535.1

^d Based on the alignment of AJ620757.1

^e Based on the alignment of DQ923122.2

^f Based on the alignment of AB327277.1

Chapter 4

Assessment Of The PCR Protocols And Subsequent Development

4.1. Overview

This chapter outlines a series of studies relating to the evaluation of the PCR protocols described in the previous chapter. The chapter is made up of three parts: the first outlines the experimental assessment of the test developed for norovirus in outbreaks of IID; the second outlines the evaluation of the selected tests for adenovirus, astrovirus, rotavirus and sapovirus for the investigation of sporadic IID; and the third part describes the results of a number of adaptations to the tests eventually implemented, which aimed to reduce the turn-around-time and cost of each of these tests without affecting their sensitivity. Evaluation in each case consists of an experimental comparison of the sensitivity of the PCR with that of alternative techniques (such as EM and EIA) available at the time of the research. The chapter concludes by summarising the results of the experiments and their implications for the laboratory diagnostic service.

4.2. Introduction

Chapter 3 outlined the PCR methods that were chosen and adapted for investigation as the potential replacements for EM in the laboratory diagnostic service for IID. This chapter describes a series of experiments relating to their assessment and, where found suitable, their subsequent evolution once implemented into the routine diagnostic service.

In the first part of the chapter the experimental evaluation of the tests developed for norovirus in outbreaks of IID is described. Initially for this service, the norovirus RT-PCR method described by Green *et al* (Green *et al*, 1995) was chosen and adapted to a SYBR green based real-time PCR method, which was implemented on the Lightcycler™ platform. The first experiment in this series thus compares this Lightcycler™ RT-PCR to the existing EM test on a panel of untested stool samples taken from outbreaks and sporadic cases of gastroenteritis.

The Lightcycler™ RT-PCR is subsequently compared to a number of other methods that emerged during the period of study (2000-2006) including a

conventional gel-based RT-PCR, two commercial EIAs and an alternative dual-labelled probe-based real-time RT-PCR.

The conventional RT-PCR method was recommended in 2003 by a European collaboration that compared a number of commonly used RT-PCR methods, including the original method described by Green *et al* (Vinje *et al*, 2003). Of the many methods assessed, the conventional RT-PCR assay using primers JV12 and JV13 was found to be most sensitive and was recommended to laboratories yet to set up a PCR service for norovirus. This assay and the Lightcycler™ RT-PCR are compared using dilution series of typed norovirus-positive samples and a panel of untested stool samples taken from outbreaks and sporadic cases of IID.

The EIA methods were compared to the Lightcycler™ RT-PCR based on the manufacturer's claim that these are a sensitive, high throughput, rapid and easy to use alternative to EM and RT-PCR. The methods are compared using panels of untested samples from both outbreaks and sporadic cases of IID.

The final study outlined in this section is a comparison of the Lightcycler™ RT-PCR with the dual-labelled probe-based real-time RT-PCR (Kageyama *et al*, 2003) selected and adapted (as described in Chapter 3) once the appropriate technology became available in the laboratory. These methods are compared on panels of outbreak and sporadic samples, and dilution series of norovirus-positive samples.

The second part of this chapter outlines the data relating to the evaluation of the PCR methods for sporadic cases of IID. The initial aim was to replace EM with a multiplex gel-based PCR for adenovirus, astrovirus, rotavirus and sapovirus alongside the norovirus assay developed for the outbreak service. The initial experiment outlines the results of the attempts made to adapt the four conventional gel-based PCR assays chosen in Chapter 3 into a multiplex RT-PCR assay. The sensitivity of the multiplex RT-PCR assay with respect to each pathogen is compared to the individual PCR assays on both dilution series of samples and panels of samples known to be positive for one of the four pathogens.

Based on the results of this experiment adaptations to the multiplex assay are described. The resultant assays are compared to antigen detection methods for astrovirus and rotavirus using a panel of clinical samples from sporadic cases of IID in children. Such methods have been shown to have sensitivity comparable or better than EM and are commonly used in routine microbiology laboratories (Anand *et al*,

2001; Eing *et al*, 2001; Kelkar *et al*, 2004; Giordano *et al*, 2005; Rabenau *et al*, 1998; Cubitt *et al*, 1999).

Finally, the gel-based PCR assays developed are compared to the dual-labelled probe-based real-time PCR methods chosen and adapted as described in Chapter 3, using panels of untested clinical samples from sporadic cases of IID and a number of dilution series of typed positive samples.

Based on these series of experiments the eventual tests implemented for the outbreak and sporadic services are described. The third part of this chapter then outlines experiments carried out in an attempt to reduce the turn-around-times and cost of these tests without affecting their sensitivity. The first examines the effect of reducing the length of the PCR cycling times on test sensitivity and rapidity. Following this is a study examining whether real-time PCR tests for DNA pathogens can be carried out using the parameters employed for RNA pathogens without a loss in test sensitivity (and hence can be carried out simultaneously). Further studies include an examination of whether pooled positive controls (i.e. a single control containing all 5 viral causes of IID) can be used in order to reduce the cost of PCR testing by allowing more clinical samples to be processed at any one time. The use of primer and probe pools (vials containing aliquoted volumes of both optimised primer and probe) is also examined to determine whether these can provide easier and more rapid test set up. The final experiment examines whether reducing the PCR reaction volumes affects the sensitivity of the PCR assays.

4.3. Assessment Of The PCR Protocols For Use In The Proposed Outbreak Service

4.3.1. Real-Time Lightcycler™ RT-PCR (SYBR Green) Vs EM

The aim of this first experiment was to compare the novel Lightcycler™ SYBR green real-time assay for norovirus described in Chapter 3 to the existing EM method employed in the laboratory. The specificity of the Lightcycler™ RT-PCR assay was first assessed using a panel made up of recognised enteric and other pathogens. It was then compared to EM using a panel of untested samples from outbreaks and sporadic cases of IID. As with all the experiments in this series the panel consisted of all samples submitted to the laboratory during a fixed period of time in order to provide a realistic cross-section of samples with which to test the two

methods. Samples from sporadic cases of IID were included since the test developed for the outbreak service was also to be used to test for norovirus in the proposed sporadic service.

4.3.1.1. Methods

Sample Panels

Specificity Panel

The specificity panel consisted of a total of 12 stool samples and 7 nucleic acid extracts containing variously: bacterial and parasitical causes of IID; non-norovirus viral pathogens known to cause IID; and other pathogens that can be detected in stool that are unrelated to IID. The bacterial and parasitical stool samples included: *Salmonella enteridis*, *Campylobacter jejuni*, *Giardia lamblia*, *E-coli 0157* and *Cryptosporidium parvum*. These samples were provided by Monklands District General Hospital. The stool samples containing non-norovirus causes of IID included: astrovirus types 1 and 3; sapovirus (Sapporo/82/JP); rotavirus types G1 and G2; and adenovirus types 41 and 40. Nucleic acid extracts of other pathogens unrelated to IID included: enterovirus; CMV; HSV-1 and 2; EBV; *Nessieria gonorrhoeae* and *Chlamydia trachomatis*. With the exception of the sapovirus sample, provided by Dr Koopmans (Bilthoven, Netherlands), the remaining samples were clinical samples stored as part of the WOSSVC collection. All samples were extracted using the nucleic acid extraction protocol described in Chapter 3.

Clinical Samples

The comparison of the Lightcycler™ RT-PCR with EM was carried out using samples from 9 suspected outbreaks of viral gastroenteritis (consisting of a total of 85 samples) together with a further 90 samples from sporadic cases of IID. As in all the experiments, and the laboratory diagnostic service itself, the information used to determine whether a sample was from an outbreak or sporadic case of IID was derived from the specimen request forms. The samples used in this experiment were all those submitted to the laboratory diagnostic service between October and December 2000. As with all other clinical samples employed in this research, details of the date of illness onset and its duration are not known since the clinician did not provide these. The conditions of transport were also not recorded. This, in fact, is true for the majority of samples submitted to the laboratory. Thus, although these factors affect the performance of the tests examined, the samples employed in the research

are representative of those expected in the ‘live’ service. Negative PCR controls (in the form of VTM) were included in each run to control for test contamination.

Laboratory Methods

The Lightcycler™ RT-PCR assay was implemented as described in Chapter 3, incorporating stool extraction (section 3.4.1.1), nucleic acid extraction (section 3.4.1.2) and the RT and PCR steps (sections 3.4.2.1 and 3.4.2.2). As part of the assessment process samples that tested positive for norovirus were then re-processed starting from stool extraction and the interpretation of the results confirmed by running the PCR product on an agarose gel.

The existing EM protocol employed uses the same stool extraction technique. Following this 2-5ml of the supernatant is spun at 100,000 rpm for 30 minutes. The supernatant is then removed and the pellet air dried for at least 10 minutes before being re-suspended. A drop of the virus suspension is then mixed with a drop of negative stain in a 50mm sterile Petri dish and placed on the ‘matt’ surface of an EM specimen grid. Grids are then examined at an instrument magnification of 60K. Each grid is examined for a period of at least 15 minutes.

4.3.1.2. Results

The LightCycler™ RT-PCR did not detect any of the specificity panel as positive, suggesting it was specific. Meanwhile the results for the clinical samples showed that, overall, 29 tested positive for norovirus using the EM technique compared to 47 using the LightCycler™ RT-PCR (Table 10).

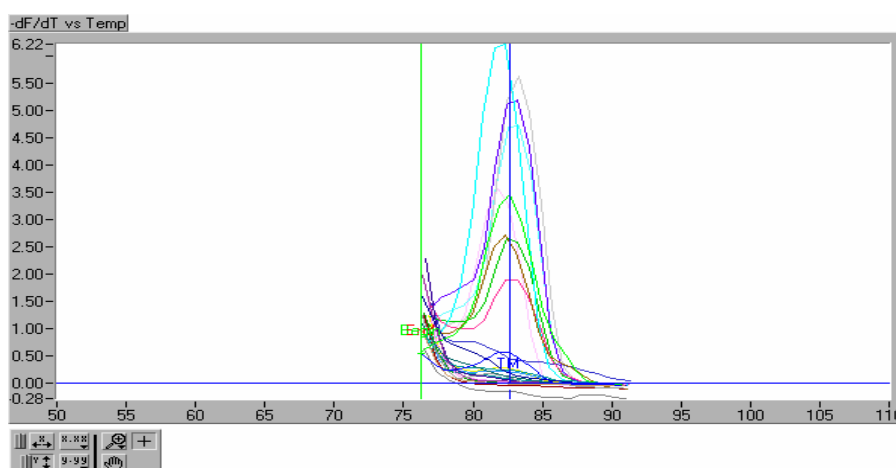
Table 10: Comparison of LightCycler™ RT-PCR and EM.

Sample Type	Number of Samples	EM Positive	LightCycler™ Positive
Outbreak 1	5	3	5
Outbreak 2	5	0	0
Outbreak 3	6	4	4
Outbreak 4	6	0	0
Outbreak 5	6	1	3
Outbreak 6	7	0	0
Outbreak 7	5	0	1
Outbreak 8	41	3	6
Outbreak 9	4	2	3
<i>Total Outbreak</i>	<i>85</i>	<i>13</i>	<i>22</i>
Sporadic	90	16	25
<i>Total samples</i>	<i>175</i>	<i>29</i>	<i>47</i>

The table shows that the Lightcycler™ RT-PCR consistently detected norovirus in more samples than the EM test, in samples from both suspected outbreaks of norovirus (from various sources) and sporadic cases of IID.

Examining the results in more detail, all samples that tested positive for norovirus using EM also tested positive using the Lightcycler™ RT-PCR. Moreover, all negative controls tested negative using the LightCycler™ RT-PCR. Examination of the melting curves of the samples that tested positive using the LightCycler™ RT-PCR showed melting point temperatures of between 81-83°C (see Figure 12 for an example) for all but two samples, which were outwith this range (80.3°C and 80.4°C). In order to corroborate the results, all Lightcycler™ RT-PCR positive results were re-investigated, by repeating the test from extraction and running the PCR products on a gel. A band of 113bp in length was observed for all 47 samples, confirming the original results.

Figure 12: Example of Lightcycler™ RT-PCR SYBR green melt curves (norovirus-positive results).



At the WOSSVC, an outbreak is confirmed as being caused by a particular pathogen if at least two of the submitted samples from a particular location are found to be positive for that pathogen. On the other hand, for an outbreak to be classified as negative at least six samples from the same outbreak have to be submitted and found to be negative for the pathogen in question. In either case, more than one sample is required before a classification is made in order to reduce the risk of false diagnoses.

Based on these criteria four of the nine outbreaks investigated were confirmed using EM compared to five using the Lightcycler™ RT-PCR assay (Table 10). Both tests in addition detected a single norovirus positive in samples from one other outbreak, which meant the outbreak could not definitely be attributed to norovirus.

4.3.1.3. Study Conclusions

The results of this study showed that the Lightcycler™ RT-PCR assay was specific and more sensitive than the existing EM protocol, detecting more outbreaks and individual cases of norovirus (Miller, Gunson and Carman, 2002). The increased sensitivity of the Lightcycler™ RT-PCR in comparison to EM confirmed the findings of earlier work (Buesa *et al*, 1996) and was subsequently substantiated further (Rabenau *et al*, 2003; Simpson *et al*, 2003). However, a possible disadvantage found was the varied melting temperature observed for the positive samples in this study (80-83°C). This highlights a potential difficulty in defining a strict melting temperature range for result interpretation, as norovirus RdRp variation may result in a PCR product with a melting temperature outwith the accepted range and thus a false negative result. Since this research was carried out other studies using SYBR green based RT-PCR assays have also highlighted melting temperature variation as a problem and have related this to norovirus type (Richards *et al*, 2004). Here, however, all samples with a melting temperature range of 80-83°C were confirmed when re-tested using agarose gel analysis to interpret the results, indicating this is an acceptable range to employ. Thus, the increased sensitivity of the Lightcycler™ RT-PCR assay combined with its increased sample capacity meant the decision was taken to implement it as the frontline test for outbreak samples in January 2001. EM was retained for testing all samples from sporadic cases of IID and as the secondary test for outbreak samples that tested negative for norovirus using the new PCR assay.

4.3.2. Real-Time Lightcycler™ RT-PCR (SYBR Green) Vs A Recommended Conventional Gel-Based RT-PCR

Following implementation of the Lightcycler™ assay as part of the new laboratory diagnostic service the primers it employs, Ni and E3, were independently assessed as part of a European collaboration study designed to identify which of the commonly used RT-PCR methods offered the most sensitive and wide ranging detection of norovirus types (Vinje *et al*, 2003). This study found that the primers Ni and E3 were unable to detect some G1 and G2 types, and that of all the RT-PCR assays examined, the most sensitive assay used the primers JV12 and JV13. As a result these were recommended to other laboratories interested in setting up a RT-PCR assay for norovirus (Vinje *et al*, 2003).

In order to investigate these findings, the Lightcycler™ RT-PCR assay was compared to the recommended gel-based RT-PCR. First, the specificity of the gel-based RT-PCR was assessed using the same panel employed earlier in assessing the Lightcycler™ RT-PCR. Subsequently, the two assays were compared using a dilution series of a G1 and G2 norovirus sample. Finally, the two PCR methods were compared using clinical samples from both the sporadic and outbreak setting.

4.3.2.1. Methods

Sample Panels

Specificity Panel

The specificity panel described in section 4.3.1.1 for the assessment of the Lightcycler™ assay was also employed in this experiment.

Clinical Samples

The gel-based RT-PCR was compared to the LightCycler™ RT-PCR method using 81 faecal samples collected from 46 sporadic cases and 7 outbreaks (containing a total of 35 samples) of suspected viral gastroenteritis. All samples were submitted to the WOSSVC during January to February 2002. Negative PCR controls (in the form of VTM) were included in each PCR run to control for test contamination. Both RT-PCR methods were also compared using a 10 fold dilution series of a G1 (White Rose) sample and a G2 (Lordsdale/93/UK X86557) norovirus-positive sample. The G1 sample was provided by Dr Marion Koopmans whereas the G2 sample was from the WOSSVC collection.

4.3.2.2. Laboratory Methods

The Lightcycler™ assay was used as previously described (section 4.3.1.1). The gel-based RT-PCR employed the same stool, nucleic acid extraction and RT methods (sections 3.4.1.1, 3.4.1.2 and 3.4.2.1). Following this, 10ul of cDNA was added to a 50ul reaction containing 10xPCR buffer (100mM tris-HCl, 15mM MgCl₂, 500mM KCL), 0.2mM of each dNTP, 0.2mM of primers JV12Y and JV13I (Koopmans *et al*, 2003). A mixture of 2U of Taq DNA polymerase, 0.028uM TaqStart antibody (Clontech Laboratories, Paolo Alto, USA) and 4xTaqStart antibody dilution buffer (Clontech Laboratories, Paolo Alto, USA) was incubated at room temperature for 10 minutes before being added to the final reaction mixture. PCR was carried out under the following conditions: 94°C for 3 mins, followed by 40 cycles of the

following: 94°C for 1 min, 37°C for 1 min, 74°C for 1 min. This is followed by a final extension step of 72°C for 7 mins. Products were then subjected to agarose gel electrophoresis. Positive bands were expected at 324bp.

4.3.2.3. Results

The gel-based RT-PCR was shown to be specific as it did not detect any of the specificity panel as positive. Results from testing the sample panel showed that, in total, the gel-based RT-PCR detected more norovirus-positive samples than the LightCycler™ RT-PCR (28 vs 19), as shown in Table 11.

6 of the 7 of the suspected outbreaks were diagnosed as being caused by norovirus using both methods. One outbreak contained 1 positive sample that was detected by both methods. Based on the criteria outlined above this outbreak could not be confirmed as being caused by norovirus. No additional outbreaks were diagnosed using the gel-based RT-PCR assay although the overall sample percentage positive rate was greater using the gel-based RT-PCR method (approximately 69% vs 49%). All negative controls tested negative using both assays.

Table 11: Comparison of LightCycler™ RT-PCR and recommended gel-based RT-PCR.

Sample Type	Number of Samples	Lightcycler™ Positive	Gel-based Positive
Outbreak 1	13	4	6
Outbreak 2	3	2	3
Outbreak 3	3	1	1
Outbreak 4	4	3	3
Outbreak 5	4	2	3
Outbreak 6	5	2	4
Outbreak 7	3	2	3
<i>Total Outbreaks</i>	<i>35</i>	<i>16</i>	<i>23</i>
Sporadic	46	3	5
<i>Total</i>	<i>81</i>	<i>19</i>	<i>28</i>

The increased sensitivity of the conventional gel-based RT-PCR was confirmed using the dilution series. Using a G2 norovirus sample the conventional RT-PCR was shown to be more sensitive than the Lightcycler™ RT-PCR assay with a detection limit of 10⁻⁴ compared to 10⁻³ (Table 12). However, similar end point detection limits were observed when testing the dilution series of the G1 (White rose) positive sample (Table 13).

Table 12: Comparison of LightCycler™ RT-PCR and recommended gel-based RT-PCR on a dilution series of a norovirus-positive sample (Lordsdale/93/UK (X86557)).

Dilution of G2 Sample	LightCycler™ Result	Gel-based Result
10 ⁻¹	+	+
10 ⁻²	+	+
10 ⁻³	+	+
10 ⁻⁴	-	+
10 ⁻⁵	-	-

Table 13: Comparison of LightCycler™ RT-PCR and recommended gel-based RT-PCR on a dilution series of a norovirus-positive sample (G1 - White Rose).

Dilution of G1 Sample	LightCycler™ Result	Gel-based Result
10 ⁻¹	+	+
10 ⁻²	+	+
10 ⁻³	+	+
10 ⁻⁴	-	-

4.3.2.4. Study Conclusions

This study demonstrated that of the two methods examined the gel-based RT-PCR method recommended by Vinje *et al* (Vinje *et al*, 2003) offered more sensitive diagnosis of individual cases of norovirus in outbreaks and sporadic cases of viral gastroenteritis (Gunson, Miller and Carman, 2003(i)). The increased sensitivity of the gel-based RT-PCR was confirmed using the dilution series of a G2 positive sample, but not using a G1 dilution panel. However, the real-time LightCycler™ RT-PCR assay did confirm an equal number of outbreaks and had the advantage of providing fewer and shorter steps. For example, the Lightcycler™ RT-PCR includes the following steps: stool extraction, nucleic acid extraction, a reverse transcriptase step and then PCR (a total of 4 steps). The gel-based RT-PCR consists of all of these plus a gel-based analysis step, which can take approximately 1-3 hours depending on the number of samples under scrutiny. Moreover, the PCR step in the Lightcycler™ test is considerably shorter (approximately 45 mins versus 2-4 hours in the gel-based assay). As discussed earlier the fewer the steps in a test the less potential for human error and the faster the process. This together with the fact that it did not miss any outbreaks meant that the LightCycler™ RT-PCR remained the assay of choice for the investigation of outbreaks of IID at the WOSSVC.

4.3.3. Real-Time Lightcycler™ RT-PCR Vs First And Second Generation Commercial EIA Methods

During the period of study a number of commercial EIA tests became available for the diagnosis of norovirus. The manufacturers of these assays claim they are a rapid, high throughput and sensitive alternative for laboratories with no access to EM or RT-PCR techniques. However, few data exist comparing these antigen detection methods to RT-PCR methods. In this study, first and second-generation EIA assays were compared with the LightCycler™ RT-PCR on samples from outbreaks and sporadic cases of IID. The first generation EIA (IDEIA, DAKO Cytomation, Cambridgeshire, UK) uses polyclonal antibodies to detect and differentiate between G1 and G2 norovirus strains. In 2003, it was replaced by a second generation EIA. This second generation EIA (IDEIA NLV, Dako Ltd, Ely, Cambridgeshire, UK) uses a cocktail of genotype-specific monoclonal antibodies to detect and differentiate between the antigens of G1 and G2 norovirus strains.

4.3.3.1. Methods

Sample Panels

Comparison Of First Generation EIA With Lightcycler™ RT-PCR

The two assays were compared using 35 faecal samples from 7 outbreaks of gastroenteritis and 46 from sporadic cases of gastroenteritis sent to the WOSSVC during October to November 2002. Each sample was tested within 3 days of collection from the patient in line with the EIA manufacturer's requirements. Samples for which the two tests produced different results were re-tested using the gel-based RT-PCR assessed in the previous experiment in order to corroborate the results. The specificity of both assays was assessed using 7 VTM vials.

Comparison Of Second Generation EIA With Lightcycler™ RT-PCR

The two assays were compared using 42 faecal samples from 6 suspected outbreaks of gastroenteritis and 28 from sporadic cases of gastroenteritis sent to the WOSSVC during March to April 2003 (Gunson, Miller and Carman, 2003 (ii)). Each sample was tested within 3 days of collection from the patient in line with the EIA manufacturer's requirements. Samples for which the two tests produced different results were re-tested using the gel-based RT-PCR assessed in the previous

experiment in order to corroborate the results. The specificity of both assays was assessed using 7 VTM vials.

4.3.3.2. Laboratory Methods

The Lightcycler™ RT-PCR and gel-based RT-PCR were used as described in the previous sections.

First Generation Commercial EIA For Norovirus

The reagents were reconstituted according to the manufacturer's instructions (DAKO Cytomation, Cambridgeshire, UK). Briefly, approximately 0.1g of faecal specimens was added to 1ml of sample diluent and mixed thoroughly. For the genogroup 1 assay, the appropriate number of microtitre wells for samples and controls was selected from the kit. 100ul of G1 cut off control was added to the appropriate well. 100ul of the negative control was added to two wells. 100ul of the samples to be tested were added to the appropriate wells. 100ul of G1 conjugate was then added to each well and the plate was incubated at 20-30°C for 120 minutes. The wells were then washed 5 times using working strength wash buffer. 100ul of substrate was then added to the wells. This was then incubated at 20-30°C for 30 minutes. 100ul of stopping solution was then added to the well. The colour change was then measured photometrically at 450nm with 600nm reference. According to the manufacturer, negative controls must have a mean absorbance value of under 0.15 A_{450} units. Positive controls must have a cut off value of more than 0.18 A_{450} units. Samples with A_{450} units above the positive control threshold are considered positive. Any sample with an A_{450} value of within 0.010 of the cut off control should be considered equivocal and retested, or interpreted alongside clinical and epidemiological data.

For the genogroup 2 assay the appropriate number of microtitre wells was removed from the kit. 100ul of G2 cut off control was added to the appropriate well. 100ul of the negative control was added to two wells. 100ul of the samples to be tested were added to the appropriate wells. 100ul of G2 conjugate was then added to each well and the plate was incubated at 20-30°C for 120 minutes. The wells were then washed 5 times using working strength wash buffer. 100ul of substrate was then added to the wells. This was then incubated at 20-30°C for 30 minutes. 100ul of stopping solution was then added to the well. The colour change was then measured photometrically at 450nm with 600nm reference. Negative controls must have an A_{450}

value of less than 0.15. Positive controls must have a cut off value of more than 0.18 A_{450} units. Samples with A_{450} units above the positive control threshold are considered positive. Any sample with an A_{450} value of within 0.010 of the cut off control should be considered equivocal and retested, or interpreted together with clinical and epidemiological data.

Second Generation Commercial EIA For Norovirus

The EIA for norovirus was carried out as per the manufacturer's instructions (DAKO Cytomation, Cambridgeshire, UK). Briefly, approximately 0.1g of faecal specimens was added to 1ml of sample diluent and mixed thoroughly. For the genogroup 1 assay, the appropriate number of microtitre wells was selected from the kit. 100ul of G1 cut off control was added to the appropriate well. 100ul of the negative control was added to two wells. 100ul of the samples to be tested were added to the appropriate wells. 100ul of G1 conjugate was then added to each well and the plate was incubated at 20-30°C for 120 minutes. The wells were then washed 5 times using working strength wash buffer. 100ul of substrate was then added to the wells. This was then incubated at 20-30°C for 30 minutes. 100ul of stopping solution was then added to the well. The colour change was then measured photometrically at 450nm with 600nm reference. Negative controls must have a mean A_{450} value of less than 0.15. Positive controls must have a cut off value of over 0.18 A_{450} units. Samples with A_{450} units above the positive control threshold are considered positive. Any sample with an A_{450} value of within 0.010 of the cut off control should be considered equivocal and retested, or interpreted alongside clinical and epidemiological data.

For the genogroup 2 assay the appropriate number of microtitre wells were selected. 100ul of G2 cut off control was added to the appropriate well. 100ul of the negative control was added to two wells. 100ul of the samples to be tested were added to the appropriate wells. 100ul of G2 conjugate was then added to each well and the plate was incubated at 20-30°C for 120 minutes. The wells were then washed 5 times using working strength wash buffer. 100ul of substrate was then added to the wells. This was then incubated at 20-30°C for 30 minutes. 100ul of stopping solution was then added to the well. The colour change was then measured photometrically at 450nm with 600nm reference. Negative controls must have an A_{450} value of under 0.15. Positive controls must have a cut off value of more than 0.18 A_{450} units. Samples with A_{450} units above the positive control are considered positive. Any

sample with an A₄₅₀ value of within 0.010 of the cut off control should be considered equivocal, and retested or interpreted together with clinical and epidemiological data.

4.3.3.3. Results

Comparison Of First Generation EIA With Lightcycler™ RT-PCR

In total, the Lightcycler™ assay detected norovirus in more samples than the EIA (18 versus 6), as shown in Table 14. All of the Lightcycler™ positives were confirmed using the gel-based RT-PCR, compared to 5 of the 7 EIA positives. A total of 4 norovirus-positives were detected by both assays

Table 14: Comparison of LightCycler™ PCR and first generation EIA.

Sample Type	Number of Samples	LightCycler™ Positive	EIA Positive
Outbreak 1	13	4	2
Outbreak 2	3	2	0
Outbreak 3	3	1	1
Outbreak 4	4	3	0
Outbreak 5	4	1	0
Outbreak 6	5	2	0
Outbreak 7	3	2	1
<i>Total Outbreak</i>	<i>35</i>	<i>15</i>	<i>4</i>
Sporadic	46	3	2
<i>Total</i>	<i>88</i>	<i>18</i>	<i>6</i>

The EIA detected norovirus-positive samples in 3 of the 7 suspected outbreaks. However, in 2 of these outbreaks only 1 sample tested positive. As discussed earlier, the WOSSVC criteria state that a norovirus outbreak must contain at least 2 positive samples in order to be confirmed as positive, as the presence of 1 positive sample could be due to a sporadic case of norovirus. Using this criterion here, only 1 outbreak could be confirmed as being caused by norovirus. The LightCycler™ RT-PCR, in comparison, confirmed norovirus as the cause in 5 outbreaks.

Amongst the sporadic cases of IID the LightCycler™ RT-PCR detected norovirus in 3 samples. All 3 results were confirmed using the gel-based RT-PCR. In contrast, the EIA test detected norovirus in 2 sporadic samples, neither of which could be confirmed using the gel-based RT-PCR. In terms of specificity the EIA detected one of the VTM samples as positive. Testing with the gel-based RT-PCR, however, confirmed that the VTM sample was norovirus-negative. Meanwhile all negative controls tested negative using the Lightcycler™ assay.

Comparison Of Second Generation EIA With Lightcycler™ RT-PCR

In total, the LightCycler™ RT-PCR detected norovirus in more samples than the EIA (26 versus 9), as shown in Table 15. All of the Lightcycler™ positive results were confirmed using the gel-based RT-PCR. A total of 8 samples tested positive for norovirus using both assays. The EIA detected norovirus in 2 samples that tested negative using the LightCycler™ RT-PCR assay. However, neither of these positive results could be confirmed using the second RT-PCR. One sample was classified as equivocal using EIA but tested positive for norovirus using the Lightcycler™ RT-PCR.

In total 6 suspected outbreaks of viral gastroenteritis were investigated. Using the WOSSVC criteria, the EIA technique diagnosed norovirus as the cause of 1 of the 6 outbreaks (outbreak 6), whereas the LightCycler™ RT-PCR established norovirus as the cause in all outbreaks.

Amongst the sporadic cases of IID the LightCycler™ RT-PCR detected norovirus in 5 samples. All 5 results were confirmed using the gel-based RT-PCR. In contrast, the EIA test detected norovirus in 3 sporadic samples, only one of which could be confirmed using the gel-based RT-PCR. In terms of specificity all negative controls tested negative using the Lightcycler™ assay and the EIA.

Table 15: Comparison of Lightcycler™ PCR and EIA

Sample Type	Number of Samples	Lightcycler™ Positive	EIA Positive
Outbreak 1	5	2	0
Outbreak 2	6	4	1
Outbreak 3	8	4	1
Outbreak 4	11	3	0
Outbreak 5	6	4	1
Outbreak 6	6	4	3
<i>Total Outbreak</i>	<i>42</i>	<i>21</i>	<i>6</i>
Sporadic	28	5	3
<i>Total</i>	<i>70</i>	<i>26</i>	<i>9</i>

4.3.3.4. Study Conclusions

These results show that both the first and second generation EIA were inadequate for norovirus detection. The LightCycler™ RT-PCR detected norovirus in more samples and confirmed more norovirus outbreaks than either EIA. In both comparisons all of the extra cases of norovirus detected by the LightCycler™ RT-PCR were confirmed using the alternative (more sensitive) gel-based RT-PCR. In contrast both EIAs produced positive results that could not be confirmed using the

gel-based RT-PCR. It is possible that these samples tested negative using the gel-based method due to primer mismatches or inhibition. However, given that these samples also tested negative using the Lightcycler™ assay, the EIA results may also have been false positives and therefore the specificity of these assays should be questioned. The fact that in the first study a VTM negative control tested positive for norovirus using the EIA points toward this conclusion.

As these EIA tests are relatively new, there are few comparisons with PCR available in the literature. Richards *et al* compared the first generation (polyclonal based) EIA with a RT-PCR assay on patient samples taken from outbreaks of IID (Richards *et al*, 2003). Overall they found the EIA to be less sensitive and specific than the RT-PCR method. These results confirmed those of the above study. However, Richards *et al* concluded their report by stating that EIA is a suitable alternative to EM as an initial screening tool before testing all samples found to negative using PCR or EM. This, however, is likely to result in longer turn-around-times since a significant proportion of samples are likely to test negative using EIA and thus require a second phase of testing. Moreover, the lower specificity of the EIA found in this research may result in the false diagnosis of norovirus outbreaks leading to unnecessary implementation of costly infection control procedures. It is worth noting that the first generation EIA was withdrawn from the market in 2003.

A recent study by Burton-MacLeod further substantiated the results of the above study examining the second generation (monoclonal based) EIA. In the reported study the EIA was assessed on a panel of known types of norovirus and other causes of viral IID (Burton-MacLeod *et al*, 2004). They found the EIA could not detect all G2 strains and had an overall sensitivity of 39% (albeit with a specificity of 100%). They concluded that the Dako assay could not be used as a frontline test for norovirus. Other recent studies have also found the Dako assay to have lower sensitivity and specificity than RT-PCR tests (Wilhelmi *et al*, 2007; Castriciano *et al*, 2007; Dimitriadis, Bruggink and Marshall, 2006; Dimitriadis and Marshall, 2005). For example, Wilhelmi *et al* assessed the EIA using 117 samples that tested positive for norovirus using RT-PCR. They found the EIA to have a sensitivity of 76.9% and a specificity of 85%. Similar results were obtained by Castriciano *et al* and Dimitriadis *et al*.

Based on the findings of this research the EIA method was not implemented in the WOSSVC and the Lightcycler™ RT-PCR remained the frontline assay for outbreaks of norovirus.

4.3.4. Real-Time Lightcycler™ RT-PCR Vs Dual-Labelled Probe-Based Real-Time PCR

As discussed earlier, in 2003 the WOSSVC obtained a number of real time PCR platforms that could support other real time chemistries including dual-labelled probes. Compared to the Lightcycler™ platform, these platforms offer greater throughput (72-96 vs 32 per run) and potentially, therefore, more rapid turn-around-times. Dual-labelled probe-based real-time PCR methods may also offer improved specificity in comparison to a SYBR green based approach due to the incorporation of an additional probe complimentary to the target sequence. As a result such assays do not require a melting curve step, which aids rapidity and offers easier result interpretation not reliant on melting temperature.

The aim of this experiment, therefore, was to compare the Lightcycler™ RT-PCR to the dual-labelled probe-based RT-PCR selected and adapted as described in Chapter 3. First the specificity of the new assay was tested using the panel employed earlier in assessing the Lightcycler™ assay. The sensitivity of the two real-time assays was then compared using clinical samples from outbreaks and sporadic cases of IID and dilution series of both a G1 and G2 positive sample.

Resource constraints meant that corroboratory testing using an alternative method was not carried out at the time. However, all samples for which the two tests produced different results were re-tested using an alternative nested RT-PCR in 2007 (Gallimore *et al*, 2007). The assay described by Gallimore *et al* was selected since it targets parts of both the RdRp and the ORF-2 regions. This extra testing was carried out in order to confirm the initial results obtained in 2004 and to ensure that the correct service decisions were made regarding the routine diagnostic service.

4.3.4.1. Methods

Sample Panels

Specificity Panel

The specificity panel described in section 4.3.1.1 for this original assessment of the assay was again employed in this experiment.

Clinical Samples

The dual-labelled probe-based real-time RT-PCR and the LightCycler™ RT-PCR were compared using 217 faecal samples collected from 6 suspected outbreaks of IID (n=64 samples), and 153 community managed cases of acute gastroenteritis. All samples were submitted to the WOSSVC during October to December 2003. Finally, each method was compared using a dilution series of a known G1 (Birmingham (AJ277612)) sample and a known G2 (Lordsdale/93/UK (X86557)) sample. The G1 sample was provided by Dr M Koopmans whereas the G2 sample was part of the WOSSVC sample collection.

Laboratory Methods

The LightCycler™ RT-PCR assay was implemented as described earlier. The dual-labelled probe-based assay was implemented as described in section 3.4.2.2 of Chapter 3.

Nested RT-PCR For Norovirus

Extracted RNA (12.5ul) was amplified using a combined one step RT-PCR kit (Qiagen, Crawley, UK) using primers G2FBN and G2SKR (25 pmoles/reaction) under the following conditions. The RT step was carried at 50°C for 30 minutes followed by the first round PCR step at 95°C for 15 minutes. This was followed by 35 cycles of: 95°C for 40 seconds; 45°C for 40 seconds and 72°C for 1 min. 2ul of first round product was then transferred to a 48 ul second round reaction (Qiagen) containing primers G2FBN2 and G2SKR (50 pmoles/ reaction) and subjected to 40 cycles of: 94°C for 40 seconds; 45°C for 25 seconds and 72°C for 1 minute 30 seconds. This was followed by a hold step 72°C for 10 min. Products were visualised using agarose gel electrophoresis. A PCR product of 357bp was expected if norovirus was successfully amplified from the sample.

4.3.4.2. Results

The dual-labelled probe-based RT-PCR was shown to be specific since it did not detect any of the specificity panel as positive. When compared on the sample panel the new real-time assay detected norovirus in more samples than the LightCycler™ RT-PCR in total (41 vs 13) as shown in Table 16. All extra positives detected by the dual-labelled probe-based assay were confirmed as containing norovirus by re-testing with the nested RT-PCR.

Using the standard WOSSVC criteria, the probe-based method diagnosed norovirus to be the cause of 4 outbreaks (outbreaks 1, 3, 4 and 5). In comparison, the LightCycler™ RT-PCR diagnosed norovirus as the cause of only one outbreak (outbreak 3). In both tests a single positive sample was detected in one other suspected outbreak (in outbreak 2 using the dual-labelled probe-based assay and outbreak 4 using the LightCycler™ RT-PCR), neither of which could be confirmed as being caused by norovirus due to the lack of multiple positive samples. All of the samples from one outbreak tested negative for norovirus using both methods (outbreak 6).

Table 16: Comparison of dual-labelled probe-based PCR and LightCycler™ RT-PCR.

Sample Type	Number of Samples	Dual-labelled Probe-based Positives	Lightcycler™ Positives
Outbreak 1	21	3	0
Outbreak 2	5	1	0
Outbreak 3	7	7	6
Outbreak 4	5	2	1
Outbreak 5	13	13	0
Outbreak 6	13	0	0
<i>Total Outbreak</i>	<i>64</i>	<i>26</i>	<i>7</i>
Sporadic	153	15	6
<i>Total</i>	<i>217</i>	<i>41</i>	<i>13</i>

Amongst the sporadic cases, the dual-labelled probe-based assay detected norovirus in more samples (15 vs 6).

Finally, the greater sensitivity of the dual-labelled probe-based RT-PCR was confirmed using the dilution panels of a G1 and G2 positive sample (made up of 10 fold dilutions). The results are shown in Table 17 and Table 18. The dual-labelled probe-based assay was found to have a lower detection limit for both norovirus type G1 (10^{-5} vs 10^{-2}) and G2 (10^{-6} vs 10^{-4}).

Table 17: Comparison of dual-labelled probe-based RT-PCR and LightCycler™ RT-PCR on a dilution series of a norovirus positive sample (G1-Birmingham AJ277612).

Dilution of G1 Sample	LightCycler™ Result	Dual-labelled Probe-based Result (Ct)
10^{-1}	+	+ (23.2)
10^{-2}	+	+ (27.0)
10^{-3}	-	+ (30.2)
10^{-4}	-	+ (33.4)
10^{-5}	-	+ (36.2)
10^{-6}	-	-

Table 18: Comparison of dual-labelled probe-based RT-PCR and LightCycler™ RT-PCR on a dilution series of a norovirus positive sample (G1-Lordsdale/93/UK X86557).

Dilution of G2 Sample	LightCycler™ Result	Dual-labelled Probe-based Result (Ct)
10 ⁻¹	+	+ (20.1)
10 ⁻²	+	+ (23.4)
10 ⁻³	+	+ (27.1)
10 ⁻⁴	+	+ (30.2)
10 ⁻⁵	-	+ (33.6)
10 ⁻⁶	-	+ (37.0)
10 ⁻⁷	-	-

4.3.4.3. Study Conclusions

Overall, the results showed that the adapted version of the dual-labelled probe-based test described by Kageyama *et al* was specific and consistently more sensitive than the Lightcycler™ assay (Gunson and Carman, 2005). This was confirmed by repeat testing of the discrepant results using an alternative PCR assay in 2007. The dual-labelled probe-based PCR also had the potential for increased throughput since it could test more samples per run than achievable on the Lightcycler™ method. As mentioned above the Lightcycler™ assay consisted of four steps (stool extraction, nucleic acid extraction, RT, and PCR) whereas the dual-labelled probe-based assay consists of just three steps (the RT and PCR steps are combined). Based on these results it was used to replace the Lightcycler™ assay as the frontline test for norovirus in outbreaks of IID in January 2004.

4.4. Assessment Of The PCR Protocols For Use In The Proposed Sporadic Service

4.4.1. Gel-based Multiplex RT-PCR For Adenovirus, Astrovirus, Rotavirus And Sapovirus Vs Individual PCR Assays For Each

The aim of the first experiment in this series was to compare the sensitivity of the novel multiplex assay described in Chapter 3 with that of the individual assays from which it was developed, to ensure the multiplex RT-PCR was at least as sensitive. Of particular interest were the results for adenovirus, to determine if the addition of an RT step affected the sensitivity of the test. The final multiplex was then assessed for specificity using a panel of samples containing recognised gastrointestinal pathogens.

4.4.1.1. Methods

Sample Panels

Clinical Samples

Both methods were initially compared using 10-fold dilution series of four different samples, each known (via testing by EM) to be positive for one of the four viral pathogens of interest. Testing with a dilution series is an important first step in the assessment of multiplex assays prior to the assessment using clinical samples, in order to determine if there are any cross-reactions between the primers that reduce test sensitivity. The clinical panel in this case consisted of 74 samples from sporadic cases of IID containing 20 that tested positive for adenovirus (using the EIA method Adenoscreen (Microgen) at the Royal Hospital for Sick Children (RHSC) in Glasgow, 10 samples that tested positive for astrovirus using EM at the WOSSVC, 20 rotavirus-positive samples tested using an EIA method (Microgen), at the RHSC and 24 sapovirus-positive samples containing representatives of both G1 and G2 sapoviruses (provided by Dr M Koopmans, Bilthoven, Netherlands).

Specificity Panel

The specificity panel described in section 4.3.1.1 was also employed in this experiment, with the addition of 7 norovirus types for cross-testing purposes. The 7 norovirus types consisted of 5 G1 types (Birmingham AJ277612), White Rose, Malta, Musgrove and Mikkelo) and 2 G2 types (Lordsdale/93/UK X86557 and Leeds/90/UK AJ277603) and were sourced from Dr M Koopmans, Bilthoven, Netherlands and the G2 types were part of the WOSSVC sample collection.

4.4.1.2. Laboratory Methods

All samples were processed using the faecal extraction and nucleic acid extraction protocols outlined in Chapter 3 (section 3.4.1.1 and 3.4.1.2). Where employed, the reverse transcriptase step was the standard laboratory method described in Chapter 3 (section 3.4.2.1). The multiplex RT-PCR for adenovirus, astrovirus, rotavirus and sapovirus is also described in Chapter 3 (section 3.4.2.3).

Individual PCR For Adenovirus

10ul of cDNA was added to a 50ul reaction containing 10xPCR buffer (100mM tris-HCl, 15mM MgCl₂, and 500mM KCl), 0.2mM of each dNTP and 0.2mM of primers ADJC1 and ADJC2 for adenovirus (Cooper *et al*, 1999). A mixture

of 2U of Taq DNA polymerase, 0.028uM TaqStart antibody (Clontech Laboratories, Paolo Alto, USA) and 4xTaqStart antibody dilution buffer (Clontech Laboratories, Paolo Alto, USA) was incubated at room temperature for 10 minutes before being added to the final reaction mixture. The PCR conditions were 94°C for 3 mins, followed by 40 cycles of: 94°C for 30 secs, 47°C for 40 secs, 72°C for 30 secs; with a final extension step of 72°C for 7 mins. Products were then subjected to agarose gel electrophoresis. Positive bands were expected at 140bp for adenovirus.

Individual RT-PCR For Astrovirus

10ul of cDNA was added to a 50ul reaction containing 10xPCR buffer (100mM tris-HCl, 15mM MgCl₂, 500mM KCl), 0.2mM of each dNTP and 0.2mM of primers mon269 and mon270 for astrovirus (Noel *et al*, 1995). A mixture of 2U of Taq DNA polymerase, 0.028uM of TaqStart antibody (Clontech Laboratories, Paolo Alto, USA) and 4xTaqStart antibody dilution buffer (Clontech Laboratories, Paolo Alto, USA) was incubated at room temperature for 10 minutes before being added to the final reaction mixture. PCR conditions were 94°C for 3 mins, 40 cycles of: 94°C for 1min, 50°C for 1 mins, 72°C for 1 min; with a final extension step of 72°C for 7 mins. Products were then subjected to agarose gel electrophoresis. Positive bands were expected at 449bp for adenovirus.

Individual RT-PCR For Rotavirus

10ul of cDNA was added to a 50ul reaction containing 10xPCR buffer (100mM tris-HCl, 15mM MgCl₂, 500mM KCl), 0.2mM of each dNTP, 0.2mM of each primer con1 and con2 (Gentsch *et al*, 1992; Rasool *et al*, 2002). A mixture of 2U of Taq DNA polymerase, 0.028uM TaqStart antibody™ (Clontech Laboratories, Paolo Alto, USA) and 4xTaqStart antibody dilution buffer (Clontech Laboratories, Paolo Alto, USA) was incubated at room temperature for 10 minutes before being added to the final reaction mixture. PCR conditions were 94°C for 5 mins, 40 cycles of: 94°C for 30 secs, 42°C for 1 minute, 72°C for 1 minute 30 secs; with a final step of 72°C for 7 mins. Products were then subjected to agarose gel electrophoresis. Positive bands were expected at 220bp for rotavirus.

Individual RT-PCR For Sapovirus

10ul of cDNA was added to a 50ul reaction containing 10xPCR buffer (100mM tris-HCl, 15mM MgCl₂, 500mM KCl), 0.2mM of each dNTP, 0.2mM of

primers JV66 and SR80 for sapovirus (Vinje *et al*, 2000). A mixture of 2U of Taq DNA polymerase, 0.028uM TaqStart antibody (Clontech Laboratories, Paolo Alto, USA), and 4xTaqStart antibody dilution buffer (Clontech Laboratories, Paolo Alto, USA) was incubated at room temperature for 10 minutes before being added to the final reaction mixture. PCR conditions were 94°C for 3 mins, 40 cycles of: 94°C for 1 min, 37°C for 1 min 30 secs, 74°C for 1 minute; with a final extension step of 72°C for 7 min. Products were then subjected to agarose gel electrophoresis. Positive bands were expected at 349bp for sapovirus.

4.4.1.3. Results

Results for the dilution series (Table 19) showed significant reductions in the end point detection limits for astrovirus, rotavirus and sapovirus the multiplex version of the assay. In fact, the multiplex assay failed to detect rotavirus at any of the concentrations tested.

Assuming that these decreases in sensitivity were due to cross-reactions between the primers for each pathogen, each set of primers was removed in turn from the multiplex assay in order to investigate the source(s) of the problem. All other constituents and parameters in the multiplex assay remained the same.

Removing the adenovirus PCR assay from the multiplex RT-PCR assay did not improve the end point detection limit of the astrovirus, rotavirus or sapovirus RT-PCR tests (Table 20). Similarly, removing the astrovirus RT-PCR and did not improve the rotavirus and sapovirus RT-PCR (Table 21). However, removing the rotavirus RT-PCR resulted in the remaining multiplex RT-PCR assay producing similar levels of sensitivity to the corresponding individual PCR assays (Table 22).

Table 19: Comparison of multiplex PCR for adenovirus, astrovirus, rotavirus and sapovirus with singleton assays on dilution series.

Dilution	Adenovirus		Astrovirus		Rotavirus		Sapovirus	
	Multi Result	Single Result	Multi Result	Single Result	Multi Result	Single Result	Multi Result	Single Result
10 ⁻¹	+	+	+	+	-	+	+	+
10 ⁻²	+	+	-	+	-	+	+	+
10 ⁻³	+	+	-	-	-	+	-	+
10 ⁻⁴	+	+	-	-	-	-	-	-
10 ⁻⁵	-	-	-	-	-	-	-	-

Table 20: Comparison of multiplex PCR for astrovirus, rotavirus and sapovirus with singleton assays on dilution series.

Dilution	Astrovirus		Rotavirus		Sapovirus	
	Multi Result	Single Result	Multi Result	Single Result	Multi Result	Single Result
10 ⁻¹	+	+	-	+	+	+
10 ⁻²	-	+	-	+	+	+
10 ⁻³	-	-	-	+	-	+
10 ⁻⁴	-	-	-	-	-	-
10 ⁻⁵	-	-	-	-	-	-

Table 21: Comparison of multiplex PCR for adenovirus, rotavirus and sapovirus with singleton assays on dilution series.

Dilution	Adenovirus		Rotavirus		Sapovirus	
	Multi Result	Single Result	Multi Result	Single Result	Multi Result	Single Result
10 ⁻¹	+	+	-	+	+	+
10 ⁻²	+	+	-	+	+	+
10 ⁻³	+	+	-	+	-	+
10 ⁻⁴	+	+	-	-	-	-
10 ⁻⁵	-	-	-	-	-	-

Table 22: Comparison of multiplex PCR for adenovirus, astrovirus and sapovirus with singleton assays on dilution series.

Dilution	Adenovirus		Astrovirus		Sapovirus	
	Multi Result	Single Result	Multi Result	Single Result	Multi Result	Single Result
10 ⁻¹	+	+	+	+	+	+
10 ⁻²	+	+	+	+	+	+
10 ⁻³	+	+	-	-	+	+
10 ⁻⁴	+	+	-	-	-	-
10 ⁻⁵	-	-	-	-	-	-

When tested using the 74 clinical samples, moreover, this version of the multiplex assay detected all cases of adenovirus, astrovirus and sapovirus in the panel. In addition, it did not detect any samples in the specificity panel as positive and hence was shown to be specific.

In all versions of the multiplex assay the inclusion of an RT step was shown to have no effect on the sensitivity of the adenovirus detection. The data also showed that changing the original annealing temperature as part of the multiplexing process had little effect on the individual assays' sensitivity.

4.4.1.4. Study Conclusions

The results from this experiment showed that it was not possible to multiplex all four assays without seriously affecting their sensitivity. Instead, the four chosen PCR assays were reduced to two tests: a single RT-PCR assay for rotavirus and a novel multiplex assay for the detection of adenovirus, astrovirus and sapovirus. Although not as efficient as a single four-way test, this still represents a saving in the cost and rapidity of testing in comparison to four separate tests since fewer steps will be carried out, less reagents will be used and less technician time is required. However, on balance it was felt that the benefits of a dual PCR test solution were not enough to warrant the replacement of the EM service for sporadic IID at this stage.

4.4.2. Developed Gel-Based PCR Methods Vs EIA Methods

As described earlier, EIA methods are popular assays in laboratories without access to EM or PCR. Studies have shown these to be similar in sensitivity to EM (Giordano *et al*, 2005; Rabenau *et al*, 1998; Putzker *et al*, 2000) although few studies exist comparing them to PCR methods. The advantage of such methods is that they are considered simple to implement, easy to use and are rapid and high throughput.

The aim of this second study, therefore, was to measure the sensitivity of commercial EIA methods in comparison to the RT-PCR assay for rotavirus and the multiplex RT-PCR for adenovirus, astrovirus and sapovirus developed in the previous experiment. Should the EIA methods prove as sensitive as the PCR assays, or more so, this and the relative ease of their implementation in comparison to a dual PCR solution would mean these were potential candidates as the replacement for EM in the sporadic service.

The multiplex RT-PCR assay was compared to an EIA for astrovirus, whilst the rotavirus RT-PCR was compared to both an ICG and an EIA for the detection of rotavirus. EIA methods for the detection of adenovirus and sapovirus were not included in the comparison since an adenovirus EIA was not available in the laboratory during the period of study and a commercial sapovirus EIA had yet to be developed. Similarly, an ICG test for astrovirus was not available in the laboratory during this time. Both types of test were assessed using faecal samples taken from children with clinically diagnosed IID.

Subsequently, in 2007, all samples for which the two tests produced different results were re-tested using the dual-labelled probe-based real-time RT-PCR assays

described in section 3.4.2.3 of Chapter 3 once these became available. These are useful methods for confirmation since they target *alternative* regions of the rotavirus and astrovirus genome (Pang *et al*, 2004; Le Cann *et al*, 2004) to the gel-based methods. This extra testing was carried out in order to confirm the initial results obtained in 2004 and to ensure that the correct service decisions were made regarding the routine diagnostic service.

4.4.2.1. Methods

Sample Panels

Comparison Of Rotavirus RT-PCR With Commercial EIA And ICG

The single gel-based RT-PCR assay was compared with a commercial ICG and EIA on 107 faecal samples collected from children under 5 years old with sporadic IID. All samples were from patients clinically diagnosed as having gastroenteritis (as shown on the specimen request forms) and were submitted to Monklands District General Hospital (MDGH), Lanarkshire between February and April 2002. All samples had initially been tested at MDGH using an ICG test for rotavirus (Rotascreen Dipstick, Microgen Bio Products). Samples were then immediately sent to the WOSSVC and tested using both the RT-PCR and a commonly used EIA (Rotascreen II EIA, Microgen Bio Products). All samples were tested within 7 days of collection from the patient in line with the EIA manufacturer's requirements. Negative (VTM) PCR controls were included in each PCR run.

Comparison Of Multiplex RT-PCR With Commercial EIA For Astrovirus

In total, 48 faecal samples were used to compare the EIA with the multiplex RT-PCR for astrovirus, adenovirus and sapovirus. All samples had originally been sent to Monklands District General Hospital (MDGH), Lanarkshire in February to April 2002, from children under 5 years old with clinically diagnosed community managed gastroenteritis. All samples had tested negative for rotavirus (using ICG) and for other microbiological pathogens at the MDGH. They had also tested negative at the WOSSVC for adenovirus, rotavirus and sapovirus using the singleton gel-based RT-PCR assays described earlier and norovirus using the Lightcycler™ RT-PCR test. All samples were tested within 7 days of collection from the patient in line with the EIA manufacturer's requirements. Negative (VTM) PCR controls were included in each PCR run to control for test contamination.

4.4.2.2. Laboratory Methods

All samples to be tested using both the rotavirus RT-PCR and the multiplex RT-PCR were processed using the faecal extraction and nucleic acid extraction protocols outlined previously in Chapter 3 (Section 3.4.1.1 and 3.3.1.2). The rotavirus RT-PCR is described previously (section 4.4.1.1) and the multiplex developed in the previous experiment is described below. The real-time PCR methods for rotavirus and astrovirus are also described in Chapter 3 (section 3.4.2.3).

Multiplex RT-PCR For Adenovirus, Astrovirus And Sapovirus

10ul of cDNA was added to a 50ul reaction containing 10xPCR buffer (100mM tris-HCl, 15mM MgCl₂, 500mM KCl), 0.2mM of each dNTP, 0.2mM of each set of primers for each of the different pathogens. A mixture of 2U of Taq DNA polymerase, 0.028uM TaqStart antibody (Clontech Laboratories, Palo Alto, USA), and 4xTaqStart antibody dilution buffer (Clontech Laboratories, Palo Alto, USA) was incubated at room temperature for 10 minutes before being added to the final reaction mixture. PCR conditions were 94°C for 3 mins, 40 cycles of: 94°C for 30 secs, 47°C for 40 secs, 72°C for 30 secs; with a final extension step of 72°C for 7 min. Products were then subjected to agarose gel electrophoresis. Positive bands were expected at 140bp for adenovirus, 449bp for astrovirus and 349bp for sapovirus.

Commercial EIA Assay for Rotavirus (Microgen)

The commercial antigen detection assay used for rotavirus was the Rotascreen II EIA test supplied by Microgen Bio-Products, Camberley, Surrey, UK. Before carrying out the EIA test all reagents were made up as per the manufacturer's instructions. The commercial EIA was then carried out according to its manufacturer's instructions (Microgen Bio-Products, Camberley, Surrey, UK). Briefly, approximately 0.1g of faecal specimens was added to 1ml of sample diluent and mixed thoroughly. The appropriate number of microtitre wells for samples and controls was selected from the kit. 50ul of positive control was then added to the appropriate positive control well. 50ul of negative control was added to the appropriate negative control well. 50ul of the sample/s were then added to the appropriate wells. 50ul of conjugate was then added to each well. The plate was then incubated at room temperature for 30mins. The wells were then washed three times using the wash buffer. 100ul of substrate was then added to all wells before a second incubation step at room temperature for 20mins. 100ul of stop solution was then

added to each well. The wells were read at absorbance 450nm. According to the manufacturer's instructions, the positive control must have an absorbance value of more than 0.6 A_{450} units. The negative control must have an absorbance value of less than 0.15 A_{450} units. Samples are regarded as positive if the A_{450} value is greater than the A_{450} value of the negative control plus 0.1 absorbance units. It is recommended that faecal samples giving absorbance values within 10% of the cut off should be retested.

Commercial EIA For Astrovirus

The EIA used for astrovirus used an amplified IDEIA test (DAKO Cytomation, Cambridgeshire, UK) and was carried out in accordance with the manufacturer's instructions. Approximately 0.1g of faecal specimens were added to 1ml of sample diluent and mixed thoroughly. The appropriate number of microtitre wells was selected from the kit. 100ul of positive control was added to the appropriate positive control well. 100ul of negative control was added to the appropriate negative controlwell. 100ul of clinical sample was added to the appropriate well. 100ul of conjugate was then added to all wells. Wells were incubated at 25°C for 60 minutes and then washed five times using wash buffer. 100ul of substrate was then added to each well and incubated at 25°C for 10 minutes. 100ul of stopping solution was added. Wells were read photometrically at 450nm. According to manufacturers instructions the positive control must have an absorbance value of more than 0.5 absorbance units. Negative controls should have absorbance values of less than 0.15 absorbance units. Cut off values are calculated by adding 0.1 absorbance units to the negative control value. Positive samples must have absorbance units above the cut off value. A result within 0.1 absorbance units of the cut off value should be interpreted cautiously taking into account the clinical and epidemiological data.

4.4.2.3. Results

Comparison Of Rotavirus RT-PCR With EIA And ICG

The RT-PCR assay detected rotavirus in 58 samples whereas the ICG detected it in 29 and the EIA in 28 (Table 23-Table 25). Rotavirus was detected in the same 17 samples by both antigen detection methods. These, plus all other EIA and ICG positive results were confirmed using the RT-PCR. All negative controls tested negative using the RT-PCR assay and the EIA and ICG methods. In 2007, all of the

RT-PCR positive results were confirmed as positive using the dual-labelled probe-based RT-PCR.

Table 23: Comparison of RT-PCR and EIA for the detection of rotavirus.

	RT-PCR	
EIA	Positive	Negative
Positive	28	0
Negative	30	49

Table 24: Comparison of RT-PCR and ICG for the detection of rotavirus.

	RT-PCR	
ICG	Positive	Negative
Positive	29	0
Negative	29	49

Table 25: Comparison of ICG and EIA for the detection of rotavirus.

	EIA	
ICG	Positive	Negative
Positive	17	12
Negative	11	67

Comparison Of Multiplex RT-PCR With Commercial EIA For Astrovirus

Using the multiplex RT-PCR, initial testing of the sample panel provided results with numerous non-specific bands that could not be read with confidence (Figure 13). At this stage it was not clear if this were due to human error or a more serious problem with the robustness of the multiplex RT-PCR. Repeat testing rectified the problem so that the results could be examined.

Following repeat testing the multiplex RT-PCR detected astrovirus in 18 samples compared to 8 that tested positive using the EIA (Table 26). Astrovirus was detected by both tests in 7 cases. The other sample that tested positive for astrovirus using the EIA tested negative using the multiplex RT-PCR. 29 samples tested negative for astrovirus using both assays. No other viral causes of IID were detected in these samples using the multiplex RT-PCR, confirming the findings of the singleton assays and the Lightcycler™ RT-PCR. All extra RT-PCR positive results were confirmed as positive using the dual-labelled probe-based RT-PCR. The sample that tested positive for astrovirus using the EIA assay but negative using the multiplex RT-PCR was also found to be negative using the dual-labelled probe-based assay.

Figure 13: Example Of Non-Specific Banding Patterns Observed With The Multiplex RT-PCR.

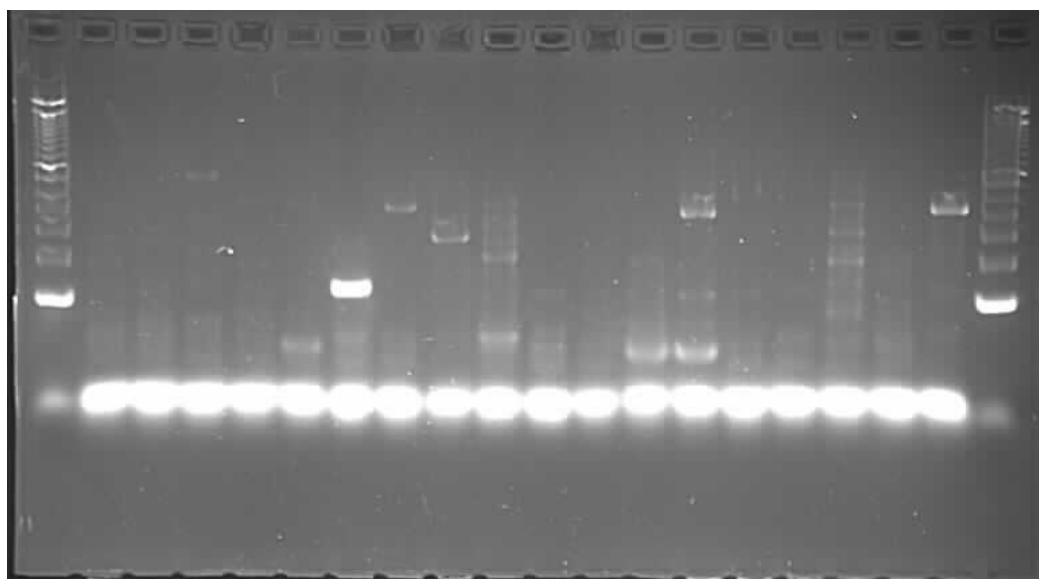


Table 26: Comparison of RT-PCR And EIA For The Detection Of Astrovirus.

EIA	RT-PCR	
	Positive	Negative
Positive	7	1
Negative	11	29

4.4.2.4. Study Conclusions

The results showed that both the rotavirus and the multiplex RT-PCR methods were more sensitive than either of the antigen detection methods examined (Gunson *et al*, 2003(iii); Gunson *et al*, 2003(iv)). Both detected the relevant pathogen in more samples than the antigen detection methods (The improved sensitivity of these assays was confirmed by re-testing the extra positive samples using real-time PCR in 2007). These results are similar to those found for the assessment of the norovirus EIA methods in the previous section.

The poorer sensitivity of the EIA methods meant these were rejected as replacements for the existing EM-based service for sporadic cases of IID. However, the numerous steps involved in a dual PCR solution together with concerns over the robustness of the multiplex RT-PCR (since repeat testing was required in this experiment to establish clear result bands) meant that, at this stage, the decision was also taken not to proceed with the implementation of the gel-based assays as the frontline tests for sporadic cases of IID. EM remained the test employed in routine

service until 2004, when dual-labelled probe-based technology was introduced. This is described in the following section.

4.4.3. Developed Gel-Based PCR Methods Vs Dual-Labelled Probe-Based Real-Time PCR Methods

As mentioned earlier, in 2003 the WOSSVC implemented a number of PCR platforms that could support real-time chemistries such as dual-labelled probes.

This introduced the possibility of implementing real-time PCR tests for adenovirus, astrovirus, rotavirus and sapovirus in the diagnostic service for sporadic cases of IID, a two test gel-based PCR solution having previously been rejected on the basis of involving too many steps. Commercial antigen detection methods had also been rejected due to their limited sensitivity.

The aim of the third experiment in this series, therefore, was to assess the sensitivity of the real-time assays for adenovirus, astrovirus, rotavirus and sapovirus selected and developed as described in Chapter 3 in comparison to the two gel-based PCR tests. The assays were compared using a number of dilution series and a panel of 322 stool samples taken from children with clinically diagnosed sporadic IID. The dual-labelled probe-based assays were also assessed using the sample specificity panel described earlier.

In 2007, all samples for which the two test types of produced different results were re-tested using a number of gel-based PCR methods. These methods were chosen because they targeted alternative regions of the viral genome to that targeted by the real-time PCR methods. In addition some of these were nested protocols - a type of assay that, although rejected for routine use due to the numerous steps involved, has been shown to be sensitive. Although confirmatory testing had no effect on the service decisions made at the time, it provided reassurance that the correct decisions were taken.

All discrepant adenovirus results were tested using the assay described by Xu *et al* (Xu *et al*, 1990). This is a universal adenovirus test that targets an alternative part of the hexon region to the others under examination. For astrovirus discrepant results, a hemi nested RT-PCR was used that targets the capsid region (Gallimore *et al*, 2005). The assay uses the same primers (mon 269 and mon 270) as described by Noel *et al* in the first round and uses an adapted mon 269 as the internal primer in the second nested step. This assay had already been shown to be more sensitive than the single

round RT-PCR described by Noel *et al.* For rotavirus, all discrepant results were tested using the nested RT-PCR described by DiStefano *et al.* (Distefano *et al.*, 2005). This assay targets the VP7 region of the rotavirus genome. Finally for all sapovirus discrepant results, the universal nested RT-PCR described by Okada *et al.* was used (Okada *et al.*, 2006). This assay targeted both the RdRp and Capsid region.

4.4.3.1. Methods

Sample Panels

Specificity Panel

The same specificity panel as used in section 4.4.1.1 was used to assess the specificity of the dual-labelled probe-based real-time PCR methods.

Dilution Series

In order to establish end point detection limits of each test 8 separate dilution series were tested. The following pathogens were represented (each in a separate dilution series): rotavirus types G1 and G2; adenovirus types 40 and 41; astrovirus types 1 and 3 and sapovirus types G1 (Sapporo/82/JP U65427) and G2 (Bristol/98/UK AJ249939).

Clinical Samples

A total of 322 faecal specimens were collected from children (aged less than 5 years) with clinically diagnosed gastroenteritis. All samples were submitted to Monklands General District Hospital (MDGH), Lanarkshire by GPs in the Lanarkshire area between December 2000 and March 2001. Samples were subsequently sent to the WOSSVC for PCR testing. The first phase of the study tested all samples using the developed gel-based RT-PCR for rotavirus, the Lightcycler™ RT-PCR for norovirus, and the gel-based multiplex RT-PCR assay for adenovirus, astrovirus, and sapovirus. Samples were then stored at -70°C . Subsequently (in 2004) all samples were tested using the dual-labelled probe-based real-time PCR for adenovirus, astrovirus, rotavirus and sapovirus described in Chapter 3. Following this second testing, samples were stored at -70°C before confirmatory testing in 2007.

4.4.3.2. Laboratory Methods

All samples to be tested using the gel-based and real-time PCR methods were processed using the faecal extraction and nucleic acid extraction procedures outlined previously. The gel-based RT-PCR technique for the detection of rotavirus and the

multiplex RT-PCR assay for astrovirus, adenovirus and sapovirus are described in the previous experiment. The dual-labelled probe-based methods for adenovirus, astrovirus, rotavirus and sapovirus are described in Chapter 3 (section 3.4.2.3).

Confirmation PCR Methods

PCR For Adenovirus

Extracted DNA (25ul) was amplified using a 25ul volume of the Qiagen PCR master mix (Qiagen, Crawley, UK) using primers AD1 and AD2 (both at 50 pmoles/ μ l per reaction). The plate was incubated in the Applied Biosystems PCR machine using the programme 94°C for 4 min, 40 cycles of: 94°C for 1 min, 54°C for 45 sec and 72°C for 2 min; followed by a hold step of 72°C for 10 min. Products were visualised using agarose gel electrophoresis. For the hexon assay a PCR product of 482bp was expected if adenovirus was successfully amplified from the sample.

Nested RT-PCR For Astrovirus

Extracted RNA (12.5ul) was amplified using a combined one step RT-PCR kit (Qiagen, Crawley, UK) using primers Mon 269 and Mon 270 (25 pmoles/ reaction). The plate was incubated in the Applied Biosystems PCR machine using the programme 50°C for 30 min, 95°C for 15 min and 35 cycles of: 95°C for 40 sec, 45°C for 40 sec and 72°C for 1 min. 2ul of first round product was transferred to a 48 ul second round reaction (Qiagen) containing primers mon269N (an adapted primer internal to mon 269) and mon270 (50 pmoles/reaction). This was then subjected to 40 cycles of: 94°C for 40 sec, 45°C for 25 sec and 72°C for 1 min 30 sec. This was followed by a hold step 72°C for 10 min. Products were visualised using agarose gel electrophoresis. A PCR product of 424bp was expected if astrovirus was successfully amplified from the sample.

Nested RT-PCR For Rotavirus

Extracted RNA (12.5.ul) was amplified using a combined one step RT-PCR kit (Qiagen, Crawley, UK) and the published forward and reverse primers (25 pmoles/ reaction). The plate was incubated in the Applied Biosystems PCR machine using the programme 50°C for 30 min, 95°C for 15 min and 35 cycles of: 95°C for 40 sec and 45°C for 40 sec. This was followed by 72°C for 1 min. 2ul of first round product was then transferred to a 48 ul second round reaction (Qiagen) containing the published internal primers (50 pmoles/reaction) and subjected to 40 cycles of: 94°C for 40 sec, 45°C for 25 sec and 72°C for 1 min 30 sec. This was followed by a hold step of 72°C

for 10 min. Products were visualised using agarose gel electrophoresis. A PCR product of 365bp was expected if rotavirus was successfully amplified from the sample.

Nested RT-PCR For Sapovirus

Extracted RNA (12.5.µl) was amplified using a combined one step RT-PCR kit (Qiagen, Crawley, UK) using primers F13/14 and R13/14 (25 pmoles/reaction). The plate was incubated in the Applied Biosystems PCR machine using 50°C for 30 min, 95°C for 15 min and 25 cycles of: 95°C for 40 sec, 45°C for 40 sec and 72°C for 1 min. 2µl of first round product was then transferred to a 48 µl second round reaction (Qiagen) containing primers F22 and R2 (50 pmoles/reaction) and subjected to 40 cycles of: 94°C for 40 sec, 45°C for 25 sec and 72°C for 1 min 30 sec. This was followed by a hold step of 72°C for 10 min. Products were visualised using agarose gel electrophoresis. A PCR product of 420bp was expected if sapovirus was successfully amplified from the sample.

4.4.3.3. Results

None of the real-time assays detected any of the specificity panel as positive indicating that they were specific.

For each of the dilution series tested the real-time PCR methods were shown to have a lower limit of detection than the conventional gel-based (Table 27-Table 30). For both rotavirus types the real-time RT-PCR assay was shown to be approximately 3 log more sensitive. The improvement was by a factor of 1-2 log for the real-time RT-PCR for astrovirus types 1 and 3, and for the adenovirus real-time PCR it was 1 log. The sapovirus real-time RT-PCR was more sensitive on the G1.1 dilution series by approximately 2 log (Gunson, Collins and Carman, 2006(i)). However, it failed to detect any of the G2.1 dilutions. This was as anticipated based on the developmental data reported in Chapter 3, where sequence alignment showed the test was unlikely to detect G2 types.

Results from testing the sample panel showed that the gel-based RT-PCR assays detected one or more pathogens in 209 (65.0%) of the 322 faecal samples investigated (Table 31). All of these positive results were also detected using the real-time dual-labelled probe-based methods. In addition, a further 36 viral pathogens were detected using the real-time methods. In total therefore, the real-time PCR methods detected viral pathogens in 245 samples (76.1%). A viral pathogen could not be

detected in 77 faecal specimens using these methods. Greater numbers of positive detections were observed for all of the individual real-time PCR tests in comparison to the conventional gel-based PCR assays. The greatest increase in detections was observed for the rotavirus, adenovirus and astrovirus tests. A smaller number of extra positive detections was observed for the dual-labelled probe-based assay for sapovirus. In 2007, upon re-testing with the gel-based confirmatory assays, all the extra real-time PCR results were confirmed as positive.

Table 27: Comparison of real-time PCR for adenovirus with multiplex RT-PCR assay on an adenovirus dilution series.

Dilution	Real-Time PCR Result		Multiplex RT-PCR Result	
	Ad40	Ad41	Ad40	Ad41
10 ⁻¹	+	+	+	+
10 ⁻²	+	+	+	+
10 ⁻³	+	+	+	+
10 ⁻⁴	+	+	+	+
10 ⁻⁵	+	+	+	-
10 ⁻⁶	+	-	-	-
10 ⁻⁷	-	-	-	-

Table 28: Comparison of real-time RT-PCR for astrovirus with multiplex RT-PCR assay on an astrovirus dilution series.

Dilution	Reat-Time RT-PCR Result		Multiplex RT-PCR Result	
	Astrovirus Type 1	Astrovirus Type 3	Astrovirus Type 1	Astrovirus Type 3
10 ⁻¹	+	+	+	+
10 ⁻²	+	+	+	+
10 ⁻³	+	+	+	+
10 ⁻⁴	+	+	+	-
10 ⁻⁵	+	+	-	-
10 ⁻⁶	+	-	-	-
10 ⁻⁷	+	-	-	-
10 ⁻⁸	-	-	-	-

Table 29: Comparison of real-time RT-PCR for rotavirus with gel-based RT-PCR on a rotavirus dilution series.

Dilution	Reat-Time RT-PCR Result		Gel-Based RT-PCR Result	
	Rotavirus G1	Rotavirus G2	Rotavirus G1	Rotavirus G2
10 ⁻¹	+	+	+	+
10 ⁻²	+	+	+	+
10 ⁻³	+	+	+	+
10 ⁻⁴	+	+	-	+
10 ⁻⁵	+	+	-	-
10 ⁻⁶	+	+	-	-
10 ⁻⁷	-	+	-	-
10 ⁻⁸	-	-	-	-

Table 30: Comparison of real-time RT-PCR for sapovirus with multiplex RT-PCR assay on a sapovirus dilution series.

Dilution	Reat-Time RT-PCR Result		Multiplex RT-PCR Assay Result	
	Sapovirus G1.1	Sapovirus G2.1	Sapovirus G1.1	Sapovirus G2.1
10 ⁻¹	+	-	+	+
10 ⁻²	+	-	+	+
10 ⁻³	+	-	+	-
10 ⁻⁴	+	-	-	-
10 ⁻⁵	+	-	-	-
10 ⁻⁶	-	-	-	-

Table 31: Comparison of real-time PCR and gel-based PCR detection rates for adenovirus, astrovirus, rotavirus and sapovirus.

Aetiological IID (s)	Gel-Based PCR	Real-Time PCR
Rotavirus	81 (25.2%)	95 (30%)
Astrovirus	70 (21.7%)	82 (25.5%)
Adenovirus	30 (9.3%)	38 (11.8%)
Sapovirus	11 (3.4%)	13 (4.0%)
Rotavirus and astrovirus	8 (2.5%)	8 (2.5%)
Adenovirus and astrovirus	2 (0.6%)	2 (0.6%)
Adenovirus and rotavirus	5 (1.5%)	5 (1.5%)
Sapovirus and rotavirus	1 (0.3%)	1 (0.3%)
Astrovirus, adenovirus and rotavirus	1 (0.3%)	1 (0.3%)
<i>Total viral pathogens detected</i>	<i>209 (65.0%)</i>	<i>245 (76.1%)</i>
Total negative	113 (35.0%)	77 (23.9%)
Total samples	322	322

4.4.3.4. Study Conclusions

The dual-labelled probe-based real-time PCR methods were shown to be specific and more sensitive than their conventional gel-based counterparts. The real-time PCR methods detected more viral pathogens in more samples in the sample panel and had a lower end point detection limit in most of the series tested. The increased detection rate was confirmed by re-testing with gel-based methods in 2007.

The only test where real-time PCR was not found to be an improvement was the dual-labelled probe-based RT-PCR for sapovirus, which failed to detect the G2.1 type. This confirms the development data presented in Chapter 3, which showed that the primers and probe developed were specific to G1 sapoviruses only and would fail to detect sapoviruses from the other genogroups. However, despite this the real-time PCR assay detected more cases of sapovirus in the sample panel than the gel-based RT-PCR, which detects sapoviruses from several genogroups (G1-3). This suggests that G1 types were more common in this panel and that the new dual-labelled probe-based assay was still useful.

The 2-test gel-based solution incorporates more steps than using 4 separate dual-labelled probe-based methods. For example, to test one sample using both the rotavirus RT-PCR and the multiplex RT-PCR would require 7 separate steps (including universal steps for stool extraction, nucleic acid extraction and reverse transcriptase, together with the PCR steps and agarose-gel analysis steps for each assay). To test a single sample using all real-time PCR methods, on the other hand, would require 6 separate steps (including universal steps for stool extraction, nucleic

acid extraction, and four separate RT-PCR steps for each of the 4 assays). Moreover, in the gel-based methods the RT and PCR steps together can take 4-5 hours, compared to 1-1.5 hours using the real-time methods. The agarose gel analysis step in addition can take 2-3 hours, depending on the number of samples under scrutiny

The improved sensitivity of the real-time PCR methods, coupled with the reduced duration and number of steps involved, resulted in these assays being implemented as the frontline tests for the detection of adenovirus, astrovirus, rotavirus and sapovirus sporadic cases of IID in November 2004 (and as the secondary tests for outbreaks of IID that were negative for norovirus). These methods replaced EM, which was retained solely for examining outbreaks classified as negative for all 5 main viral pathogens using the real-time PCR methods.

4.5. Experiments Aimed At Improving the Efficiency and Cost Of Real-Time PCR In The Routine Diagnostic Setting

Based on the research described, from 2004 dual-labelled probe-based real-time PCR methods have been employed at the WOSSVC as the frontline tests for both sporadic cases and outbreaks of IID. In this third section, a number of experiments aimed at reducing the turn-around-time of these real-time PCR methods and increasing sample throughput are described. Some of these methods are also aimed at reducing the cost of carrying out a real-time PCR test. The experiments described examined the effect on test sensitivity of variously reducing the length of PCR parameters; carrying out DNA PCR under RT-PCR conditions; using pooled positive controls; using primer/probe pools and reducing the overall volume of PCR reagents.

4.5.1. Reducing The PCR Cycling Times

Most probe-based real-time PCR assays consist of the following steps: a Taq DNA polymerase activation step of 95°C for 2-15 minutes (depending on the exact Taq DNA polymerase used) and 40-50 cycles of the following steps: a denaturation step of 95°C for 15-30 seconds, and an annealing/extension step of 60°C for 60 seconds. If the target is RNA then a reverse transcriptase step of 50°C for 30 minutes is required prior to these steps. Based on these parameters the overall turn-around-time of most real-time PCR assays ranges from 2-2.5 hours (excluding extraction). Any technique that can be used to reduce this duration without affecting the sensitivity of the test represents a saving, both in terms of the result turn-around-times

and the economic cost of the test (since shorter duration means more efficient use of laboratory equipment, allowing more testing within the working day). The aim of this first experiment, therefore, was to examine whether the PCR cycling times in the implemented tests could be reduced without affecting their sensitivity.

The dual-labelled probe-based real-time PCR methods for astrovirus, adenovirus, norovirus, rotavirus and sapovirus were compared using normal and reduced PCR cycling conditions. As a first approximation the cycling times were reduced by 50%. The reduced PCR parameters consisted of the following: the RT step (where in use) was reduced from 30 minutes to 15 minutes; the denaturation step from 15 seconds to 8 seconds; and the annealing/extension step was altered from 60 seconds to 30 seconds. The Taq DNA polymerase activation step was not changed as it was felt that this would result in sub-optimal Taq DNA polymerase activity and was most likely to reduce sensitivity.

4.5.1.1. Methods

The effect of employing different cycling time parameters was assessed using (10 fold) dilution panels of adenovirus, astrovirus, norovirus, rotavirus and sapovirus positive samples. The cycle thresholds of each dilution and the end point were recorded and compared.

4.5.1.2. Results And Study Conclusions

The results of this study (Table 32) showed that reducing the duration of the reverse transcription, the denaturing and annealing/extension steps by 50% reduced the reaction run time of the assay significantly without any concurrent loss in sensitivity. Overall the reaction run time was reduced to approximately 60 minutes from 100 minutes for DNA real-time PCR, and to 75 minutes from 120 minutes for RNA real-time PCR. As a result, the reduced PCR cycling time parameters were introduced into the routine service in January 2005.

Table 32: Comparison of real-time PCR sensitivity with normal and reduced PCR cycling parameters.

Dilution	Adenovirus Ct Value		Astrovirus Ct Value		Norovirus Ct Value		Rotavirus Ct Value		Sapovirus Ct Value	
	Normal Parameters	Reduced Parameters	Normal Parameters	Reduced Parameters	Normal Parameters	Reduced Parameters	Normal Parameters	Reduced Parameters	Normal Parameters	Reduced Parameters
10 ⁻¹	25.1	25.7	27.1	27.5	22.8	21.9	26.1	25.5	21.7	21.4
10 ⁻²	30.6	29	30.8	30.4	26.3	25.9	28.8	27.5	25.1	24.9
10 ⁻³	33.4	33.6	34.2	34.1	29.8	30.0	32.7	30.4	28.9	29.0
10 ⁻⁴	-	-	-	-	34.8	33.7	-	-	32.8	33.0
10 ⁻⁵	-	-	-	-	-	-	-	-	36.2	37.2
10 ⁻⁶	-	-	-	-	-	-	-	-	-	-
TRT (mins)	100	60	120	75	120	75	120	75	120	75

4.5.2. Simultaneous RNA and DNA Testing

Where purchased from the same supplier, the kits for most DNA and RNA real-time PCR tests contain the same reagents (Taq DNA polymerase, buffers etc.), which has the advantage that the same temperature(s) can be used to activate the enzymes contained in them. Thus, where the PCR parameters are also the same across tests, multiple assays can be processed simultaneously on the same plate. This increases the efficiency of platform use and helps reduce sample turn-around-times.

In the service for sporadic cases of IID, the RNA assays implemented for astrovirus, rotavirus and sapovirus employed the same kit and PCR parameters, and as a result were routinely processed on the same plate to ensure the platform was always used to its maximum capacity. (The norovirus RNA assay employed a different annealing temperature and as a result was always carried out separately.)

The second experiment in this series sought to examine whether the real-time DNA assay for adenovirus could also be carried out on the same plate as these RNA assays. The adenovirus assay also employed the same reagents and PCR conditions as the tests for astrovirus, rotavirus and sapovirus - with the exception that there was no RT step. The RT step in this case involved an extra temperature hold of 15 minutes at 50°C (reduced from 30 minutes following the results of the previous experiment). The aim of this experiment, therefore, was to examine whether the adenovirus assay could be implemented using these (RNA) conditions without affecting its sensitivity, and thus could be carried out at the same time as the RNA assays.

4.5.2.1. Methods

A dilution series of an adenovirus-positive control was tested using the adenovirus PCR under both DNA and RNA conditions (see section 3.4.2.3 in Chapter 3). The endpoint Ct value was recorded and compared.

4.5.2.2. Results And Study Conclusions

The results of this study (Table 33) showed that the adenovirus assay did not suffer any loss in performance when executed using the RNA cycling conditions. As a result, from January 2005, where otherwise the platform would be operating at less than maximum capacity, the adenovirus test was run simultaneously with those for astrovirus, rotavirus and sapovirus under RNA conditions.

Table 33: Comparison of adenovirus real-time PCR under DNA and RNA testing conditions.

Dilution	Adenovirus PCR Ct (Under DNA Conditions)	Adenovirus PCR Ct (Under RNA Conditions)
10 ⁻¹	19.8	20.0
10 ⁻²	23.5	23.7
10 ⁻³	26.5	26.7
10 ⁻⁴	29.9	29.6
10 ⁻⁵	33.0	33.3
10 ⁻⁶	36.7	36.8

4.6. Use Of Pooled Positive Controls

Positive and negative controls are an essential part of any diagnostic service. A positive control i.e. a sample known to be positive for the pathogen of interest (and in the case of real-time PCR tests, with a known Ct value) is included in the process from sample preparation onwards. In a stable system the test result for this control should always be consistent, providing reassurance in each run that the test is working properly. This, in turn, increases the confidence in the negative results provided, indicating that they are not due to a loss in sensitivity of the assay.

In the implemented IID service a positive control was included for each pathogen that samples could potentially be tested for (6 in total including G1 and G2 for norovirus). This meant, however, that 6 of the 96 wells in each nucleic extraction run were required for positive controls alone. Positive controls were also required on the PCR reaction plate(s) for each pathogen tested for on that particular plate.

The aim of this experiment, therefore, was to examine whether all positive controls could be pooled into one tube, thereby freeing up both extraction wells and PCR wells for actual clinical samples. Since extraction costs in particular are a substantial component of the overall test cost this would, if successful, help reduce the cost of the service as well as improving sample throughput and thus turn-around-times.

4.6.1.1. Methods

To make up the pooled control, 6 samples containing variously adenovirus, astrovirus, norovirus (G1 and G2), rotavirus and sapovirus were diluted to give a Ct of between 28-31. From this, the dilution that would give a Ct value approximately 1 log above this value was made. The 6 controls were then added together and the volume made up with VTM to make a 1:10 dilution of this (returning the Ct to between 28-

31). Following this the pooled control was aliquoted into single use vials. In order to test its effectiveness, the pooled control was then repeatedly tested over a period of time using the relevant PCR assays (from sample preparation onwards) to check that the Ct value detected for each pathogen in the pooled control was stable over time. The control was tested on the day of manufacture, at week 4, week 8 and week 24, and when not in use was stored at -70°C .

4.6.1.2. Results And Study Conclusions

Experimental data showed that pooling all positive controls into a single pool resulted in a stable control that could reliably be used to monitor a PCR reaction (Table 34). As a result pooled controls were implemented in the IID service in January 2005.

Table 34: Effect of using pooled positive controls on test sensitivity.

	Adenovirus Result (Ct)	Astrovirus Result (Ct)	Norovirus G1 Result (Ct)	Norovirus G2 Result (Ct)	Rotavirus Result (Ct)	Sapovirus Result (Ct)
Day of Manufacture	29.2	31.2	29.7	31.2	28.2	29.5
Week 4	29.4	31.8	29.9	31.0	28.6	29.4
Week 8	29.1	30.9	28.9	31.2	28.9	29.9
Week 24	30.0	31.4	29.5	31.9	28.3	29.3

4.7. Use of Primer/Probe Pools

All PCR methods utilise a number of reagents, which have to be added together before use. Although the real-time PCR methods implemented in the IID service utilised commercial RNA and DNA kits that have most PCR reagents added together in a ready-to-use mix (including buffer, dNTPs, Mg^{2+} and, in the case of DNA mastermixes, Taq Polymerase), the primers, probe and sometimes water still had to added separately in addition to the sample and, where appropriate, the RT-PCR enzyme. However, the greater the number of pipetting steps there are in a test the more likely it is that an error may occur.

The aim of this experiment, therefore, was to examine whether the necessary primers, probes and water for a given assay could be added together in large quantities in pre-prepared batches and then frozen, stored and defrosted as required without affecting the sensitivity of the test. Batch processing in this way has several potential advantages. First, preparing the mastermix, RT enzyme and primer/probe pools in

large quantities ensures that the concentrations of each are the same between runs (i.e. it facilitates reproducibility). It also reduces the number of steps required in each individual run of an assay, thereby reducing the potential for human error and speeding up the process. The proposed batches were of 2-3 aliquots, each containing enough volume for 24 PCR reactions, and stored at -20°C .

4.7.1.1. Methods

The use of primer/probe pools was tested using the dual-labelled probe-based assays for adenovirus (an example of a DNA test) and astrovirus (an example of an RNA test). First, for each, a positive control was tested using unpooled primers/probes (as in the implemented service) to establish a baseline Ct value for the control. Following this, batches of the appropriate Mastermix (and RT enzyme in the case of astrovirus) were prepared by aliquoting these separately into 24-reaction volumes. The relevant primers, probes and water were then also pooled into 24-reaction aliquots.

To test the effect of pooling on test sensitivity the positive control was then re-tested using the pooled primers/probes to check the Ct value detected was the same as in the original test. The primer/probe pools were then stored at -20°C and the process repeated at week 4, week 6 and week 8 to examine whether the performance of the primer/probe pools was affected by the storage and de-frosting process.

4.7.1.2. Results And Study Conclusions

Examination of the Ct values for the astrovirus and adenovirus controls showed no difference when tested at baseline, day of manufacture, 4, 6 and 8 weeks (Table 35). Use of primer/probe pools did not affect the sensitivity of either test and these were equally effective after storage and defrosting. Based on these results the use of primer/probe pools for all real-time PCR methods was implemented in January 2005.

Table 35: Effect of using primer/probe pools on test sensitivity.

	Adenovirus Result (Ct value)	Astrovirus Result (Ct value)
Before Pooling	28.6	31.5
Day of Manufacture	28.7	31.2
Week 4	29.0	32.2
Week 6	27.9	31.5
Week 8	28.4	30.8

4.7.2. Reducing The PCR Reagent Volume

Most published real-time PCR reactions are carried out in 50ul volumes. This was also the case in the original implementation of the real-time tests used in the new IID service. Reducing this volume could significantly reduce the cost of the test, since more tests could then be carried out per commercial kit. The aim of this study, therefore, was to examine whether reducing the PCR reaction volume would affect test sensitivity.

4.7.2.1. Method

The possibility of reducing reagent volumes was examined using the dual-labelled probe-based assay for adenovirus (as an example of a DNA test) and norovirus types G1 and G2 (an example of an RNA test). For each a positive control was diluted 10 fold. Each dilution series was then tested in triplicate using PCR reaction volumes of 50ul, 25ul and 15ul. The endpoint detection limits of each test using the different volumes were then examined to look for any loss in sensitivity.

4.7.2.2. Results And Study Conclusions

The results of this experiment showed that reducing the PCR reaction volume had no significant effect on the endpoint detection limit of the real-time PCR methods examined (Table 36). As a result reaction volumes in the implemented tests were reduced from 50ul to 25ul in January 2005 and further to 15ul in January 2007 once it was confirmed in routine service that the initial reduction in volume had no detrimental effects.

Table 36: End point detection limits of tests for adenovirus and norovirus using different PCR volumes.

Dilution	Adenovirus Result (Ct)			Norovirus G1 Result (Ct)			Norovirus G2 Result (Ct)		
	50ul	25ul	15ul	50ul	25ul	15ul	50ul	25ul	15ul
10-1	19.2	18.8	19.3	22.1	21.8	22.1	26.2	26.5	25.8
10-2	22.5	21.8	22.0	25.7	25.4	26.0	29.8	30.0	29.5
10-3	26.0	25.8	25.9	29.0	29.1	29.4	33.0	34.2	33.5
10-4	29.3	28.5	29.1	32.4	33.0	33.2	36.2	37.2	38.3
10-5	33.0	32.9	33.3	36.4	37.2	36.8	-	-	-
10-6	37.0	36.2	37.6	-	-	-	-	-	-
10-7	-	-	-	-	-	-	-	-	-

4.8. Summary

This chapter presented a series of studies that assessed the sensitivity of the PCR methods selected and described in Chapter 3 in relation to possible alternatives were adapted for routine diagnostic use. The experiments were split into three parts: those concerned with the development of the norovirus outbreak service, those relating to the sporadic service, and finally, experiments aimed at improving the efficiency of the tests implemented in both services.

Results from the first experiment relating to the outbreak service showed that the SYBR green based Lightcycler™ RT-PCR developed as described in Chapter 3 was more sensitive than EM for the detection of norovirus, in both samples from outbreaks and sporadic cases of IID (Miller, Gunson and Carman, 2002). This method was implemented as the frontline test for outbreak samples in January 2001. It was subsequently compared to two commercial EIA methods for norovirus and was found to be both more sensitive and specific than either (Gunson, Miller and Carman, 2003(ii)). The Lightcycler™ method was also compared to a recommended gel-based RT-PCR that was held to be the most sensitive of the methods available at that time. However, although the Lightcycler™ RT-PCR assay was in fact shown to be less sensitive it did not miss any outbreaks and, moreover, involved fewer steps (Gunson, Miller and Carman, 2003(i)). As a result the decision was taken to retain it as the frontline test for norovirus in the outbreak service at this stage. The final experiment in this series, carried out in 2003 once the technology became available, showed that the new dual-labelled probe-based test for norovirus was also more sensitive than the Lightcycler™ RT-PCR, which in this case missed both individual cases and outbreaks of norovirus (Gunson and Carman, 2005). The dual-labelled probe-based RT-PCR also had the potential to offer better throughput and rapidity. As a result this assay

was implemented in 2004 and remains the frontline test for norovirus in the outbreak service at the time of writing.

The initial experiments relating to the sporadic service focused on multiplexing the individual tests chosen in Chapter 3 for use in a single multiplex assay capable of testing for adenovirus, astrovirus, rotavirus and sapovirus. The result was two gel-based assays: a rotavirus RT-PCR and a multiplex RT-PCR for adenovirus, astrovirus and sapovirus. Rotavirus could not be included in the multiplex since this resulted in a loss in sensitivity in both rotavirus and astrovirus detection. In the second experiment the two assays developed were then compared to antigen detection methods for astrovirus and rotavirus, and shown to be more sensitive than either (Gunson, Collins and Carman, 2006(i)). However, the number and duration of the steps required to carry out the two gel-based assays, plus concerns over the appearance of non-specific products in the multiplex RT-PCR, meant that these were not implemented in the sporadic service at this stage. In the final experiment in this series, again carried out once the necessary technology was available, the two gel-based RT-PCR methods were compared to individual dual-labelled probe-based real-time PCR assays on dilution series and a panel of clinical samples. In each case the dual-labelled probe-based real-time PCR assay detected a lower dilution of pathogen, and detected more clinical samples as positive than the relevant gel-based RT-PCR assay. The dual-labelled probe-based RT-PCR approach also involved fewer steps and had the potential to offer better throughput and rapidity than the EM test still used in the routine service. As a result these assays were implemented alongside the norovirus RT-PCR in November 2004 in the service for sporadic cases of IID.

Once dual-labelled probe-based PCR assays were implemented in both the outbreak and sporadic services a series of experiments aimed at improving the efficiency of these tests was carried out (Gunson, Collins and Carman, 2006(ii)). In the first experiment it was shown that reducing PCR cycling times had no effect on test sensitivity and improved rapidity. The second experiment showed that the DNA test for adenovirus could be carried out under the same conditions (i.e. on the same PCR plate) as any of the RNA tests for astrovirus, rotavirus or sapovirus without affecting its sensitivity (the norovirus assay involved a different annealing temperature to any of the other tests and as a result was always kept separate). Further studies then showed that pooled positive controls, primer/probe pools and reduced

reagent volumes could all be employed without affecting test performance. Use of these measures was therefore implemented in January 2005.

Chapter 5

Effects Of Introducing The PCR Protocols On The Laboratory Diagnostic Service

5.1. Overview

This chapter examines the effects of implementing the PCR assays described in the previous chapter as the frontline tests for the investigation of outbreaks and sporadic cases of gastroenteritis on the laboratory diagnostic service offered by the WOSSVC.

First, the test monitoring system introduced following implementation of the PCR assays is described. The impacts of the changes on the routine laboratory service is then examined through the analysis of the WOSSVC laboratory records from 1999-2006, together with a Scotland-wide survey of laboratory testing protocols for cases of IID, which was first carried out in 2004 and updated in 2006. These data are examined to determine whether the implementation of the new assays provided any benefits to the routine laboratory service in terms of key measures of performance including sample throughput, test sensitivity and turn-around-times. Data on each of these aspects are presented at key stages during the period of change (1999-2006) and the implications for the routine laboratory service are discussed.

5.2. Introduction

As a result of the research described in Chapter 4, several changes were made to the WOSSVC routine laboratory service (these are summarised in Table 37). This chapter examines the effects of these changes on the laboratory service, including the need to implement a test monitoring system, and discusses whether or not the changes have led to benefits in terms of improved sample throughput, test sensitivity and turn-around-times.

Table 37: Summary of testing protocols for outbreaks and sporadic cases of IID in the WOSSVC between 1999-2006.

Year	Outbreaks Of Gastroenteritis	Sporadic Cases Of Gastroenteritis In Children <10 Years And Immunocompromised Patients
Pre-2001	EM on all stool samples.	EM on all stool samples.
2001-2003	Lightcycler™ real-time RT-PCR for norovirus introduced in January 2001. If test result negative, EM.	
2004	Dual-labelled probe-based real-time RT-PCR for norovirus introduced in January 2004. If test result negative, EM.	Dual-labelled probe-based real-time RT-PCR for rotavirus, astrovirus, adenovirus, norovirus and sapovirus introduced in November 2004. Once weekly batch testing used.
2005	Primer/probe pools, reduced cycling conditions, pooled positive controls and reduced PCR reagent volumes introduced in January 2005.	Primer/probe pools, reduced cycling times, pooled positive controls and reduced PCR reagent volumes introduced in January 2005.
2006	If negative, testing for rotavirus, astrovirus, adenovirus and sapovirus using dual-labelled probe-based real-time RT-PCR. If test result negative for all of the above, EM.	Frequency of testing increased to at least three times weekly from January 2006.

5.3. PCR Test Monitoring

Unlike commercial tests such as EIA, PCR assays developed in the laboratory do not have their own quality control systems. As a result, following routine implementation of such assays, it is important to monitor the tests carefully to detect any contamination, or sudden or long-term loss in sensitivity that could lead to false negative or positive results.

The real-time PCR assays implemented in the new diagnostic service for viral causes of IID were monitored in a number of ways. Firstly, positive and negative controls were included in each PCR test. For each assay a single positive control was included to ensure that the entire testing protocol (including the extraction, reverse transcriptase and PCR steps) was optimally sensitive. Initially the positive control used in all the PCR assays described here was a sample that had tested positive for the pathogen in question using EM and had been diluted to the endpoint detection limit of the PCR test. For a run to be passed as valid the positive control had to be detected as positive. If the control was found to be negative then the run had to be repeated. Subsequently, from 2005, all dual-labelled probe-based real-time PCR assays utilised a single positive control containing all viral pathogens to be tested. For a run to be

passed as valid the Ct value of the positive control had to fall within a pre-determined range (plus or minus three standard deviations from the pre-determined mean, which was determined by testing the control at least 20 times). Again, if the control did not fall within the pre-determined range then the run was repeated. The Ct of the positive control was monitored using Westgard rules, which are designed to detect inter-test variation and test drift (Niesters, 2002; Gray *et al*, 1995; Gray, 1999).

All PCR runs also included negative controls, which took the form of a viral transport medium for every eight extractions. Negative controls were included to detect test contamination, which can result in a false positive result. For a PCR run to be passed as valid all negative controls had to test negative using the PCR assays. If any of the negative controls tested positive then all samples and controls had to be re-extracted and the test repeated.

The real-time PCR assays were also monitored to check for the emergence of variant viruses containing nucleotide base changes in the areas of the genome targeted by the primers or probes in the test, and which may result in a loss in test sensitivity. This is a particular problem in the case of norovirus, rotavirus and sapovirus where new variants have been shown to emerge in the past (Lopman *et al*, 2004; Gallimore *et al*, 2006). For norovirus, the number of negative outbreaks occurring over time was measured. An increase in the number of negative outbreaks would suggest that the test had become insensitive and may need to be re-designed or replaced by an alternative test. For the remaining pathogens, this kind of assessment is more difficult and relied upon awareness of the latest developments in the literature.

5.4. Data Collection

5.4.1. WOSSVC Laboratory Data (1999-2006)

All WOSSVC laboratory data for viral gastroenteritis testing from 1999-2006 was transferred from the routine laboratory record database (Telepath) and downloaded into an Excel™ spreadsheet for examination. The data collected for each sample included:

- the patient name and date of birth;
- the date the sample was received in the laboratory and the date the final result was reported (from which the turn-around time can be deduced);
- the sample origin (e.g. GP, hospital, laboratory or health board);

- whether the sample was from a recognised outbreak or was a sporadic case of gastroenteritis (this was determined via the clinical details outlined on the specimen request form);
- the test/s carried out and;
- the final result/s.

Based on these data, the data for outbreak samples and sporadic samples were analysed separately and summary statistics for each year were calculated, including:

- the total number of samples tested;
- the total number of positive detections made;
- the total number of outbreaks confirmed as being caused by norovirus (or another pathogen) and the number of outbreaks classified as negative for all viral pathogens (outbreak samples only);
- the turn-around-times for each test/sample (the time taken in days from when the sample was received in the laboratory and the date the final result was reported);
- the distribution of Health Boards of origin and;
- the age distribution of the patients tested.

These data were then cross-referenced with the changes in the routine service, described above and summarised in **Error! Reference source not found.**, in order to determine the effects of the changes on each of these aspects.

The total number of samples tested each year is used as an indicator of the usefulness of the tests in place, since increased use suggests that clinicians find the test useful. The sample submission data was also compared with the turn-around-times to examine whether changes in the numbers of samples submissions affected test rapidity. Ideally the assays should be able to process large or small numbers of samples with little effect on turn-around-times.

The total number of positive detections achieved for each virus type is a measure of sensitivity, and was measured for both outbreak and sporadic samples each year. A sensitive test is more likely to provide an etiological diagnosis, which in turn will be useful in the clinical and public health setting as the result is more likely to influence patient management and infection control. Measuring the number of laboratory-confirmed positive and negative outbreaks was used as an additional method of assessing test sensitivity. At WOSSVC, an outbreak is confirmed as being caused by a particular pathogen if at least two of the submitted samples from a

particular location are found to be positive for that pathogen using a laboratory method (Richards *et al*, 2003; Duizer *et al*, 2007). On the other hand, for an outbreak to be classified as negative at least six samples from the same outbreak have to be submitted and found to be negative for the pathogen in question. In either case, more than one sample is required before a classification is made in order to reduce the risk of false diagnoses. Outbreak samples that do not meet either set of criteria e.g. because there are too few, remain unclassified. Improvements in test sensitivity should result in increased positive outbreak classifications while ensuring that the number of outbreaks classified as negative remains low.

The effect of each new test or test amendment on test rapidity was measured by measuring the turn-around-times for outbreak and sporadic samples. Ideally turn-around-times should be as short as possible so that the results can usefully influence patient and outbreak management. Test rapidity is particularly important in the outbreak setting.

The sample submission data broken down by health board was examined to determine whether the number of health boards using the WOSSVC service increased in response to service developments (again, an indication that users are finding the service useful).

The age of the patients from which samples were submitted was also examined to determine whether submission patterns had changed in line with the new test developments.

5.4.2. Survey Of Laboratory Methods And Reporting Protocols

In March 2004, as part of a study examining the effect of laboratory protocols on national surveillance systems, a telephone survey was carried out in order to ask laboratories to outline their current testing and reporting protocols for viral gastroenteritis. In total, 22 laboratories were contacted representing all NHS boards in Scotland except Orkney, Shetland, the Western Isles, Fife and the Borders (laboratories from these health boards declined to take part). The questions that were asked are outlined in Table 38.

The survey results were used in addition to the laboratory data to determine how Scottish microbiology laboratories used the WOSSVC diagnostic service for viral IID. This included whether or not any laboratories used the sporadic service offered by the WOSSVC on a routine basis and whether the WOSSVC outbreak

molecular service was used either as the frontline test for outbreaks of IID or as a confirmatory test.

Information on the types of tests used by the laboratories for both sporadic cases and outbreaks of gastroenteritis was then updated in 2006 in order to determine whether changes in the WOSSVC diagnostic service had led to service developments in other laboratories.

Table 38: Survey of laboratory testing protocols for sporadic cases and outbreaks of viral gastroenteritis.

Questions
Do you test for viral pathogens in sporadic cases of IID?
What viral pathogens do you test for in sporadic cases?
What age groups do you test in sporadic cases?
What diagnostic tests do you use in sporadic cases?
What viral pathogens do you test for in outbreaks of viral gastroenteritis?

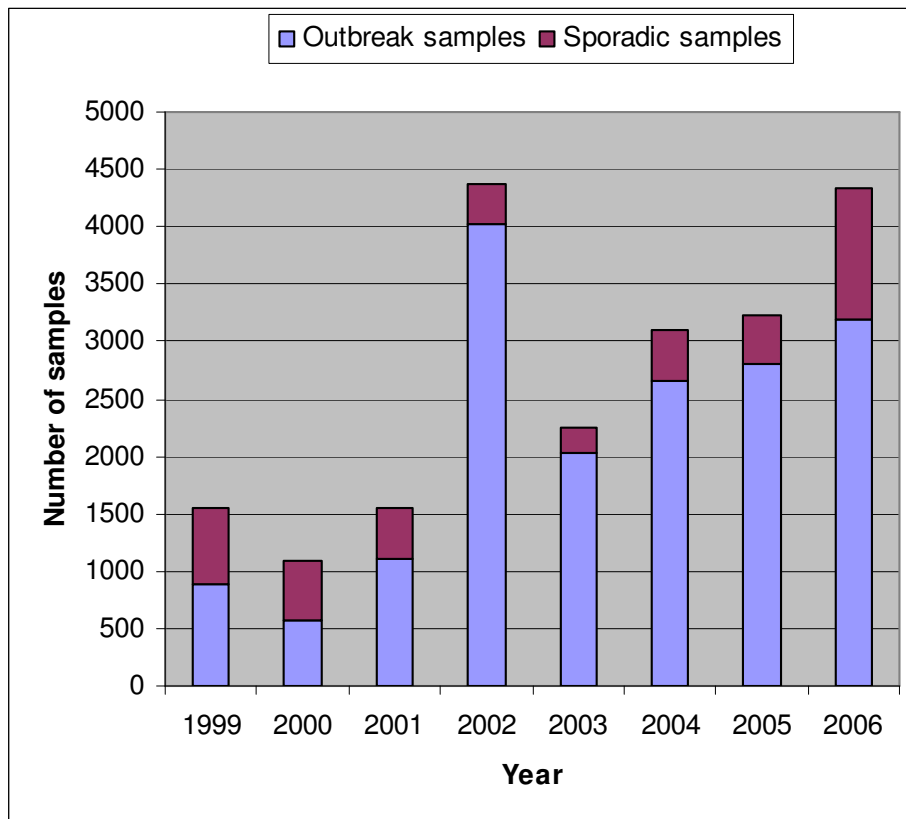
5.5. Results

5.5.1. WOSSVC Laboratory Data 1999-2006

5.5.1.1. Total Number Of Samples Tested

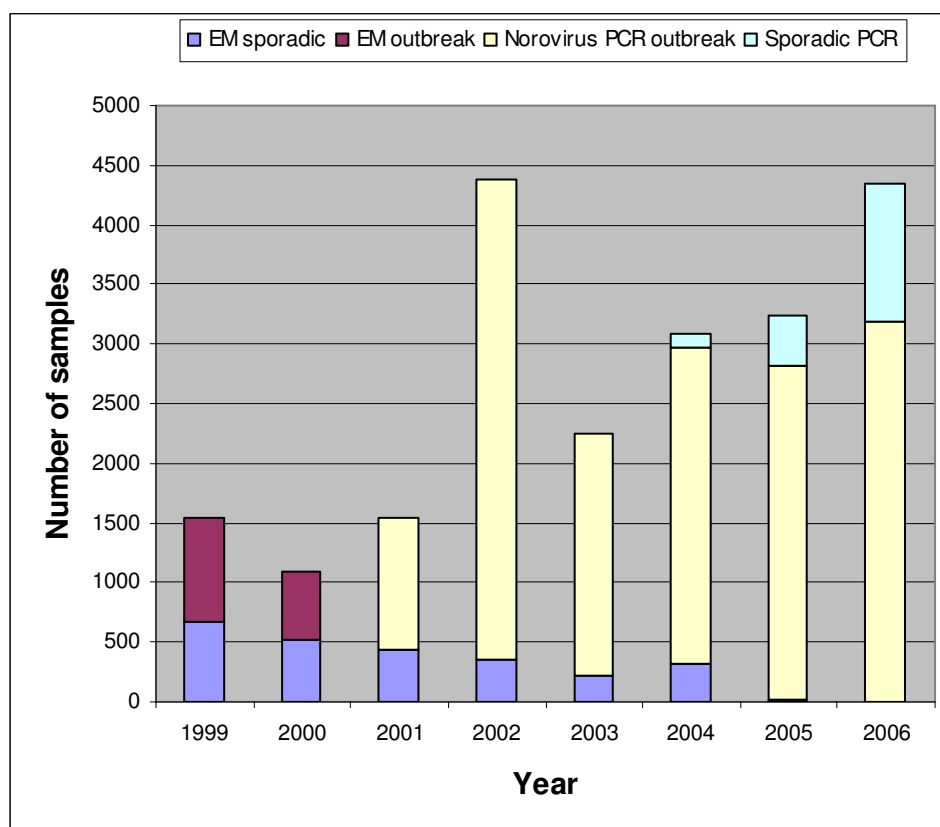
Figure 14 shows the total number of samples tested annually for IID at WOSSVC in the period 1999-2006 (incorporating both sporadic and outbreak cases). Figure 15 shows the same data but with the figures broken down by the test type employed.

Figure 14: Total number of IID samples tested (1999-2006).



From Figure 14 it can be seen that from 2001 the total number of samples tested for IID at the WOSSVC increased, with a sharp peak observed in 2002. This increase in sample numbers is likely to be due to the emergence of a new norovirus variant (see Chapter 2). The increase continued to be seen year on year. Examining this data by sample source (outbreak or sporadic) shows that this increase was largely driven by increases in the number of outbreak samples submitted, which from 2001 made up the majority of the IID samples submitted to the WOSSVC. Samples from sporadic cases of IID were similar in all years examined except 2006 where the number submitted more than doubled compared to previous years. This increase was also due to the emergence of a new norovirus variant (Gallimore *et al*, 2007).

Figure 15: Total number of IID samples tested by test type (1999-2006).



Examining this same data by type of test carried out shows that the increase in submissions of outbreak samples coincided with the implementation of the Lightcycler™ real-time RT-PCR for norovirus as the frontline test for the investigation of samples of this type in 2001 (Figure 15). After its implementation the number of samples tested from outbreaks of gastroenteritis using the Lightcycler™ real-time PCR assay increased in each year. Moreover, the increase in the total number of samples tested continued after the implementation of the dual-labelled probe-based real-time RT-PCR in 2004.

The number of sporadic samples tested by EM remained similar in all years of its use (1999-2003). The molecular service for sporadic samples was implemented in 2004. After its implementation, initially the numbers tested were similar to that tested by EM. However, in 2006 the number of samples tested from sporadic cases rose to over a thousand (N=1147). This was the largest number of submissions in all years of the study. Implementation of the new test for sporadic cases of IID also resulted in a further drop-off in the number of samples tested using EM, since use of this test was now limited to the investigation of PCR negative outbreaks of gastroenteritis.

5.5.1.2. Total Number Of Positive Detections

Figure 16 shows the norovirus detection rate in samples from outbreaks during the period 1999-2006. EM was the test used from 1999-2000, the Lightcycler™ real-time RT-PCR was employed from January 2001, and subsequently, the dual-labelled probe-based real-time PCR was introduced in late 2004. Figure 17 shows the total number of positive viral detections made from sporadic cases of IID between 1999-2006. EM was the frontline test for sporadic cases between 1999 and late 2004 and dual-labelled probe-based real-time PCR assays replaced EM from November 2004 onwards. For each figure the percentages shown were calculated relative to the number of samples tested using this method in each year.

Figure 16: Detection rates for norovirus in outbreak samples (1999-2006).

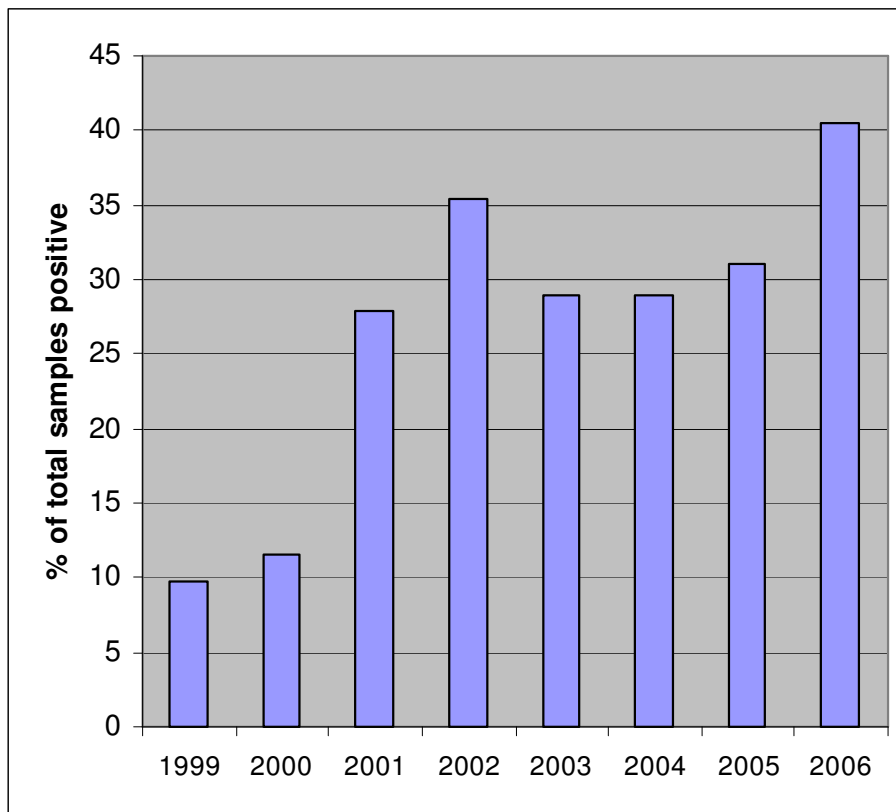
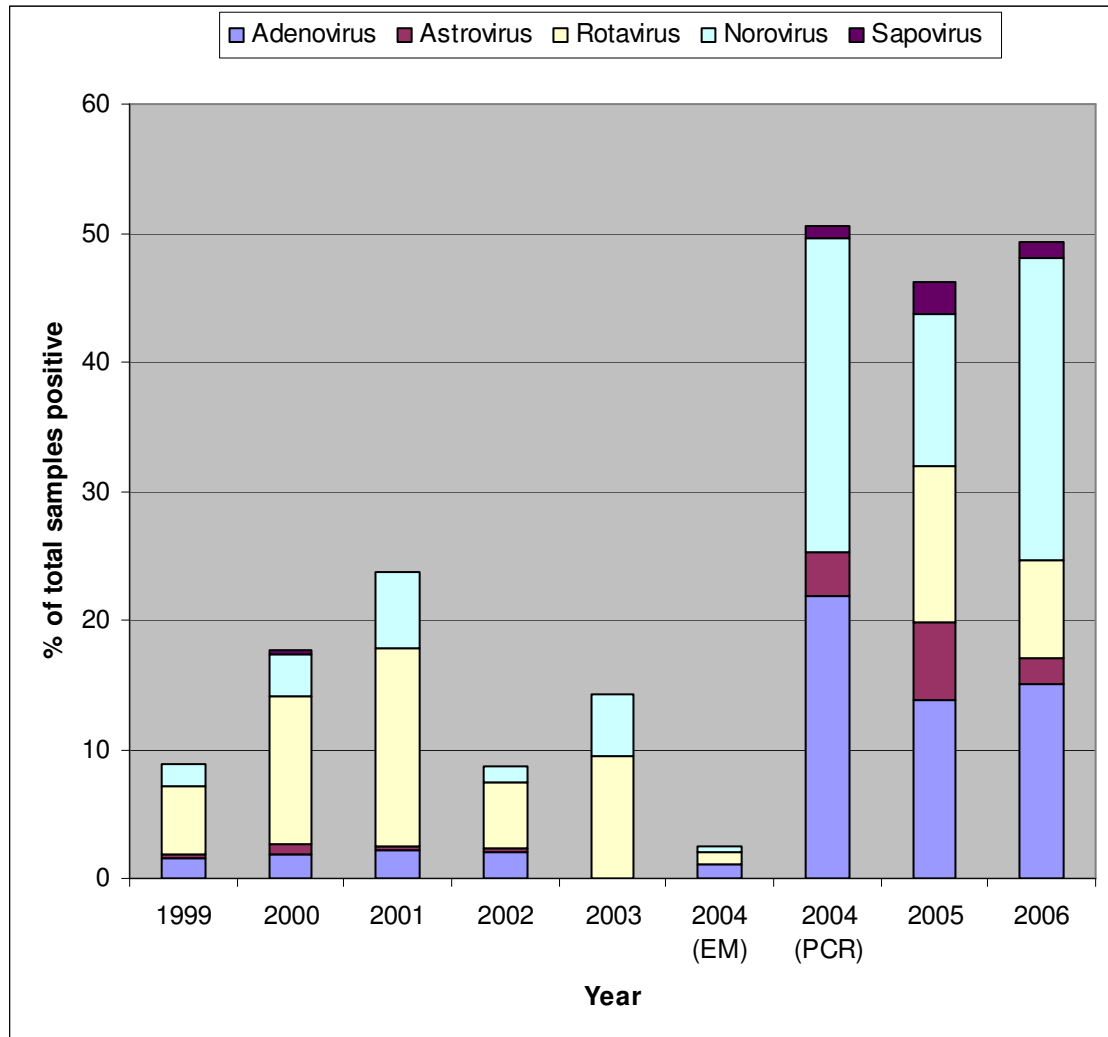


Figure 16 shows that using EM, norovirus was detected in between 10-12% of all outbreak samples submitted. The results obtained when the Lightcycler™ and dual-labelled probe-based real-time RT-PCR were introduced demonstrate a substantial improvement on this detection rate, with norovirus identified in between 28% and 41% of the samples tested. A similar pattern of increased detection was

observed for sporadic cases of IID after the implementation of the molecular service for samples of this type (Figure 17).

Figure 17: Detection rate achieved in sporadic samples.



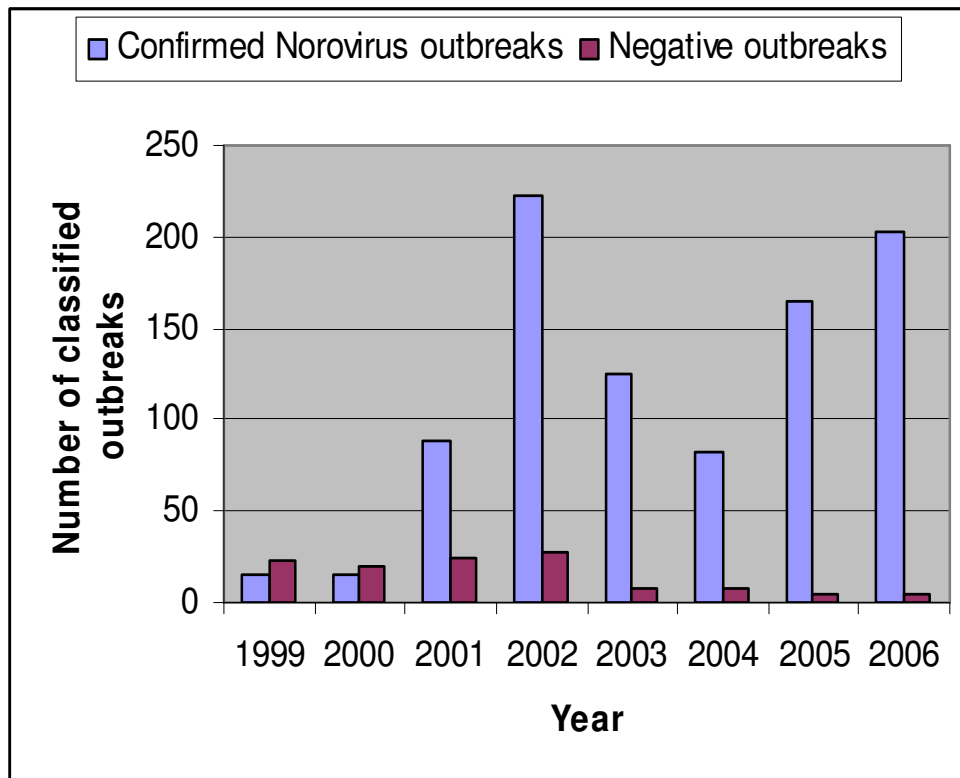
Between 1999 and October 2004 for example, when EM was the frontline test for sporadic cases of IID, adenovirus was detected in less than 2% of all samples tested. When, however, the dual-labelled probe-based real-time PCR service was implemented in November 2004 the detection rate for adenovirus rose to 14-22%. Similarly, after the implementation of the molecular service the detection rate for norovirus in sporadic cases of gastroenteritis increased from 1-7.4% to 12-24%. Increases in the detection rate were also observed for astrovirus and sapovirus. Rotavirus was the only viral pathogen for which the implementation of PCR did not result in a significant improvement in the detection rate. For the years examined the detection rates achieved by EM ranged between 1-15% whereas those achieved by

dual-labelled probe-based real-time RT-PCR were 0-12%. Overall, however, the detection rate in the sporadic setting ranged between 48-52% in the years following implementation of the molecular tests. This is significantly higher than that achieved with EM, where the overall percentage positive rates varied from just 2-23%.

5.5.1.3. Total Number Of Outbreaks Confirmed Caused By Norovirus

Figure 18 shows the number of clinically diagnosed outbreaks of gastroenteritis that were confirmed as being caused by norovirus during the period 1999-2006, together with the number that met the criteria for a norovirus-negative outbreak i.e. could not be attributed to norovirus using the tests employed.

Figure 18: Total number of outbreaks confirmed positive and negative for norovirus.



It can be seen from Figure 18 that the number of outbreaks confirmed as being caused by norovirus increased considerably after the implementation of the Lightcycler™ real-time RT-PCR in 2001. In 1999 and 2000 the numbers of outbreaks confirmed by EM as being caused by norovirus were 15 and 16 respectively. After the introduction of the Lightcycler™ real-time RT-PCR in 2001 the number of norovirus-positive outbreaks detected quickly increased to 83. Moreover, in each of the following five years the number of outbreaks in which norovirus was confirmed as

the cause followed an upward trend and exceeded the level detected previously by EM. Of note is 2002, in which an exceptionally high number of norovirus-positive outbreaks was detected (N=223). This is likely to have been due to the emergence of a new norovirus variant, which in other countries was also associated with a large increase in cases and outbreaks of norovirus (Lopman *et al*, 2004). A large number of norovirus outbreaks was also detected in 2006. As shown earlier, this is also associated with the emergence of a new norovirus G2.4 variant (Gallimore *et al*, 2007).

Figure 18 also shows that the number of outbreaks that were confirmed negative for norovirus has also fallen steadily since the implementation of the LightcyclerTM real-time RT-PCR in 2001. In 1999 and 2000 the number of norovirus-negative outbreaks was 20 and 23 (representing 60% and 55% of all classified outbreaks tested by EM). In 2001 and 2002, the proportion of classified outbreaks that tested negative for norovirus using the LightcyclerTM real-time PCR fell to 21% and 27%, respectively. From 2003 and after the implementation of the dual-labelled probe-based real-time RT-PCR in 2004 the number of norovirus negative outbreaks fell to very low levels. In these years the total number of classified outbreaks that were confirmed negative for norovirus using PCR was between just 4 and 8 (representing between 2-8% of all classified outbreaks tested).

5.5.1.4. Turn-Around-Times

Figure 19 and Figure 20 summarise the WOSSVC turn-around-times for outbreak and sporadic samples between 1999-2006.

It is clear from these figures that the use of real-time RT-PCR has improved the turn-around-times of samples tested both in the outbreak and sporadic setting. For example, when EM was the frontline test for outbreaks of gastroenteritis in 1999 and 2000, 50% of results were reported between 5 to 7 days after arriving in the laboratory, whilst 90% were reported between 11 to 13 days after submission (Figure 19). The implementation of the LightcyclerTM real-time RT-PCR for norovirus from 2001-2003 resulted in a considerable improvement in these turn-around-times, with 50% of results available by day 5 after submission and 90% reported by day 8 in all years (Figure 20). The implementation of the dual-labelled probe-based real-time RT-PCR in 2004 resulted in further improvements, with 50% of results reported just 3 days after submission and 90% of sample results reported by day 6 during this year. A

further improvement in turn-around-times was then again observed in 2005 when primer/probe pools, reduced PCR cycling times, and pooled positive controls were implemented. In this year 50% of results were reported by day 2 and 90% by day 5. This improvement was maintained in 2006.

Examining the turn-around-times for sporadic samples shows that between 1999 and 2004, use of EM resulted in 50% of samples being processed by days 5 to 9, but 90% was not achieved until days 11 to 23. This is longer than that achieved using EM for outbreak samples and was despite the fact that, overall, fewer samples were tested using EM during this period. In 2004, after the implementation of the molecular service, 50% of results were processed by day 3, whilst 90% were reported by day 13. However, these figures were achieved on a very small number of samples (n=119). In 2005, when larger numbers of samples were tested, 50% of results were available by day 9 and 90% by day 23. This was slower than when EM testing was employed. In 2006, however, the numbers of samples of this type submitted to the laboratory increased and as a result batch testing was increased to at least three times a week. As a result the turn-around-times improved substantially, with 50% of results available after day 3 and 90% by day 6.

Figure 19: Turn-around-times achieved for outbreak samples (1999-2006).

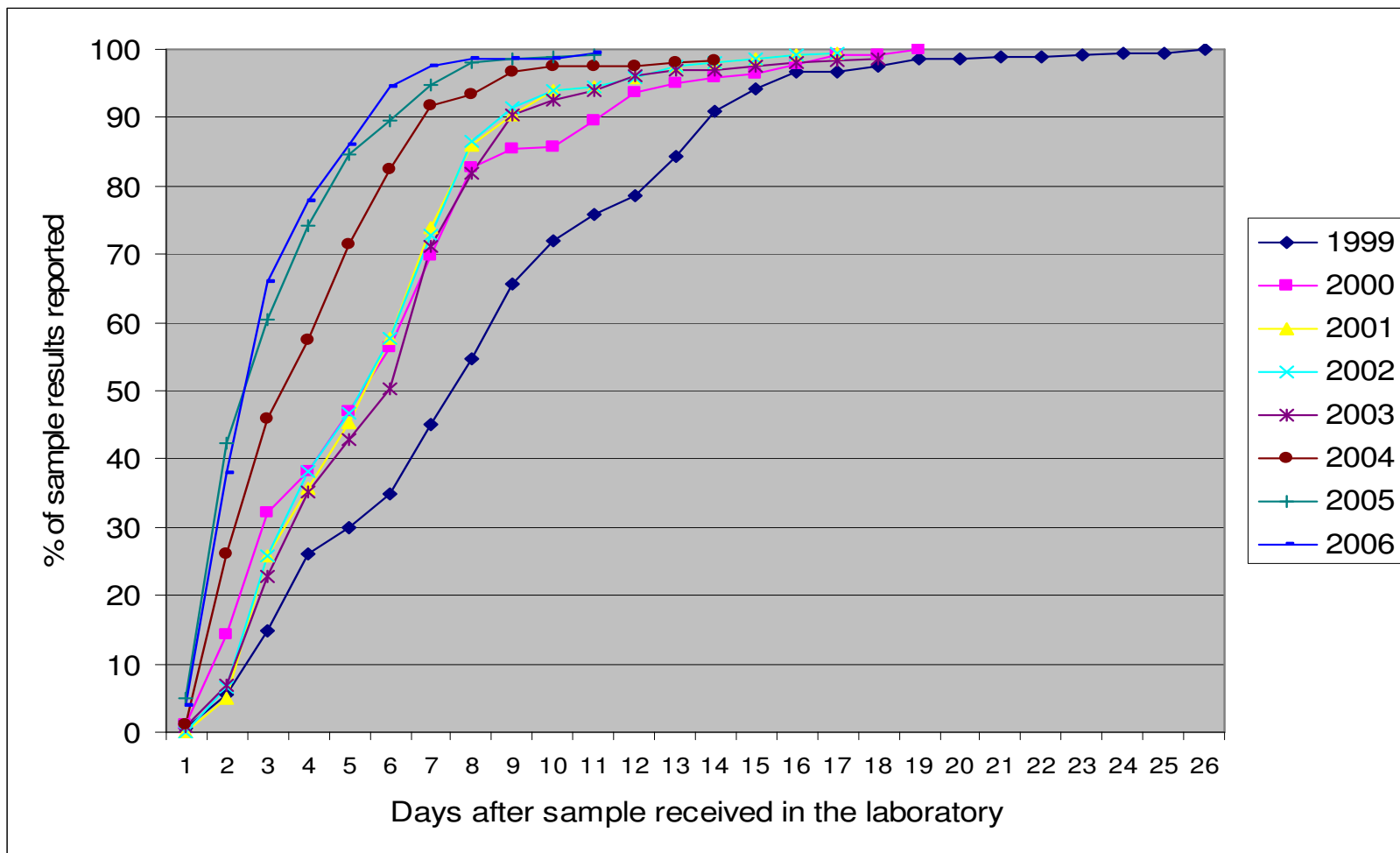
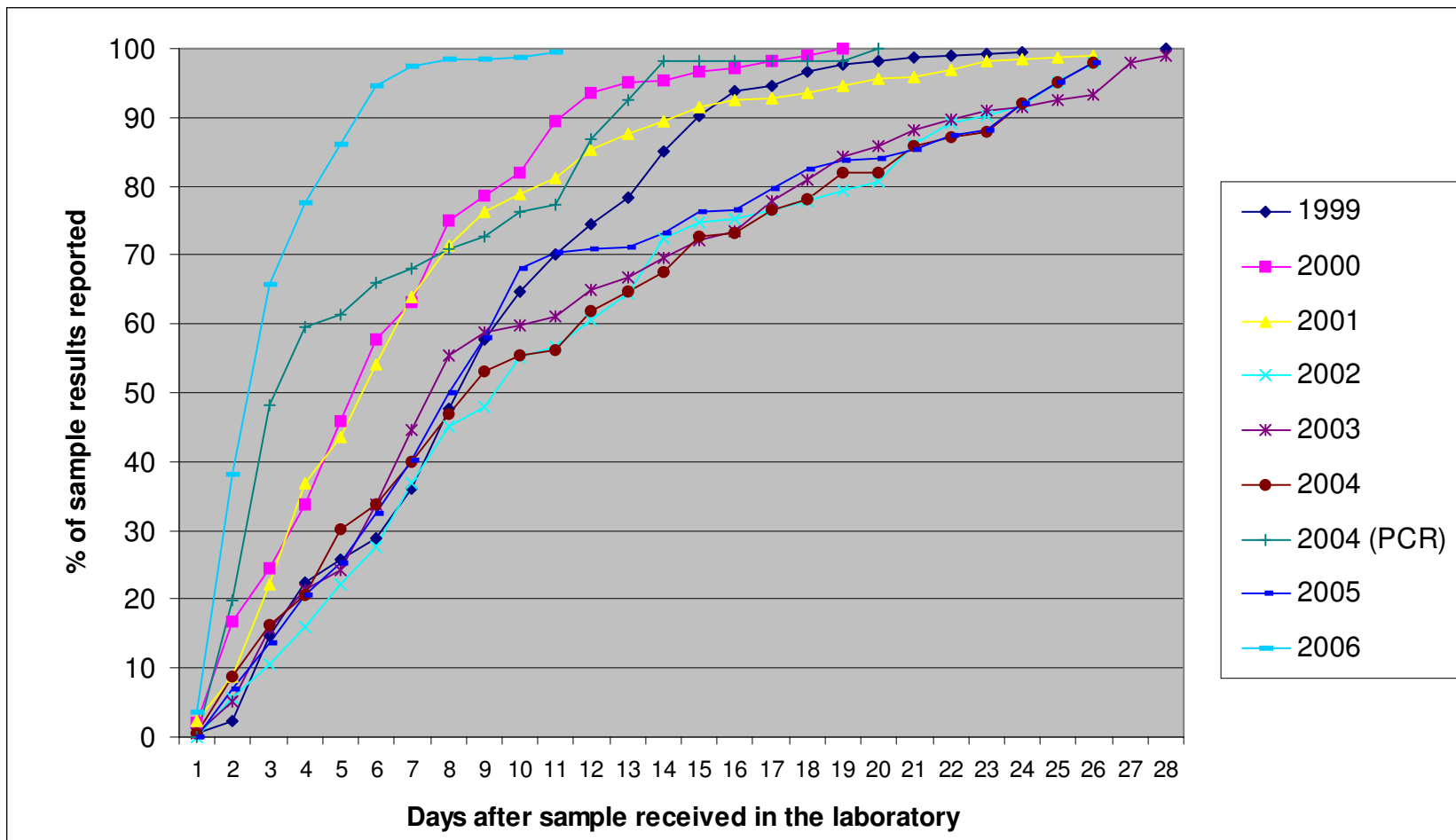


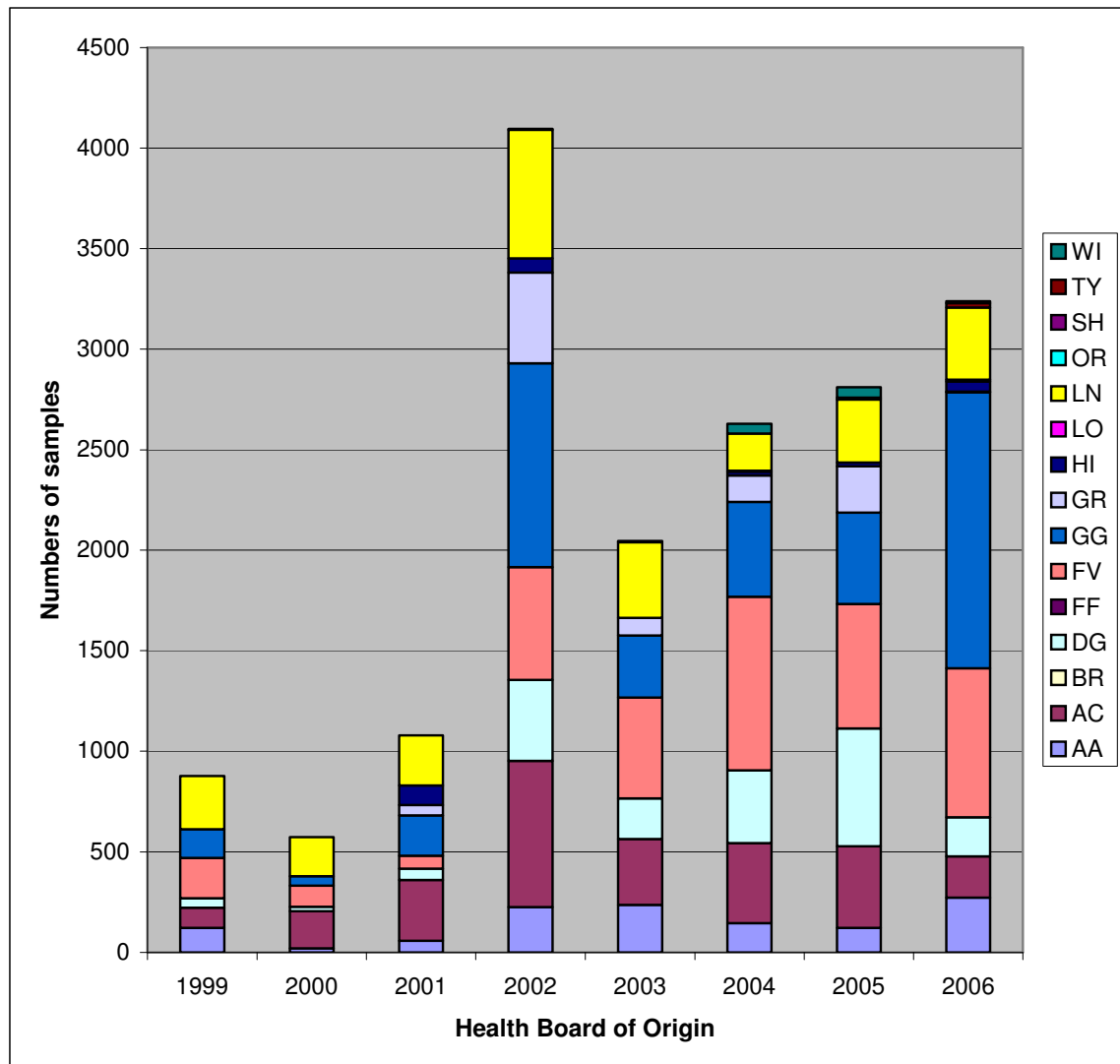
Figure 20: Turn-around-times for samples from sporadic cases of IID (1999-2006).



5.5.1.5. Health Board Of Origin

Figure 21 and Figure 22 show the total number of samples from outbreaks and sporadic cases of IID submitted for testing to the WOSSVC during the period 1999-2006, broken down by health board of origin.

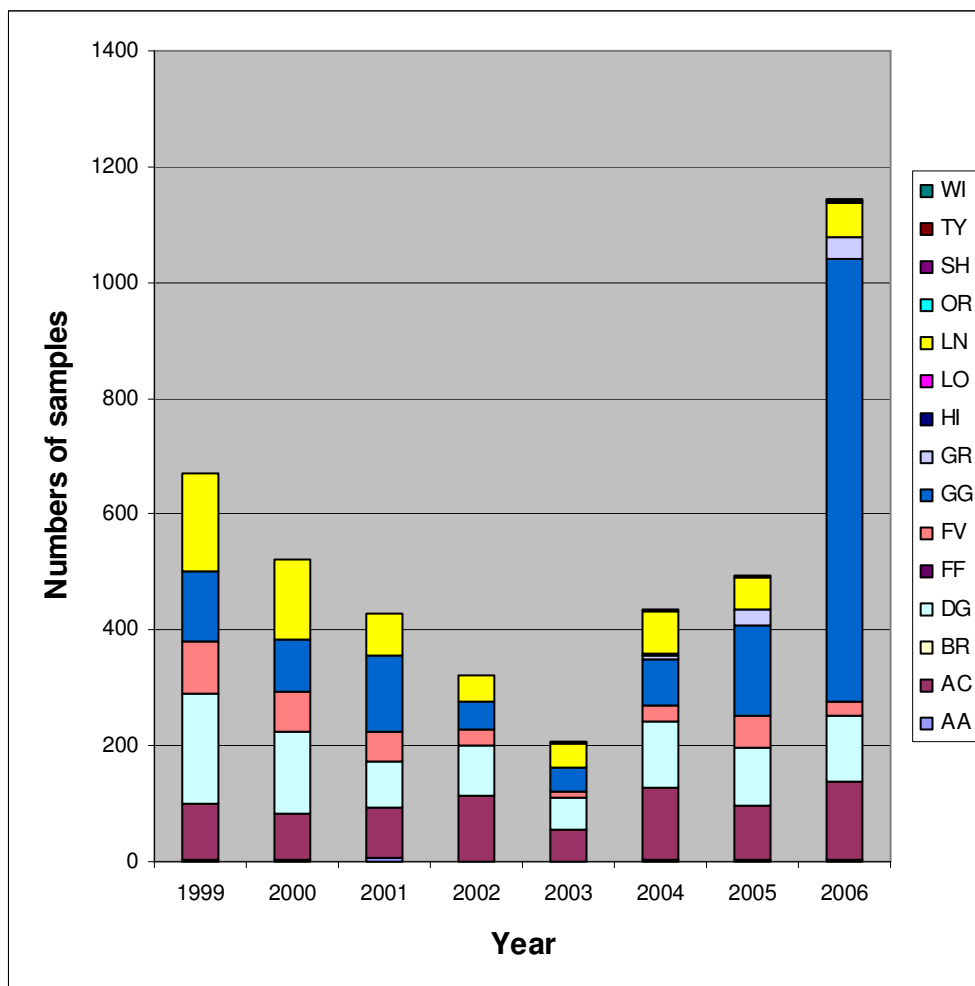
Figure 21: Total outbreak samples by health board (1999-2006).



In total there are fifteen health boards in Scotland. Figure 21 shows that in 1999 and 2000, when EM was the frontline test for outbreak samples, the WOSSVC received outbreak samples from six of these health boards: Argyshire and Arran (AA), Argyle and Clyde (AC); Dumfries and Galloway (DG); Forth Valley (FV); Greater Glasgow (GG); and Lanarkshire (LN). Lanarkshire and Argyle and Clyde were the biggest contributors during this time.

Post-2001, when RT-PCR was introduced for norovirus testing in outbreaks, two other health boards also submitted substantial numbers of samples: Highlands and Islands (HI); and Grampian (GR). Results from the telephone survey (Section 5.5.2) show that the laboratories in these two health boards utilised the WOSSVC service as either a back up test to EIA or as the frontline test for the investigation of samples from outbreaks. Tayside (TY) and the Western Isles (WI) also submitted small numbers of samples. Lothian (LO), Fife (FF), the Borders (BR) and Orkney (OR) and Shetland (SH) submitted no samples to the WOSSVC during the time period studied. This was because these health boards utilised the EM service available in East of Scotland Virology Centre in Lothian.

Figure 22: Total number of sporadic samples by health board (1999-2006).



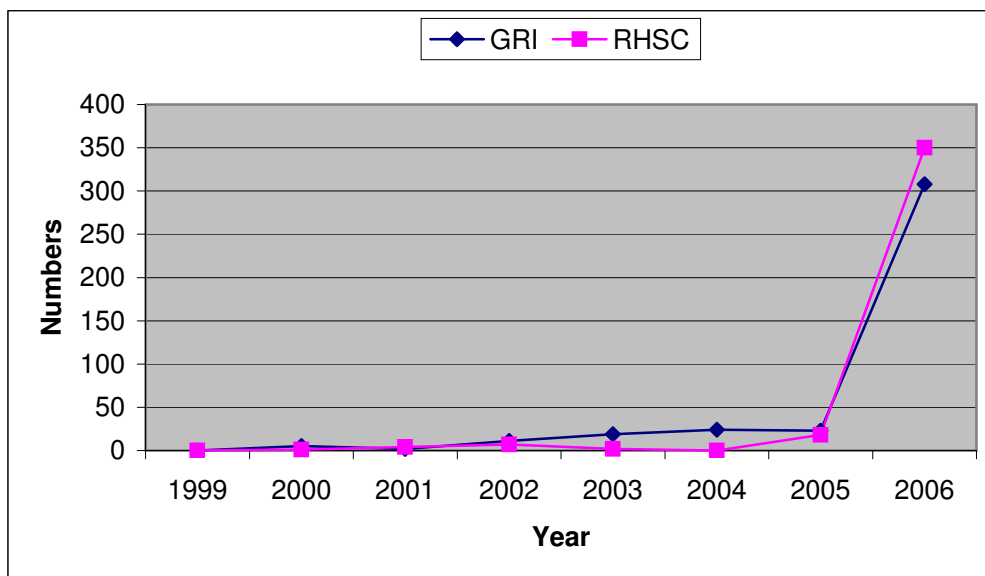
Examination of the samples submitted for sporadic testing shows that from 1999-2004 samples were received from five health boards: Argyle and Clyde, Dumfries and Galloway, Forth Valley, Greater Glasgow and Lanarkshire (Figure 22).

From late 2004 when PCR was implemented as the frontline test additional samples were also sent from Grampian.

In 2006 a large increase in sample submissions was observed from Greater Glasgow Health Board. Closer examination showed that the majority of these samples had been submitted by Bone Marrow Transplant (BMT) wards at the Royal Hospital for Sick Children and the Glasgow Royal Infirmary (Figure 23). Both of these wards submitted only a few samples between 1999-2005. However, in 2006 both wards sent over 300 samples (308 from the GRI unit and 350 from the RHSC). The reasons for this are discussed in section 5.6.2.

Overall, therefore, the number of IID samples submitted for testing to the WOSSVC laboratory has increased steadily since the introduction of the first molecular tests in 2001, partly as a result of more health boards using the service, and partly as a result of those who already do so increasing their usage.

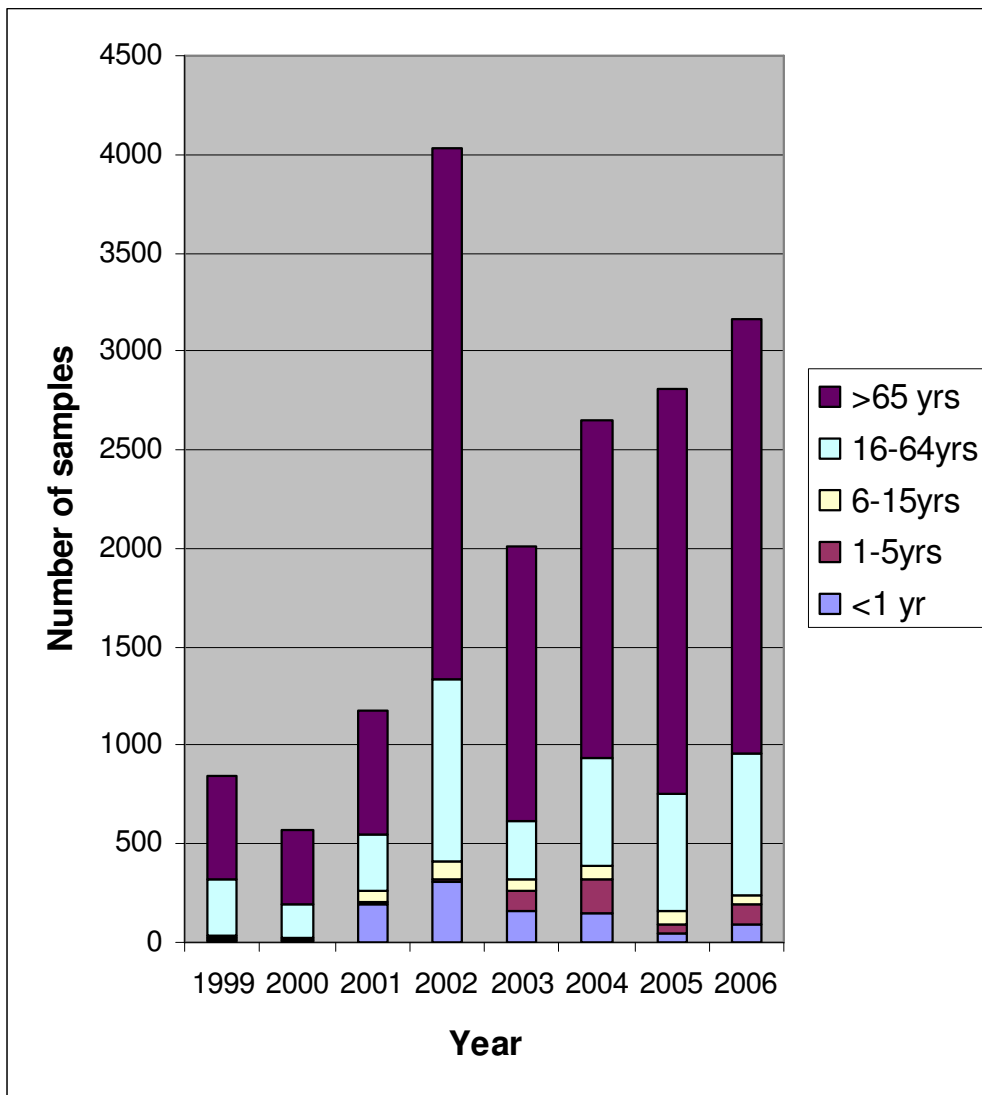
Figure 23: Number of sporadic samples submitted from the RHSC and GRI bone marrow transplant wards (1999-2006).



5.5.1.6. Age Of Patients

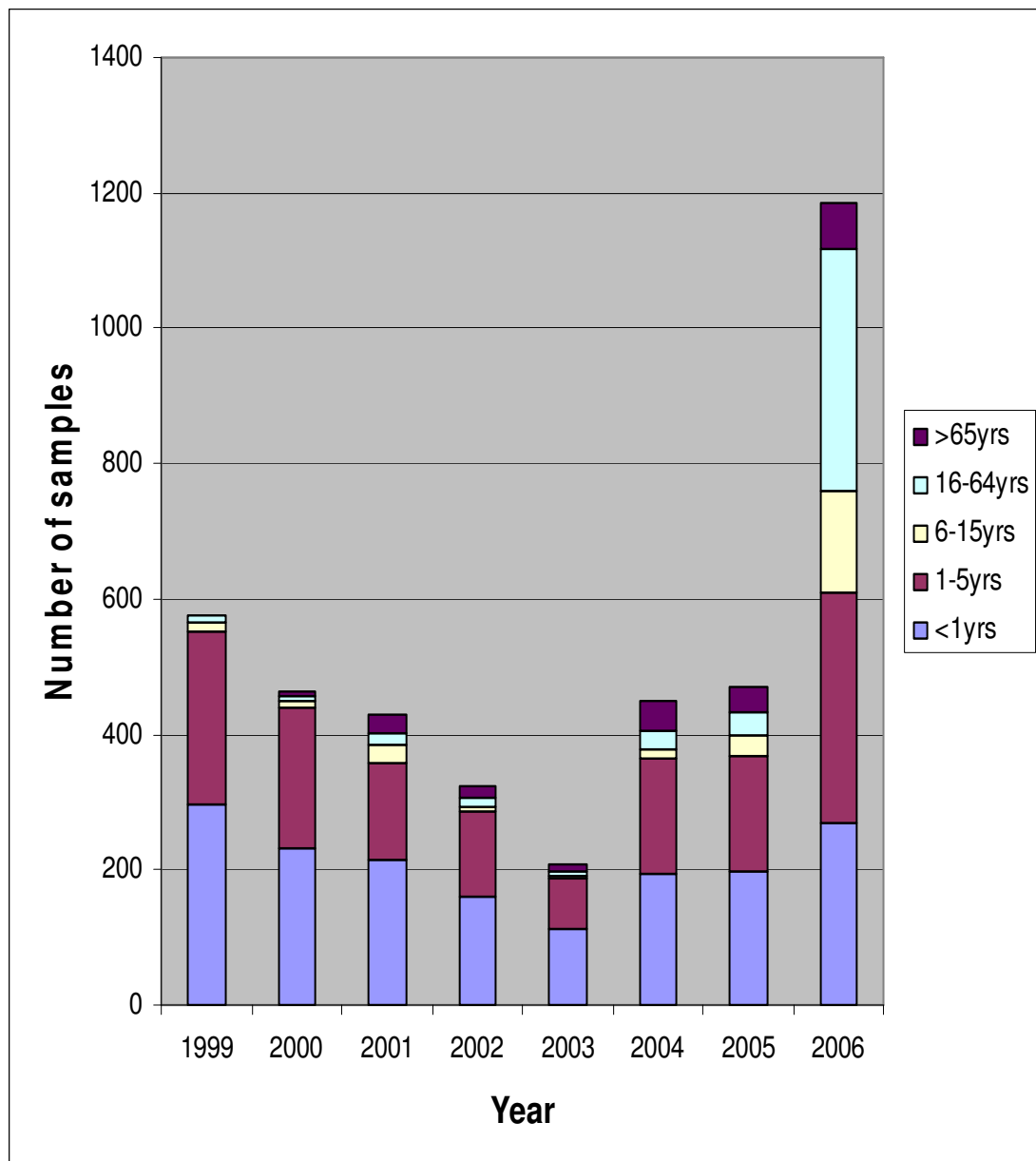
Figure 24 and Figure 25 show the total number of samples from outbreaks and sporadic cases of IID submitted for testing to the WOSSVC during the period 1999-2006, broken down by the age of the patient.

Figure 24: Total outbreak samples by age group (1999-2006)



Examination of the data from outbreak samples shows that, each year, although samples were submitted from all age groups the majority were from the 65 years and over age group (Figure 24). This is in line with the known epidemiology of norovirus outbreaks. The data also show that after the norovirus outbreak service was introduced in 2001, the large increases in sample submission rates outlined earlier have come predominantly from the 65 years and over age group.

Figure 25: Total number of sporadic samples by age group (1999-2006).



Examining the samples submitted from sporadic cases of gastroenteritis shows that the majority of samples in each year were from the under 5 years age group. For the period of 1999-October 2004 this is in line with the coding protocol in place at the time since the service was only offered on a routine basis for children under 10 years of age. After the implementation of the molecular service in November 2004 no significant change was observed until 2006 when a large increase in submission was observed in the 16-64 year age group. This was largely due to the large increase in submissions from the adult BMT patients (outlined in section 5.5.1.5).

5.5.2. Survey Of Laboratory Testing Practice

5.5.2.1. Testing Protocols For Outbreaks Of Viral IID

In March 2004, three years after the implementation of Lightcycler™ real-time RT-PCR at the WOSSVC, 20 of the 22 laboratories surveyed (91%) were shown to utilise the WOSSVC service for investigating samples from outbreaks of suspected viral IID (Table 39). One laboratory (5%) utilised EM and carried out testing for the health boards Lothian, Fife, the Borders and Orkney. One other laboratory was found to have no protocol for outbreaks of IID (5%).

Of the laboratories that utilised the WOSSVC outbreak service, 17 (85%) were found to use it as their frontline test. These were from the health boards Ayrshire and Arran, Argyll and Clyde, Dumfries and Galloway, Highlands and Islands, Forth Valley, Greater Glasgow and Lanarkshire. Three laboratories (15%) used the molecular service as a back up to their own testing system. These laboratories carried out testing for Grampian and Tayside. In each case norovirus was first tested for using an EIA (DAKO Cytomation, Cambridgeshire, UK), which replaced EM during 2003. Each stated that samples from all EIA negative outbreaks that tested negative for norovirus by EIA were submitted to the WOSSVC for testing by PCR.

In 2006, laboratories were contacted again by email and asked to outline whether their testing policies had changed. Several changes were outlined. In 2005, the virus laboratory at Aberdeen Royal Infirmary switched from EIA to real-time RT-PCR for norovirus as the frontline test for outbreaks of gastroenteritis (the same dual-labelled probe-based assay as used at the WOSSVC) as the frontline test for outbreaks of gastroenteritis. The laboratories at Ninewells Hospital, Dundee, Perth Royal Infirmary, Dumfries Royal Infirmary, Edinburgh Royal Infirmary and Wishaw General all stated that they plan to implement this test in their own laboratory as their frontline test for outbreaks of gastroenteritis in 2007.

5.5.2.2. Testing Protocols For Sporadic Viral IID

The results of the questionnaire (Table 39) showed that in March 2004, 14 (64%) of the laboratories questioned had their own testing protocol for investigating sporadic viral IID. The eight laboratories with no testing protocol (36%) included Nuffield Hospital (Greater Glasgow Health Board), Oban (Argyll and Clyde), Glasgow Royal Infirmary (Greater Glasgow), Hairmyres Hospital (Lanarkshire), the

Western Infirmary (Greater Glasgow), the Southern General Hospital (Greater Glasgow), Vale of Leven Hospital (Argyle and Clyde), and Dumfries Royal Infirmary (Dumfries and Galloway). No laboratory used the WOSSVC service for sporadic cases of IID.

With regards to the pathogens tested for in the sporadic setting, it was found that 10 laboratories tested for rotavirus only (71%). The remaining four laboratories tested for rotavirus as well as other non-rotavirus viral pathogens (26%): two of which (13%) tested for norovirus, adenovirus and rotavirus and two of which tested for adenovirus and rotavirus (13%). No laboratories tested for sapovirus or astrovirus.

Of the 14 laboratories with a testing policy, 9 (64%) tested only children (i.e. those aged under 5 years) for viral causes of IID whilst two (13%) tested both children and elderly patients (65yrs and over). None of the laboratories tested samples from the adult population. Three laboratories (21%) did not state their testing policy with regards to the age group tested.

Overall 13 laboratories used antigen detection methods (93%) for testing sporadic samples of IID. Four laboratories used EIA (26%), three used Latex agglutination (20%), and six used ICG (40%). PAGE was utilised in one laboratory (7%). None of the laboratories were using molecular tests for the investigation of sporadic IID.

The laboratories were contacted again in 2006 and asked whether the testing service had changed. All laboratories stated that the testing protocol for sporadic cases of IID remained the same as in 2004.

Table 39: Responses to the questionnaire on laboratory testing practice.

Laboratory	NHS board	Outbreak IID	Sporadic IID			
		What viral pathogens tested for?	Test for viral pathogens?	What viral pathogens tested for?	Age group tested?	Tests used?
Aberdeen Royal Infirmary	Grampian	Dako EIA for norovirus ^a . If negative, norovirus at WOSSVC ^b	Yes	Rotavirus, adenovirus	Not stated	EIA
Falkirk District Royal Infirmary	Forth Valley	Norovirus at WOSSVC	Yes	Rotavirus	<5yrs, >65yrs	ICG
Inverclyde Hospital	Argyle & Clyde	Norovirus at WOSSVC	Yes	Rotavirus	<5yrs, >65 yrs	ICG
Wishaw Hospital	Lanarkshire	Norovirus at WOSSVC ^c	Yes	Rotavirus	<5yrs	ICG
Raigmore Infirmary	High&s & Isl&s	Norovirus at WOSSVC	Yes	Rotavirus	<5yrs	Latex
Stirling Royal Infirmary	Forth Valley	Norovirus at WOSSVC	Yes	Rotavirus	<5yrs	ICG
Ninewells Hospital, Dundee	Tayside	Dako EIA for norovirus ^a . If negative, norovirus at WOSSVC ^c	Yes	Rotavirus, adenovirus, norovirus	Not stated	EIA
Perth Royal Infirmary	Tayside	Dako EIA for norovirus ^a . If negative, norovirus at WOSSVC ^c	Yes	Rotavirus, adenovirus, norovirus	Not stated	EIA
Monklands District General Hospital	Lanarkshire	Norovirus at WOSSVC	Yes	Rotavirus	<5yrs	Latex
Crosshouse Hospital	Ayrshire & Arran	Norovirus at WOSSVC	Yes	Rotavirus	<5yrs	Latex
Nuffield Hospital	Greater Glasgow	None	No	-	-	-
Edinburgh Royal Infirmary	Lothian	EM ^c	Yes	Rotavirus	<5yrs	PAGE
Royal Hospital for Sick Children, Glasgow	Greater Glasgow	Norovirus at WOSSVC	Yes	Rotavirus, adenovirus	<5yrs	EIA
Oban Hospital	Argyle & Clyde	Norovirus at WOSSVC	No	-	-	-
GRI	Greater Glasgow	Norovirus at WOSSVC	No	-	-	-
Hairmyres Hospital	Lanarkshire	Norovirus at WOSSVC	No	-	-	-
Western Infirmary	Greater Glasgow	Norovirus at WOSSVC	No	-	-	-
Southern General Hospital	Greater Glasgow	Norovirus at WOSSVC	No	-	-	-
Royal Alexandria Hospital	Argyle & Clyde	Norovirus at WOSSVC	Yes	Rotavirus	<5yrs	ICG
Vale of Leven Hospital	Argyle & Clyde	Norovirus at WOSSVC	No	-	-	-
Victoria Infirmary	Greater Glasgow	Norovirus at WOSSVC	Yes	Rotavirus	<5yrs	ICG
Dumfries Royal Infirmary	Dumfries & Galloway	Norovirus at WOSSVC ^c	No	-	-	-

^a EIA implemented in late 2003 in place of EM

^b Replaced by dual-labelled probe-based real-time PCR in 2005 (as used at WOSSVC)

^c Planned replacement by dual-labelled probe-based real-time PCR in 2007 (as used at WOSSVC)

5.6. Discussion

Since the introduction of molecular tests at WOSSVC for detection of viral pathogens in cases of gastroenteritis the number of samples tested by the routine diagnostic service has risen steadily, as have the detection rates for each of the main viral causes. Furthermore, this has been achieved at the same time as a substantial reduction in sample turn-around-times. The reduced turn-around-times were maintained despite fluctuations in sample submissions. These improvements in the WOSSVC laboratory service have been observed for both outbreaks and sporadic cases of gastroenteritis.

5.6.1. Effects On The Diagnostic Service For Outbreaks Of IID

The improvements outlined above have been most telling for norovirus diagnosis in outbreak samples. Since the implementation of Lightcycler™ real-time RT-PCR in 2001, followed by the dual-labelled probe-based real-time RT-PCR in 2004, there has been a significant increase in the numbers of outbreak samples tested at the WOSSVC, with the number continuing to rise each year. The increase in the number of samples submitted is due to steadily increasing use by health boards who were already using the service pre-2001, together with an increase in the number of health boards utilising the service (from 6 to 9), both of which suggest that users find the service useful (although the increases in sample submissions will also be a reflection of outbreak activity). Laboratories were shown to use the service in two ways. The majority of those questioned in 2004 used the WOSSVC norovirus service as their frontline test for outbreak samples. A small number at this time (three) utilised the service as a back up to their own frontline norovirus test (EIA). From 2007, moreover, a number of the laboratories questioned plan to implement the dual-labelled probe-based real-time RT-PCR for norovirus described in this thesis in their own laboratories. This further highlights the usefulness of this test.

The implementation of the RT-PCR assays for norovirus in samples from outbreaks has led to significant improvements in the pathogen detection rate. For example, in 1999 and 2000, less than 100 norovirus detections were made using EM. After 2001, the number of positive detections increased substantially. Increases in the number of outbreaks classified as being caused by norovirus were also seen after 2001. Although this increase in detection may be a result of a sudden increase in

norovirus activity in Scotland in 2001 or improved submission of samples by clinicians, it is more likely that this is due to the use of the real-time RT-PCR. The ability of the real-time RT-PCR assays to test larger numbers of samples compared to EM has no doubt contributed to the increase in the number of detections. However, it is the increased sensitivity of the RT-PCR assays that is most important. This can be seen when comparing the overall norovirus detection rates for EM and the real-time RT-PCR methods. For samples tested by EM, the detection rate was always below 12%. However, from 2001-2006, when real-time RT-PCR was implemented, the detection rate for norovirus in outbreak samples rose to 28-41%. Interestingly, the dual-labelled probe-based real-time RT-PCR assay (introduced in 2004) resulted in a slightly greater detection rate than the LightcyclerTM real-time RT-PCR assay (28-41% vs 28-35%). This confirms the increased sensitivity of the dual-labelled probe-based real-time RT-PCR assay outlined in Chapter 4. The improved sensitivity of the PCR assays in comparison to EM can also be seen when examining the annual number of outbreaks classified as norovirus negative by the WOSSVC. Using EM, the majority of submitted outbreaks tested negative. However, after the implementation of RT-PCR the overall number and proportion of negative outbreaks fell. Two phases of reduction were observed. The first was seen after the implementation of the LightcyclerTM real-time RT-PCR in 2001. A further improvement was observed in 2004 when the dual-labelled probe-based real-time RT-PCR was implemented, again highlighting the improved sensitivity of this assay compared to the LightcyclerTM real-time RT-PCR. The low and reducing number of negative outbreaks detected in all years since 2001 also suggests that the emergence of a new norovirus variant in 2002 and again in 2006 has had no recognisable effect on either PCR's sensitivity.

Despite the increased sensitivity of both the RT-PCR assays, a fairly large proportion of the total outbreak samples submitted remained negative for norovirus and other viral pathogens. There are several possible explanations for this. Perhaps alternative nucleic extraction methods may have increased the detection rate achieved in this study. Only one method was used throughout this thesis and it had not been compared to other extraction methods (as it was already established for existing PCR methodology in the WOSSVC). Some of these may have been due to norovirus types that cannot be detected by the RT-PCR assays employed or those that are shed at levels below the detection limit of both RT-PCR assays. Vinje *et al* highlighted that the primers utilised by the LightcyclerTM real-time RT-PCR assay failed to detect

some G1 and G2 types in the panels tested and, compared to other commonly used assays, failed to detect certain norovirus types at a lower limit of detection (Vinje *et al*, 2003). The dual-labelled probe-based real-time assay has also been shown to be less sensitive for some G1 and G2 noroviruses (Jolkihumar *et al*, 2005). Therefore, use of other tests may have improved detection further. In addition a number of the outbreak samples that tested negative for viral pathogens may have been caused by non-viral pathogens and thus were diagnosed accurately.

Some of the samples may also have been negative due to the presence of PCR inhibitors. PCR inhibitors generally exert their effects through direct interaction with DNA/RNA or interference with DNA polymerases. The use of an internal control, a superior alternative to a positive control, would have provided the capability to detect these (Rosenstraus *et al*, 1998). An internal control is present in each PCR reaction to ensure it is working optimally. It is the ideal positive control as it can detect extraction failures, PCR inhibition, and technical errors *relating to each individual sample* (a positive control will detect general errors/failures that are assumed to be related to all samples at each stage rather than to the individual sample). Several internal control systems are described in the literature (Jenson *et al*, 2004; Neisters *et al*, 2002; Maroufi *et al*, 2006). Most are separate PCR assays that are multiplexed with the diagnostic assay, although some are used as separate assays altogether. (More complicated versions also exist where the PCR primers for a particular pathogen also contain regions that are weakly complimentary to the internal control. Consequently, the primers bind to the internal control in the absence of the pathogen nucleic acid.) The internal control target usually takes the form of a non-human virus, an oligonucleotide or a plasmid, which is added manually to the sample, extraction or PCR mastermix (Tedder *et al*, 2006; Jenson *et al*, 2004; Neisters *et al*, 2002). Some systems also target parts of the human genome that should be present in all clinical samples (Muller *et al*, 2007; Whiley *et al*, 2004).

Many of the newer automated nucleic acid extraction machines can be adapted to add the chosen internal control to all samples during the process of extraction. In most cases the internal control is usually added to the lysis buffer, which is then added to a “lysis plate” already containing the selected volume of clinical sample. For best control, clinical samples should be spiked with an internal control before extraction and each diagnostic test should be multiplexed with an internal control assay. However, such a system increases the number of pipetting steps involved, prolongs

hands-on time and can be difficult to standardise. An internal control was not included in this research due to concerns that it would significantly increase testing costs and would also increase technician hands-on time in order to spike samples and interpret results. However, it is something that is likely to be explored in the near future (see Chapter 6).

A number of samples are also likely to have been sent from an outbreak site from patients and HCWs that do not have specific norovirus symptoms but are feeling “unwell”. Also, in the hospital and residential home setting, a large number of patients are likely to suffer from enteric illness due to non-norovirus causes such as incontinence, reaction to medication and changes in diet (Lopman *et al*, 2002). Enteric illness due to these causes is difficult to differentiate from norovirus illness and therefore these individuals are likely to have samples submitted for testing. A negative test result in such cases is entirely reasonable. A number of the RT-PCR negative samples may also be from outbreaks that have tested negative but could not be labelled as true negative outbreaks as they did not meet the minimum criteria of six submitted samples.

One of the key objectives in introducing the new tests was the improvement of turn-around-times, since EM unlike PCR can be slow as it requires technical expertise and has been found unsuited to processing large numbers of samples (Utagawa *et al*, 2002). As mentioned previously, a rapid result is more likely to be of use to clinicians and public health officials as it will affect patient and outbreak management. The data presented here shows that, in fact, this objective has been achieved. Although sample numbers have increased significantly since the introduction of RT-PCR for norovirus, these have been processed in shorter times than achieved on smaller numbers of samples using EM. It should be noted that the turn-around-times calculated included weekends, when the laboratory was closed. Also, the endpoint used in the calculation was the date the paper report was sent out, which means that the majority of results were actually generated the previous day, at least. As a result, the turn-around-times for the tests themselves are likely to be shorter than shown.

For example, when EM was the frontline test for outbreaks, 90% of sample results were reported by day 13. In comparison, following implementation of the RT-PCR assays the same proportion of a larger number of samples was processed in just 5-8 days (depending on the RT-PCR test used). Furthermore the reductions in turn-

around-times were maintained despite fluctuations in the sample numbers submitted. This shows both real-time PCR methods used for norovirus to be robust.

Comparing the two RT-PCR assays in routine service, it was shown that the dual-labelled probe-based real-time assay resulted in better turn-around-times. Many factors contribute to this. Firstly, the dual-labelled probe-based real-time assay utilises a one step RT-PCR kit whereas the LightcyclerTM real-time RT-PCR was a two-step assay (with separate reverse transcriptase and PCR steps). Fewer technical steps not only speeds up the process but also reduces the likelihood of error, which in turn results in fewer test repeats and thus reduced turn-around-times. The utilisation of such a kit in the LightcyclerTM real-time RT-PCR assay would also have had this effect compared to the two-step process. However, during the LightcyclerTM period of use an affordable kit of this type was not available. A second reason for the improved performance of the dual-labelled probe-based real-time assay is that it can process 96 samples and controls per run, whereas the LightcyclerTM real-time RT-PCR can process only 32 samples and controls. The dual-labelled probe-based real-time assay also offers simpler result interpretation in comparison to the LightcyclerTM real-time RT-PCR assay (as outlined in Chapter 4). Again, easier interpretation reduces the number of repeat tests needed and thus helps reduce turn-around-times.

A further improvement in turn-around-times was observed after the implementation of primer/probe pools, reduced RT-PCR cycling parameters, and pooled PCR positive controls in 2005. As outlined in Chapter 4, primer/probe pools are aliquots containing pre-optimised primer and probe. These are stored in 24-reaction aliquots at -20°C . The associated mastermix and enzyme are also stored in 24-reaction aliquots at -20°C . Depending upon the amount of tests to be carried out, the desired number of aliquots are defrosted and then added together with a minimum of pipetting, therefore increasing the ease and speed of the setup process and reducing the risk of errors. In addition to this, the use of reduced RT-PCR cycling parameters was shown in Chapter 4 to halve the time required to carry out each step of the RT-PCR process compared to the manufacturer's estimate (with the exception of the Taq activation phase which remains as the manufacturers describe). As a result DNA and RNA testing times are significantly reduced (by 30-40 minutes) and more test runs can be carried out in the laboratory each day. Pooled positive controls allow more samples to be processed per run, therefore improving throughput and rapidity. These

amendments therefore contributed to the reduction in turn-around-times observed in 2005 and 2006.

5.6.2. Effects On The Diagnostic Service For Sporadic Cases Of IID

Similar improvements to those described above for the norovirus outbreak testing service were also observed for the testing of sporadic cases of gastroenteritis (although some caution should be applied in interpreting these results, since the time period involved since the introduction of these tests has been relatively short). The dual-labelled probe-based real-time PCR assays, which test for the five main causes of IID, were implemented in November 2004. In 2005 similar numbers of samples were tested as when EM was employed. However a large increase in sample submission was observed in 2006. Although this may be partly the result of increased disease activity in the community it is also likely to be due to increasing awareness of the utility of the service, especially in local BMT units (this is discussed further below). As with the norovirus outbreak service, the increased submission rates suggest users find the service useful.

When compared to the norovirus outbreak diagnostic service, it was found that fewer health boards use the sporadic service on a routine basis. This may be partly due to the fact that the service is fairly recent in nature. However, it is more probably due to the fact that most laboratories already have their own testing protocol for sporadic cases of gastroenteritis. This was indicated by the results of the laboratory survey, which highlighted that 64% of the laboratories questioned had an in-house testing service in place for sporadic gastroenteritis. In most cases laboratories tested for rotavirus only. As a result the other viral causes of sporadic IID are largely untested for, despite increasing evidence, confirmed by the results shown here, that these are common causes of illness. Examination of the health boards that did use the WOSSVC molecular service in each year suggests the service is mainly used by laboratories with no testing protocol for sporadic gastroenteritis of their own (although this was not explicitly asked in the questionnaire). These included the following health boards: Argyle and Clyde, Dumfries and Galloway, Greater Glasgow and Lanarkshire.

In 2006, most health boards submitted the same or slightly fewer sporadic samples than observed in 2005. The substantial increase in total sample numbers observed in 2006 originated primarily from Greater Glasgow health board. This was

the result of a sharp increase in the number of samples being sent from the BMT ward at the Royal Hospital for Sick Children and the equivalent adult ward at Glasgow Royal Infirmary. Anecdotal evidence from clinicians working at these units suggests that they first became aware of the new test's availability at WOSSVC in 2006 and would have made use of the service had they been aware of it in previous years (Personal communication, Dr Balfour, RHSC). Also during 2006, there were occurrences of both outbreaks and sporadic cases of norovirus in each environment, affecting multiple patients and causing long term shedding in a number of those affected. Several patients with long term shedding suffered prolonged isolation and illness and may also have subsequently infected other patients. Patients, on the other hand, who were sent home to recover posed an infectious risk to family members and others (e.g. school pupils). Similar problems were also observed with occurrences of rotavirus, adenovirus, and astrovirus. One patient died as a result of a disseminated adenovirus group F infection. Molecular methods are particularly useful in this setting as virus from immunocompromised patients is often shed at low levels, which other methods find difficult to detect. RT-PCR is more likely to detect those infected and can do so earlier than either EM or EIA (Cubitt *et al*, 1997). The increased sensitivity also decreases the likelihood of false negative results, which might otherwise lead to an infected patient remaining on the ward, causing further infections.

As a result of these issues, both wards began to screen all patients with diarrhoea in order to distinguish viral infection from other causes (e.g. other pathogens or issues relating to the management of the transplant) and to isolate those with infection from other patients. Increased monitoring of infected patients also occurred in order to determine when shedding, and therefore the presumed infection or infectiousness, had stopped. Thus, in 2006, although the Royal Hospital for Sick Children already has an EIA-based service for investigating cases of gastroenteritis, the BMT ward chose largely to bypass this service in favour of the molecular test employed at WOSSVC - highlighting its usefulness. The awareness demonstrated by clinicians at these units of the problems caused by viral IID in BMT patients, together with their recently acquired awareness of the WOSSVC molecular service is likely to result in further use of the service in future years.

Compared to the data obtained when using EM, the detection rates for most pathogens in sporadic samples showed significant increases when dual-labelled probe-based real-time PCR was implemented. These increased detection rates have led to an

increase in the overall detection rate. When EM was the frontline test the overall detection rate was between 2-23% whereas using the dual-labelled probe-based real-time PCR the figure was between 48-52%. Again this shows the increased sensitivity of the real-time PCR assays when compared to EM and confirms the findings described in Chapter 4 and published in the literature. The most significant increases were observed for adenovirus and norovirus, which were the most common pathogens detected in the sporadic setting. Adenovirus has long been recognised as a common cause of sporadic gastroenteritis. However, these data also confirm other more recently published accounts that indicate norovirus is also a common cause of gastroenteritis in the sporadic setting (Oh, Gaedicke, Schreier, 2003; Kirkwood and Bishop, 2001; Simpson *et al*, 2003; McIver *et al*, 2001; Subekti *et al*, 2002; Qiao *et al*, 1999; Phan *et al*, 2004; Bereciartu, Bok, Gomez, 2002; Sakai *et al*, 2001; Boga *et al*, 2004; Rodriguez-Baez *et al*, 2002; Phan *et al*, 2005; Buesa *et al*, 2002; de Wit *et al*, 2001; Bonn *et al*, 1999; Farkas *et al*, 2000; Pang *et al*, 2000; Froggatt *et al*, 2004; Chikhi-Brachet *et al*, 2002; Simpson *et al*, 2003; Fretz *et al*, 2005).

Although the detection rates for astrovirus and sapovirus in sporadic samples remained small, increases were observed after the implementation of the dual-labelled probe-based real-time PCR assays in comparison to the EM test used previously. At the time of writing none of the laboratories questioned use either EM or a molecular method to test cases of sporadic gastroenteritis, which means that the WOSSVC is the only laboratory currently able to test for these pathogens.

The benefits of dual-labelled probe-based real-time PCR assays in testing for adenovirus, astrovirus, norovirus and sapovirus are therefore clear. However, the utility of the assay for rotavirus is questionable based on the evidence shown, as detection rates for this pathogen did not significantly improve compared to when EM was used (1-15% vs 0-12%). This lack of improvement in detection rate may be due to the fact that most of those seeking medical attention for rotavirus infection shed virus at levels that are equally detectable by both EM and RT-PCR (Kang *et al*, 2004). The low number of rotavirus detections overall compared to norovirus and adenovirus moreover, may be explained by the fact that some of the laboratories that use the service already screen sporadic samples for rotavirus before deciding whether or not to send them to the WOSSVC. The assay remains useful however, for those health boards that do not have a sporadic testing service, and is also likely to prove valuable for detecting rotavirus in the BMT setting.

Samples from sporadic cases of IID that did not test positive for any viral pathogens are likely to result from a number of factors. Some are likely to contain PCR inhibitors and may therefore have been falsely classified as negative. Some are also likely to be from patients with intestinal illness due to other infectious causes (e.g. bacterial or parasitic infections, or other viral pathogens such as rotavirus C and enterovirus). Some cases may be due to viral infections that are not detected by the assays used (e.g. non-predominant noroviruses). For example, the sapovirus real-time RT-PCR assay is likely to miss any non-G1 sapoviruses. Perhaps using an alternative extraction protocol may also improve detection rates of all pathogens. Patients with non-infectious causes of illness may also contribute to the proportion of sporadic samples that tested negative for viral pathogens.

As with the diagnostic service for IID outbreaks, utilisation of the RT-PCR assays for sporadic cases of gastroenteritis has allowed more samples to be tested with reduced turn-around-times. When EM was the frontline test 50% of samples were reported by day 5-9, whilst 90% were reported by day 11-23. Initially, the results for the replacement RT-PCR assays were disappointing, with once-weekly batch testing leading to prolonged turn-around-times (with 90% of results not reported until day 23) since samples that needed repeat testing had to wait another week before this could take place. However, as the sample numbers increased testing was increased in frequency to at least three times a week. As a result turn-around-times improved significantly, and in 2006 90% of samples were reported by day 6.

5.7. Summary

Chapter 5 examined real effects of implementing the developed PCR assays as the frontline tests for the investigation of outbreaks and sporadic cases of gastroenteritis on the laboratory diagnostic service offered by the WOSSVC. The results showed that since the introduction of molecular tests in the laboratory for the detection of viral pathogens in cases of gastroenteritis the number of samples tested by the routine diagnostic service has risen steadily, as have the detection rates for each of the main viral causes. Furthermore, this has been achieved at the same time as a substantial reduction in sample turn-around-times. The reduced turn-around-times were maintained despite fluctuations in sample submissions. These improvements in

the WOSSVC laboratory service have been observed for both outbreaks and sporadic cases of gastroenteritis.

Chapter 6

Discussion

6.1. Summary Of Research

The research in this thesis sought to advance knowledge in the field of public health by developing and assessing PCR methods for the detection of viral causes of IID in a routine diagnostic setting, an approach that distinguishes it from previous work in this area.

As described in Chapter 2, outbreaks and sporadic cases of Infectious Intestinal Disease (IID) are a major public health issue, resulting in significant morbidity and sometimes mortality every year. Viral pathogens have been shown to be responsible for a significant proportion of the IID burden, particularly in sporadic cases in children and outbreaks in all age groups.

Laboratory diagnosis of viral IID is important, as the aetiological causes of IID (bacteria, parasites or viruses) cannot be differentiated based on clinical or epidemiological data alone. Before this research began, Electron Microscopy or EM was the frontline method used at the West of Scotland Specialist Virology Centre (WOSSVC) for the diagnosis of viral causes of IID in samples from both outbreaks and sporadic cases of IID. However, although rapid on a small number of samples, this test has limited sensitivity compared to other emerging techniques and is laborious, requiring highly specialised staff and expensive equipment. Alternative methods of detection include antigen detection methods. These offer similar sensitivity to EM but in comparison have the potential for improved rapidity, ease of use and throughput. These methods, however, are not available for all the main viral causes of IID.

In recent years, the molecular method Polymerase Chain Reaction (PCR) has been increasingly used in research on the main causes of viral IID. PCR assays are not currently offered by commercial companies but have been found to be more sensitive than either EM or antigen detection methods. However, conventional gel-based PCR methods can be expensive to implement and are technically complex, consisting of numerous steps. As a result, in the past these have not been considered suitable for use in the routine diagnostic setting, where the ideal test is one that is not only sensitive but also rapid, high throughput, and easy to use.

Real-time PCR has the potential to overcome many of the pitfalls associated with gel-based methods. Using this technique, for example, all or the majority of the numerous steps associated with PCR can be carried out in a single step. The relatively recent nature of the technology, however, means that the choice of assays of this type is limited and, prior to this research, there were no examples of its use in a diagnostic setting.

The overall aim of this research was therefore to examine whether PCR methods could be adapted for use in the routine diagnostic setting and, in comparison to the EM-based service, bring significant benefits to the laboratory service in terms of increased detection rate and throughput, and reduced turn-around-times.

Based on a review of the literature (see Chapter 2) the decision was taken to divide the proposed new service into two parts: a separate service for samples from outbreaks of gastroenteritis and one for those from sporadic cases of IID. For the outbreak service, a norovirus assay was chosen as the frontline test for sample investigation, based on an increasing number of reports showing norovirus to be the principle cause of outbreaks of IID. For the sporadic service, the decision was taken to employ this same norovirus assay together with PCR tests for adenovirus, astrovirus, rotavirus and sapovirus to investigate sporadic cases of gastroenteritis in children under 10 years of age and immunocompromised patients (the patient groups already tested using the EM-based service) since these viral pathogens have been shown to contribute significantly to IID in children and can cause severe and chronic infection in immunocompromised individuals of all age groups. In the case of the outbreak service, moreover, it was decided that samples that tested negative for norovirus should be examined further using the tests for the other non-norovirus pathogens.

Following this, the PCR and associated methods for use in the proposed service were chosen and adapted as described in Chapter 3. In each case a review of the available methods was conducted and an appropriate test selected for each pathogen based on a combination of clinical and laboratory requirements. In selecting an appropriate test evidence of its sensitivity and specificity was key (including the ability to detect a wide range of virus subtypes). However, other factors also played an important part. Based on the desire to use the test in a routine setting, for example, preference was given to single round assays over nested techniques in order to minimise test complexity and length (and hence the potential for error). Priority was also given to tests that targeted the most conserved regions of the virus, in order to

minimise the risk of sensitivity drift over time and/or sudden loss of sensitivity due to the emergence of a new virus type or subtype, which is more likely when targeting more variable areas of the genome.

With regards the outbreak service there were two phases of development. In 2000, when the initial developments took place, there were no real-time PCR methods for the detection of norovirus available in the literature. The early work, therefore, sought to adapt a published gel-based method (Green *et al*, 1995) into real-time format in order to increase its potential for rapid turn-around-times, and by association, high throughput, since speed is particularly important in an outbreak setting where rapid results may inform decisions on the infection control procedures to be used. The novel real-time assay was developed using SYBR green technology, which was the predominant real-time technology available at the time, and was implemented on the Lightcycler™ RT-PCR platform, which was one of only two PCR platforms available in the laboratory, the other being a conventional PCR block (the ABI 9700).

From 2003, several other real-time PCR platforms were obtained by the laboratory, which can be used for other real-time PCR chemistries including dual-labelled probes. Such chemistries use an additional probe, which specifically binds to the PCR product and fluoresces in direct relation to the amount of PCR product being produced. The use of a probe increases the specificity of the assay in comparison to SYBR green based methods, which bind to all dsDNA. Compared to the Lightcycler™ platform, these platforms also offer improved throughput. Consequently, from this point on, the research focused on developing a real-time PCR assay for norovirus that used dual-labelled probe-based technology. The assay described by Kageyama *et al* was chosen for investigation (Kageyama *et al*, 2003). The original publication used separate RT-PCR assays to detect G1 and G2 norovirus types. Here, as a further development the assays were multiplexed in an attempt to improve rapidity, throughput and reduce testing costs.

As with the outbreak service, the research relating to the sporadic service also took place in two stages. The first development phase took place between 2000 and 2002. The aim of the initial studies was to develop a novel gel-based multiplex RT-PCR assay for adenovirus, astrovirus, rotavirus and sapovirus. The assays chosen to be multiplexed were all single round PCR assays selected from the published literature (Gentsch *et al*, 1992; Rasool *et al*, 2002; Cooper *et al*, 1999; Vinje *et al*,

2000; Noel *et al*, 1995). Multiplexing was attempted in order to reduce the number of steps involved in the testing process and thus improve rapidity, ease of use and throughput (as well as reducing costs). Real-time PCR methods were not considered at this time since the decision had been taken to prioritise use of the only available real-time platform in the laboratory for use in the norovirus outbreak service.

In 2003, when the other real-time platforms became available in the laboratory the possibility of implementing real-time PCR for all tests in the sporadic service was examined. Here the decision was taken to move straight to dual-labelled probed-based technology, rather than SYBR green based assays, in line with the ongoing developments in tests for the outbreak service. Where possible these assays were chosen from the literature (Pang *et al*, 2004; Le Cann *et al*, 2004; Heim *et al*, 2003). However, no such assay was available for sapovirus and thus a novel in-house method was designed (Gunson, Collins and Carman, 2006(i)). The new method detected sapovirus G1 types only. However, although it was known that this test would miss non-G1 types, data from the HPA and other studies suggested that this was reasonable, given that sapovirus G1 types were the most predominant strains pre-2004 (Okada *et al*, 2002; Gallimore *et al*, 2006).

Following selection and adaptation of the appropriate tests a series of studies was then carried out to assess the sensitivity of each assay in comparison to existing and possible alternative methods (see Chapter 4). Results from the first experiment relating to the outbreak service showed that the SYBR green based Lightcycler™ RT-PCR assay developed as described in Chapter 3 was more sensitive than EM for the detection of norovirus, in both samples from outbreaks and sporadic cases of IID (Miller, Gunson and Carman, 2002). This was the first real-time RT-PCR for norovirus to be published at the time and was implemented as the routine test for outbreak samples in the WOSSVC laboratory in January 2001. It was subsequently compared to two commercial EIA methods for norovirus and was found to be both more sensitive and specific than either (Gunson, Miller and Carman, 2003(ii)). This study was one of the first to show the relatively poor sensitivity and specificity of the EIA methods, findings that have subsequently been confirmed in other studies (Wilhelmi *et al*, 2007; Castriciano *et al*, 2007; Dimitriadis, Bruggink and Marshall, 2006; Dimitriadis and Marshall, 2005). The Lightcycler™ method was also compared to a recommended gel-based RT-PCR that was held to be the most sensitive of the gel-based methods available at that time (Vinje *et al*, 2003). However, although the

Lightcycler™ RT-PCR assay was indeed shown to be less sensitive it did not miss any outbreaks and, moreover, involved fewer and shorter steps than the gel-based assay (Gunson, Miller and Carman, 2003 (i)). As a result the decision was taken to retain it as the frontline test for norovirus in the outbreak service at this stage.

The final experiment in this series, carried out in 2003 once the relevant technology became available, showed that the new multiplexed dual-labelled probe-based assay for norovirus (based on the test published by Kageyama *et al*, 2003) was also more sensitive than the Lightcycler™ RT-PCR, which in this case missed both individual cases and outbreaks of norovirus. The dual-labelled probe-based RT-PCR also had the potential to offer better throughput and rapidity than the Lightcycler™ test. As a result it was implemented in 2004 and remains the frontline test for norovirus in the outbreak service at the time of writing.

The initial experiment for the sporadic service focused on multiplexing the individual assays for adenovirus, astrovirus, rotavirus and sapovirus chosen and adapted as described in Chapter 3. The results, however, showed that the rotavirus RT-PCR could not be successfully multiplexed as the primers interacted with and reduced the sensitivity of the astrovirus and sapovirus RT-PCR assays. Instead, the multiplexing process resulted in two RT-PCR assays: a rotavirus RT-PCR and a novel multiplex RT-PCR for adenovirus, astrovirus and sapovirus.

The two assays developed were then compared to antigen detection methods for astrovirus and rotavirus, and shown to be more sensitive than either (Gunson *et al*, 2003(iii), Gunson *et al*, 2003(iv)). However, the number of steps involved in carrying out the two gel-based assays plus concerns over the appearance of non-specific products in the multiplex RT-PCR meant that these were not implemented in the sporadic service at this stage.

In the final experiment in this series, again carried out once the technology became available, the two gel-based RT-PCR methods were compared to the dual-labelled probe-based real-time PCR assays selected (or developed in the case of sapovirus) as described in Chapter 3. The results showed that for each pathogen the real-time PCR assay detected a lower dilution of pathogen and identified more clinical samples as positive than the relevant gel-based assay (Gunson and Carman, 2005). The dual-labelled probe-based approach also involved fewer, shorter steps and had the potential to offer better throughput and rapidity than the EM test still in use in the routine service at this time. As a result these assays were implemented in the service

for sporadic cases of IID in November 2004, alongside the dual-labelled probe-based norovirus RT-PCR. From this date EM was used solely to investigate outbreaks that tested negative for a viral pathogen using all of the PCR methods available.

Following implementation of the dual-labelled probe-based real-time PCR methods in the routine service a number of experiments were carried out that attempted to further modify these tests in order to reduce turn-around-times, improve throughput and reduce cost (see Chapter 4). In the first experiment it was shown that reducing PCR cycling times had no effect on test sensitivity and improved rapidity considerably (e.g. reducing turn-around-times from 100 minutes to 60 minutes). The second experiment showed that the test for adenovirus, a DNA test, could be carried out under the same conditions (i.e. on the same PCR plate) as any of the RNA tests for astrovirus, rotavirus or sapovirus without affecting its sensitivity, thus allowing more tests to be carried out per PCR run. (The norovirus assay involved a different annealing temperature to any of the other tests and as a result was always kept separate). Further studies then showed that pooled positive controls, primer/probe pools and reduced reagent volumes could all be employed without affecting test performance, thereby improving test efficiency (Gunson, Collins and Carman, 2006(ii)). Use of these measures was therefore implemented in January 2005.

The effect of implementing the various changes on the WOSSVC laboratory diagnostic service for IID was then examined, as described in Chapter 5. Sample submission data from the period 1999-2006 showed that since the introduction of molecular tests for the detection of viral pathogens in cases of gastroenteritis the number of samples tested by the routine diagnostic service has risen steadily. In 1999, when EM was the frontline test, a total of 887 IID samples from outbreaks and 668 from sporadic cases were submitted for testing at the WOSSVC. In the years following implementation of the LightcyclerTM real-time assay for norovirus in the outbreak service the number of outbreak samples tested rose to between 1112-4025 (the latter figure a peak in activity due the emergence of a new worldwide variant of norovirus in 2002). The rise in the number of outbreak samples tested continued following implementation of the dual-labelled probe-based assay in 2004, from 2033 to 3189 in 2006. Similar patterns were observed in the submission rates for samples from sporadic cases of IID, although some caution should be applied in interpreting the results for this service since the time period involved since the introduction of the new tests has been relatively short. In 1999, a total of 668 samples from sporadic

cases were tested, rising to 1153 in 2006 following introduction of real-time PCR tests to the sporadic service. The increased use of the service reflects greater use of the service by existing users (health boards), an increasing number of health boards submitting samples to the laboratory for diagnosis, and an increase in sample submission from immunocompromised patients and adult patients.

In addition to increased submission rates, the detection rates in the routine service have risen for all viral pathogens following implementation of the PCR methods, in both outbreaks and sporadic cases of gastroenteritis. For example, for samples submitted from outbreaks of IID the norovirus detection rate achieved by EM was always below 12%. However, from 2001-2003, when the Lightcycler™ RT-PCR was the frontline test, the detection rate was between 28-35%. A slight further improvement in detection rate was observed after the implementation of the dual-labelled probe-based real-time RT-PCR assay in 2004, with a detection rate of between 28-41%. The improved detection rate resulted in an increase in the number of outbreaks confirmed to be caused by norovirus, from a total of 15 and 16 in the years 1999 and 2000 respectively, compared to between 83-223 in the 6 years post-implementation of the PCR-based service. Similar improvements were observed after the implementation of the PCR service for sporadic cases of IID. For example, in the years when EM was the frontline test the overall detection rate was between 2-23%, whereas following implementation of the real-time PCR assays the figure was between 48-52%.

These improvements, moreover, were achieved at the same time as a substantial reduction in sample turn-around-times. For example, when EM was the frontline test for outbreaks of gastroenteritis in 1999 and 2000, 50% of results were reported between 5 to 7 days after arriving in the laboratory, whilst 90% were reported between 11 to 13 days after submission. Following implementation of the Lightcycler™ real-time RT-PCR for norovirus in the period 2001-2003 a considerable improvement in these turn-around-times was observed, with 50% of results available by day 5 and 90% reported by day 8 in each of these years. The implementation of the dual-labelled probe-based real-time RT-PCR in 2004 resulted in further improvements, with 50% of outbreak results reported just 3 days after submission and 90% of sample results reported by day 6 during this year. These reduced turn-around-times were maintained despite fluctuations in sample submissions. Finally, the implementation of the test amendments, e.g. pooled controls, in 2005 saw further

reductions in outbreak sample turn-around-times. The final outcome was that at the time of writing 50% of outbreak sample results are reported by day 2 with 90% reported by day 5 (this was achieved in two consecutive years) compared to best results of day 5 and day 11 respectively when EM was the frontline test for outbreaks.

Similar improvements in turn-around-times were observed for samples from sporadic cases IID. For example, between 1999 and 2004, use of EM resulted in 50% of samples being processed by days 5 to 9, but 90% was not achieved until days 11 to 23. At first, use of the new PCR-based service resulted in similar turn-around-times in 2005 (50% of results were available by day 9 and 90% by day 23). In 2006, however, the numbers of samples of this type submitted to the laboratory increased and as a result batch testing was increased to at least three times a week. This, together with the test amendments outlined above led to a substantial improvement in turn-around-times, with 50% of results available after day 3 and 90% by day 6.

6.2. Main Findings

In conclusion, the implementation of a molecular diagnostic service for viral causes of IID at the WOSSVC has enabled more stool samples to be tested in shorter periods of time, using more sensitive tests than any previously available.

Whilst it is possible that some of the increases in sample submission and detection rate observed for the PCR-based service were due to increased disease activity in the years examined, the size and consistency of the increases over a period of several years suggests this was not the determining factor. Moreover, the possibility that PCR techniques may be over-sensitive (discussed in Chapter 2) - detecting asymptomatic IID or prolonged shedding following resolution of symptoms in a minority of cases - is less likely to apply in the routine diagnostic setting, where samples are only tested as a result of patients presenting with the symptoms of IID. In any case, the detection of prolonged or asymptomatic shedding in patients may still be of value in this context, since these patients may pose an infectious risk to others. This is particularly important in an outbreak setting where detecting those shedding virus in the absence of symptoms may aid infection control procedures (Gallimore *et al*, 2004).

Although previous research has shown the improved sensitivity of PCR in comparison to alternative methods such as EM and EIA, to date these studies have

focused on use of the tests in a research setting, based on relatively small numbers of samples and carried out by highly specialised researchers. The research described in this thesis, in contrast, has demonstrated the increased sensitivity of PCR in comparison to EM based on high volumes of samples in a routine diagnostic setting where the tests are often carried out by less specialised staff.

Moreover, the research has also shown that such sensitivity can be achieved at the same time as reduced turn-around-times in comparison to EM. Studies showing the positive effect of real-time PCR methods on rapidity and throughput (and, by implication, ease of use) are largely absent from the published literature despite the fact that these factors can be very important, since they determine whether a test is suitable for a routine diagnostic setting and how clinically useful it will be (discussed in more detail in the following section).

Diagnostic laboratories have in the past been reluctant to implement PCR assays as a result of many factors, most of them associated with negative perceptions or experiences of conventional gel-based PCR (see Chapter 2 for full details). The results shown here, however, have demonstrated that real-time PCR can overcome many of these difficulties. The real-time PCR methods introduced at WOSSVC have proven to be both robust and capable of high throughput, indicating relative ease of use. (In particular, the use of a specific labelled probe in the dual-labelled probe-based assays was found to aid ease of interpretation in comparison to a SYBR green based approach.) Moreover, several techniques for improving the efficiency of the real-time tests without affecting their sensitivity have been successfully demonstrated (Gunson, Collins and Carman, 2006(ii)). Such techniques are largely ignored in the scientific literature but are of high practical value in a diagnostic setting.

One drawback of PCR remains, however, which is that the implementation of such methods still requires technical expertise in quality control (see Chapter 5). All PCR assays, and in particular real-time PCR assays, should be monitored carefully to detect any contamination, or sudden or long-term loss in sensitivity. Either could result in false negative or positive results. However, to ease this problem there are now a number of useful guides available that describe monitoring systems such as those used here (described in Chapter 5), and which are relatively easy to implement and maintain (Gunson, Collins and Carman, 2005; Niesters, 2002; Gray *et al*, 1995; Gray, 1999).

In addition to the benefits seen within the laboratory itself, the wider usefulness of the new molecular service for norovirus in outbreaks of IID is indicated by the fact that a number of other Scottish Specialist Virology Centres or SVCs (Aberdeen, Dundee and Edinburgh) have begun to use the WOSSVC service for norovirus outbreaks or plan to do so in the near future, despite having their own testing capability. Moreover, since publications relating to the research carried out here, which were among the first to show the practical advantages of both the Lightcycler™ and dual-labelled probe-based real-time RT-PCR methods, and the reduced sensitivity and specificity of commercial EIA methods for norovirus (Miller, Gunson and Carman, 2002; Gunson, Miller and Carman, 2003) a number of virology laboratories have replaced or are about to replace EM or commercial EIA methods for the detection of viral pathogens in outbreak samples with the real-time PCR test for norovirus described here.

In the sporadic setting the real-time PCR tests implemented have proven useful for laboratories with limited or no testing protocol of their own. They have also proven useful in the BMT setting for the detection of viral IID in immunocompromised patients, where the sensitivity of EM and EIA may not be good enough (Cubitt *et al*, 1999). At the time of writing no other laboratory in Scotland offers real-time PCR testing for viral causes of sporadic IID. The WOSSVC, in fact, provides the only service in Scotland that tests for all five main causes of viral IID.

It is not only virology laboratories that can benefit from use of the PCR tests described here. The WOSSVC is currently involved in a pilot study to outsource real-time PCR tests to microbiology laboratories affiliated to District General Hospitals (DGHs). The first phase of the study will examine the effect of implementing the WOSSVC real-time RT-PCR for norovirus in two DGH sites. Each site will be provided with a “kit” for the test containing the standard operating procedures, pre-optimised primer/probe pools, and PCR controls and reagents. The WOSSVC will also offer full training to laboratory staff and a troubleshooting service. To facilitate the implementation of the test the laboratories involved have obtained automated extraction equipment and PCR platforms similar to those in use at the WOSSVC. Having a norovirus service on-site at the DGH will negate the need to send samples to the WOSSVC and therefore has the potential to improve the rapidity of sample results significantly. For clinicians, access to a sensitive and rapid service for norovirus will aid patient management and infection control decisions (see below for a detailed

discussion). In the laboratory, supported implementation of the test will provide useful experience of real-time PCR methodology, which in future could be used in other areas of their service. The plan is that, if successful, the project may be widened to include other sites and tests.

Finally, awareness of the benefits of implementing real-time PCR, demonstrated in this research, has been used to improve other diagnostic services in the WOSSVC laboratory. Based on the successful experience of implementing real-time PCR in the norovirus outbreak service, for example, in 2004 conventional RT-PCR assays for the detection of viral and bacterial respiratory infections were replaced in the laboratory with a number of real-time PCR methods (Gunson *et al*, 2005). Since their implementation significant improvements have been observed in throughput, rapidity and overall detection rates. This service also benefited from the test amendments described in Chapter 4, which once implemented led to further improvements in rapidity, ease of use and cost. Similar real-time PCR systems have also been developed in the laboratory to test for viral pathogens in other sample types and/or patient groups (e.g. CSF samples and plasma from BMT patients). Plans for further use of this technology in the laboratory include extending its use to other areas of microbiology such as rapid MRSA screening (Gilpin *et al*, 2007), bacterial gastroenteritis (Wang and Mestaphai, 2007; Shannon *et al*, 2007) and bacterial sepsis (Stratidis, Bia and Edberg, 2007).

6.3. Implications For Patient Management

The changes in the WOSSVC diagnostic service for viral causes of IID introduced in this research are likely to aid patient management on a number of fronts. More sensitive testing, for example, increases the likelihood of obtaining an accurate diagnosis, which enables clinicians to confirm the initial clinical diagnosis and to exclude other, more serious causes of IID or non infectious causes of intestinal illness (Fine *et al*, 1998). A more rapid, accurate result also enables the clinician to correctly advise the patient on the likely duration of illness, its infectiousness and how they can reduce the likelihood of further spread to other members of the public (Guerrant *et al*, 2001). Patients, for example, can be advised on the sources of IID (e.g. soiled nappies) and informed that simple procedures such as frequent hand washing can reduce the risk of infection.

Accurate management based on a laboratory diagnosis is particularly important if the patient poses a public health hazard, since such patients may also require follow up testing before returning to their place of work. This applies to patients working in the food industry or the National Health Service (Bresee *et al*, 2002), children attending nursery school or elderly residents in a care home. The ability to swiftly advise a patient in such matters based on an accurate laboratory result will help prevent further infections, which in turn saves on further health care expenditure related to, for example, GP/hospital visits by newly infected patients. Furthermore, the ability to provide a result rapidly can help reduce the number of unnecessary tests that are carried out in the meantime, and in those with more serious illness may help prevent unnecessary hospitalisation or, if already hospitalised, may mean a reduction in the length of stay. All of these factors contribute to reducing unnecessary health care costs as well as improved care for the patient.

Access to a more rapid and sensitive service also has benefits in reducing the prescription of unnecessary treatments. Although not generally recommended for treating IID, particularly in children and for patients managed in the primary care setting, a recent study in Ireland found that approximately 7.5% of patients who consulted a GP for symptoms of IID received empirical therapy, which represented 15% of all treatments prescribed (Acute Gastroenteritis in Ireland, North and South - A Telephone Study, 2003). This is despite the increasing evidence that shows viruses to cause much of the illness in all age groups (Phan *et al*, 2005; Bon *et al*, 1999; Bereciartu, Bok and Gomez, 2002; Qiao *et al*, 1999; Oh, Gaedicke and Schreier, 2003; de Wit *et al*, 2001 (i); de Wit *et al*, 2001 (ii)) and the fact that antibiotics may, in some bacterial infections, result in more severe or prolonged symptoms (Armon *et al*, 2001). Those with travel associated IID, immunocompromised patients with IID and patients hospitalised due to IID are also likely to receive empirical anti-microbial treatment (Guerrant *et al*, 2001), despite data showing that viral pathogens can cause many of these cases (Keswick *et al*, 1982; Vollet *et al*, 1979; Bolivar *et al*, 1979; Steffen *et al*, 1999; Chapin *et al*, 2005). Consequently, access to a more rapid and sensitive viral diagnosis service can help encourage more judicious use of antibiotics, potentially reducing the number of patients receiving unnecessary treatment and/or reducing the duration of treatment (Armon *et al*, 2001). This will both reduce costs and help prevent the emergence of antimicrobial resistance (Holmberg *et al*, 1984; Cohen and Tauxe, 1986).

For all of this to be effective, however, it is important that clinicians receive laboratory results as early as possible after the onset of symptoms, and while the illness is still occurring. The sensitivity of the testing is key in providing an accurate diagnosis, but unless clinicians and public health managers receive test results within a clinically useful timeframe these are of little practical value either to them or to the patient.

Previously, using the EM-based service the best turn-around-times achieved for samples from cases of IID were 50% of results reported by day 5 and 90% reported by day 11 (the figures were the same for both outbreak and sporadic samples, although turn-around-times for the latter varied considerably more and could be as high as day 9 for 50% of results and day 23 for 90%). Based on these figures, it is likely that in a large proportion of cases the laboratory diagnosis was not received by clinicians until after resolution of the illness - too late to take steps to prevent the spread of the infection and/or unnecessary treatment/testing/hospitalisation. Following implementation of the new PCR-based service, on the other hand, turn-around-times for outbreak samples were reduced to days 2 and 5 respectively (50% and 90% of results reported), and days 3 and 6 for sporadic samples. Consequently, results from the new service are far more likely to be of value to clinicians in providing appropriate patient care. This increased utility is likely to have been a factor in the increased uptake of the service i.e. the increase in sample submission rates observed following introduction of the new tests.

One of the main advantages of a rapid diagnostic result in any context is that it allows the appropriate treatment, (where available) to be started when the illness is occurring, the pathogen is present and when treatment is likely to be most useful. However, historically rapid and sensitive tests for the majority of viral pathogens have not been available and as a result anti-viral therapies have been mainly restricted to the treatment of those viral pathogens known to cause chronic infections such as HBV, HCV and HIV (Waugh *et al*, 2002; O'Shea, 2007; Sandrock and Kelly, 2007). In recent years, improvements in the rapidity and sensitivity of routine diagnostic tests for some viral pathogens have led to the emergence of new therapies. For example, the availability of rapid and sensitive tests for influenza such as DIF, bedside tests and real-time PCR have led to the development of new anti-influenza therapies. Examples include oseltamivir, a neuraminidase inhibitor that has been shown to be most effective when given within 48 hours of disease onset (Waugh *et al*, 2002; Mitamura,

2007). Similarly, the availability of rapid and sensitive tests has made the use of pre-emptive therapies feasible for CMV, adenovirus and EBV-related disease in BMT patients (Cochrane, 2006; Baillie, 2006; Okano and Gross, 2007; Neofytos *et al*, 2007).

The improvement in rapidity offered by the real-time PCR service implemented in this research can thus help create demand for the development of new anti-viral agents aimed at reducing the clinical severity of IID, the duration of infection or at preventing infection altogether (Estes *et al*, 2006) – particularly since data from the telephone survey suggest that other testing centres also intend to utilise or replicate the service. Such drugs could be used, for instance, to treat those with severe disease (to reduce severity or duration of symptoms) or could be used prophylactically in the outbreak setting to reduce the number of cases and further spread. Recent studies using nitrazoxanide, a thiazolidine anti-infective agent, have shown it to reduce the duration of symptoms due to norovirus and rotavirus in paediatric patients (Rossignol and Gohary, 2006; Rossignol *et al*, 2006).

6.4. Implications For Infection Control

The new molecular service for IID is also likely to be beneficial in the outbreak setting as the increased rapidity and sensitivity of the service allows timely, focused implementation of control measures that are recommended by published guidelines and the previous experience of infection control teams (MMWR weekly, 2005). Previously, the turn-around-times of the EM-based service (where the best performance achieved for outbreak samples was 50% of results reported by day 5 and 90% reported by day 11) were such that it was of limited use in this context, since results would often be obtained too late to inform decisions on possible control measures. As discussed in Chapter 2, the quicker infection control procedures can be implemented following the start of a norovirus outbreak the more likely it is that these will be effective. One estimate, for example, is that control procedures must be implemented within 3 days if they are to be successful (Lopman *et al*, 2003). The improved turn-around-times of the PCR-based service (50% of results reported by day 2 and 90% by day 5), therefore, mean that the test results are now much more likely to be of value in this setting.

Even where results were reported 'in time' to be of use, moreover, the poor detection rate of the EM-based service for outbreaks (a total of 15-16 outbreaks confirmed in the years 1999-2000 compared to between 83-223 in the 6 years post-implementation of the PCR-based service) meant that false negative results were more likely and thus may have led to under-implementation of infection control procedures.

Based on the new real-time PCR service, swift and confident knowledge of the actual cause of an outbreak can help persuade ward managers to implement expensive control procedures such as short-term ward closure. It can also help exclude other, potentially more serious, causes of outbreaks that may require different infection control measures. Once an outbreak of norovirus has been confirmed, moreover, the improved rapidity and sensitivity of the PCR tests described here can also aid ward management in other ways. Screening, for example, can be used to aid decisions on staff deployment and patient cohorting or isolation measures, by identifying those with symptomatic and asymptomatic infection (Gallimore *et al*, 2004).

The rapid and sensitive testing provided by the PCR-based service may also allow new forms of infection control to be employed in future. A holding ward, for example, could be used to temporarily house all newly admitted patients with symptoms of gastroenteritis. A rapid test could then be carried out to exclude norovirus or other viral infection before allowing the patient to be transferred to another ward. Norovirus-positive cases could remain in the holding ward until the risk of infection was past, thereby preventing spread to others within the hospital.

In future, screening could also be considered for Health Care Workers (HCWs) before allowing them to return to work after a period of illness due to viral IID. Most current guidelines for norovirus infections suggest a worker should not return to work until 48-72 hours after clinical symptoms have abated (Joint Working Group from NHS Boards, Local Authorities and SCIEH, 2003). A further development of this would be to screen the returning HCWs to identify those with prolonged shedding, preventing these individuals from returning to work until the risk of infection had abated.

The new PCR-based service could also be used in future to test environmental surfaces for viral contamination, thus measuring the effectiveness of infection control procedures such as cleaning. Several studies have established that contaminated fomites can be responsible for prolonging or reseeding outbreaks in closed settings (Kussi, 2002; Evans, 2002), although the relationship between the presence of virus

on an inanimate surface and infectivity has yet to be fully proven. Areas at risk include much-handled items such as door handles, lockers, taps, soft furnishings, floors, walls, soiled linen and commodes (Green, 1997; Gallimore *et al*, 2006). Whilst in theory most of these areas can be cleaned using already established procedures, in practice the cleaning is often poorly executed and/or external pressures to re-open a ward can prevent the proper procedures from being carried out (Christie, 2002; Cowden, 2002). The rapidity of the new PCR service, however, means that in future the effectiveness of the cleaning process could be tested using environmental swabs before a ward is re-opened, thereby encouraging strict adherence to procedure and reducing the risk of outbreaks recurring.

6.5. Implications For Disease Surveillance

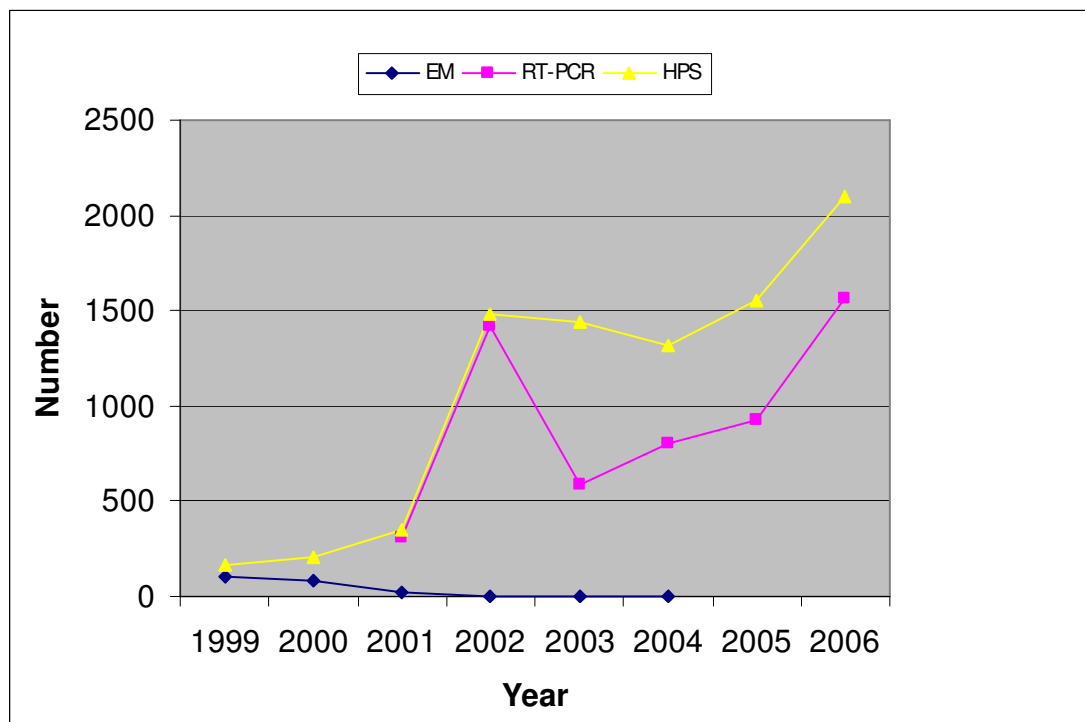
At present the surveillance of viral and other causes of IID in Scotland is carried out by Health Protection Scotland (HPS), formerly known as the Scottish Centre of Infection and Environmental Health (SCIEH). HPS collect data on cases of IID from three surveillance systems: the national surveillance scheme for laboratory confirmed infections, the national surveillance scheme for general outbreaks of IID, and the notifications of food poisoning system (Wall *et al*, 1996). Two of these systems utilise laboratory data (the national surveillance scheme for laboratory confirmed infections and the national surveillance scheme for general outbreaks of IID) and are therefore likely to have been affected by the changes in the WOSSVC service described in this thesis.

The first of these schemes, the national surveillance system for laboratory confirmed infections, is a voluntary system operated by HPS that aims to detect unusual outbreaks or new trends associated with a particular pathogen. It relies on clinical microbiology laboratories in Scotland reporting all laboratory-confirmed infections to the HPS. In each case laboratories are asked to submit the following data: patient details (surname and forename or, if these are not available, the laboratory number can be submitted instead), organism and type, the age of the patient, NHS board of origin, and clinical details. The HPS provide a standard form requesting these data, although some laboratories send their own form to the HPS. The report is sent on a weekly basis to the HPS by post, email or fax. The data collected from these reports are then examined by health board and for the nation as a

whole, and are compared to historical data in order to identify any outbreaks or new trends associated with a particular pathogen.

The data collected by the system are skewed by a number of factors. For example, not all cases of viral IID occurring in the community are likely to seek medical attention (Wheeler *et al*, 1999; Wall *et al*, 1996; WHO 7th report). Of those that do, most are likely to be suffering from severe infections and to be from particular at-risk patient groups (e.g. infants and the elderly). The GP/hospital sample submission rate is also important, as not all patients who present to GPs/hospitals have a sample submitted for laboratory investigation. Where a sample is submitted the testing protocol of the laboratory is very important. The absence of a test for a particular pathogen or the routine use of an insensitive diagnostic assay increases the chance of underestimating the presence of some pathogens in the community.

Figure 26: Number of norovirus detections made at the WOSSVC vs total reported by the HPS surveillance system (1999-2006).



Comparing the number of norovirus detections made by the WOSSVC service to that collected by the national surveillance scheme for laboratory confirmed infections (Figure 26) suggests that the WOSSVC service is a major contributor to this surveillance system. In 2001 and 2002, in fact, when real-time PCR was first introduced in the laboratory, almost all reported cases were likely to have originated from the WOSSVC. In subsequent years, once other laboratories began to replace EM

with more sensitive tests e.g. EIA (or introduce a norovirus testing protocol), the proportion of cases contributed by the WOSSVC initially fell but has since risen and continues to represent more than 50% of all reported cases. Thus the improvements observed in this research in terms of rapidity, detection rate and uptake of the service since the transition to real-time PCR are likely to substantially improve the quality of the HPS surveillance data. The increase in service sensitivity, together with the rise in the number of individuals tested from a greater range of health boards, allows a more accurate picture of the role of norovirus-related illness to be drawn, in different age groups, health board regions and in Scotland as a whole. The increased rapidity of the service, moreover, will help improve the timeliness of the surveillance data, creating a more accurate picture of when cases are occurring. Taken together, these improvements can aid the identification of unusual trends such as the large increases in disease activity observed as a result of the emergence of a new worldwide norovirus variant in 2002 and again in 2006 (Lopman *et al*, 2004; Gallimore *et al*, 2007).

The improved timeliness of the surveillance data will also increase public health officials' ability to investigate unusual outbreaks of norovirus whilst these are still occurring (and thus implement suitable control measures). Possible examples include outbreaks of norovirus where the source is a pre-packaged food that is widely distributed e.g. through a supermarket chain, and which causes illness over a wide geographic area that can only be linked via a national surveillance scheme.

For non-norovirus causes of IID the new PCR-based service in the laboratory is likely to have less impact on the data collected by the national surveillance of laboratory confirmed infections, for a number of reasons. Between 2004 and 2006, for example, when real-time PCR for non-norovirus pathogens was first introduced in the laboratory, the rotavirus and adenovirus detections reported by the WOSSVC contributed only a tiny proportion of the total number of cases reported to the HPS surveillance scheme (Figure 27). In the case of rotavirus, this can largely be attributed to the fact that many laboratories already screen for rotavirus (as shown in the results of the telephone survey described in Chapter 5) and report their cases to the HPS directly. Samples that are submitted to the WOSSVC tend to come from a small number of laboratories with no rotavirus protocol of their own, or have already been screened for rotavirus pre-submission. In the case of adenovirus, meanwhile, cases reported to the HPS include detections from all types of adenovirus-related diseases

(e.g. eye infections and respiratory infections) since the HPS system does not require respondents to distinguish between the different types. No attempt is made in the system to distinguish between trends in adenovirus related to IID and those related to other diseases. Thus, although the WOSSVC is one of the few laboratories to test for adenovirus in samples from cases of IID (as shown in the results of the telephone survey described in Chapter 5) the contribution made by these detections to the overall figures is tiny, and is too small to have a significant impact on the analysis of trends in adenovirus.

Comparing the number of astrovirus and sapovirus detections reported by the WOSSVC since the introduction of real-time PCR for their detection in late 2004 with the number collected by the HPS surveillance system in the same period (Figure 28 and Figure 29) confirms that the WOSSVC is the only laboratory currently able to test for these pathogens, as suggested by the telephone survey described in Chapter 5. (In fact, in some cases the number detected by the WOSSVC exceeded the number published by the HPS, either due to faulty reporting by the WOSSVC or failure to include all reported cases by the HPS.) Prior to this (between 1999-2003), EM was used to test for astrovirus and sapovirus in a number of laboratories including the WOSSVC, but was gradually phased out in other laboratories in favour of focusing on alternative tests (mostly EIA) for the detection of rotavirus. However, despite the fact that the WOSSVC is currently the only laboratory to test for these pathogens in sporadic cases of IID, the number of samples submitted to this service and hence the rate of detections has been, to date, too low for the HPS to determine any useful epidemiological patterns - although the situation may improve as more samples from sporadic cases of IID are submitted to the WOSSVC service, of which there were some early indications in 2006.

Figure 27: Number of rotavirus and adenovirus detections made at the WOSSVC vs total reported by the HPS surveillance system (1999-2006).

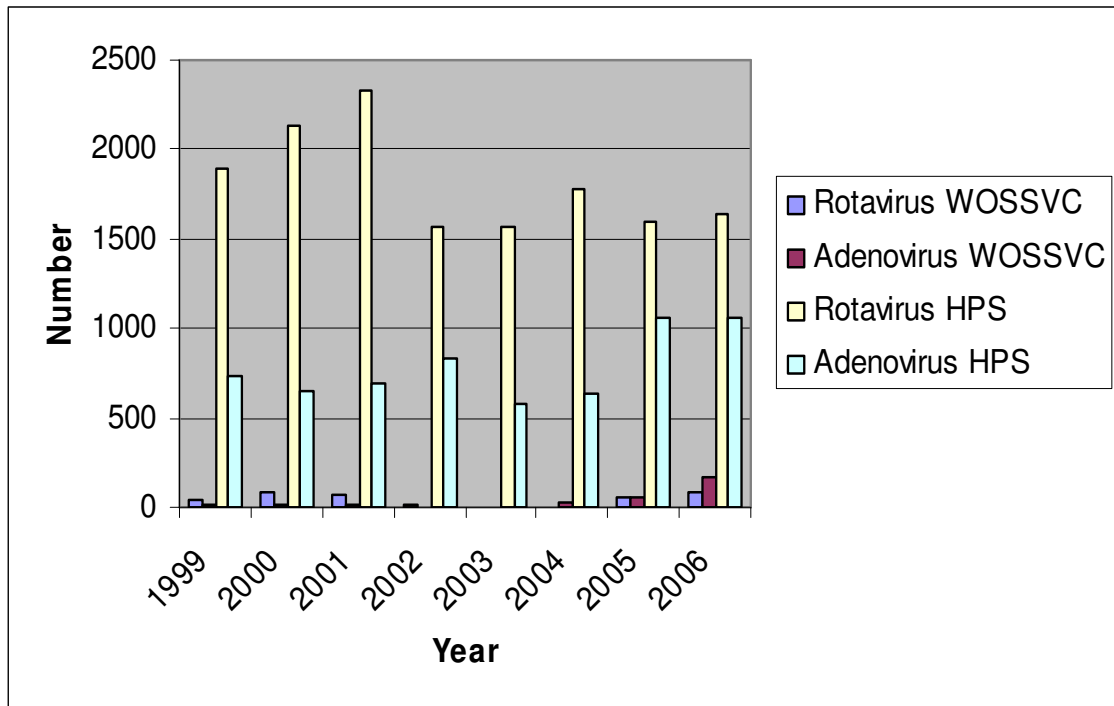


Figure 28: Number of astrovirus detections made at the WOSSVC vs total reported by the HPS surveillance system (1999-2006).

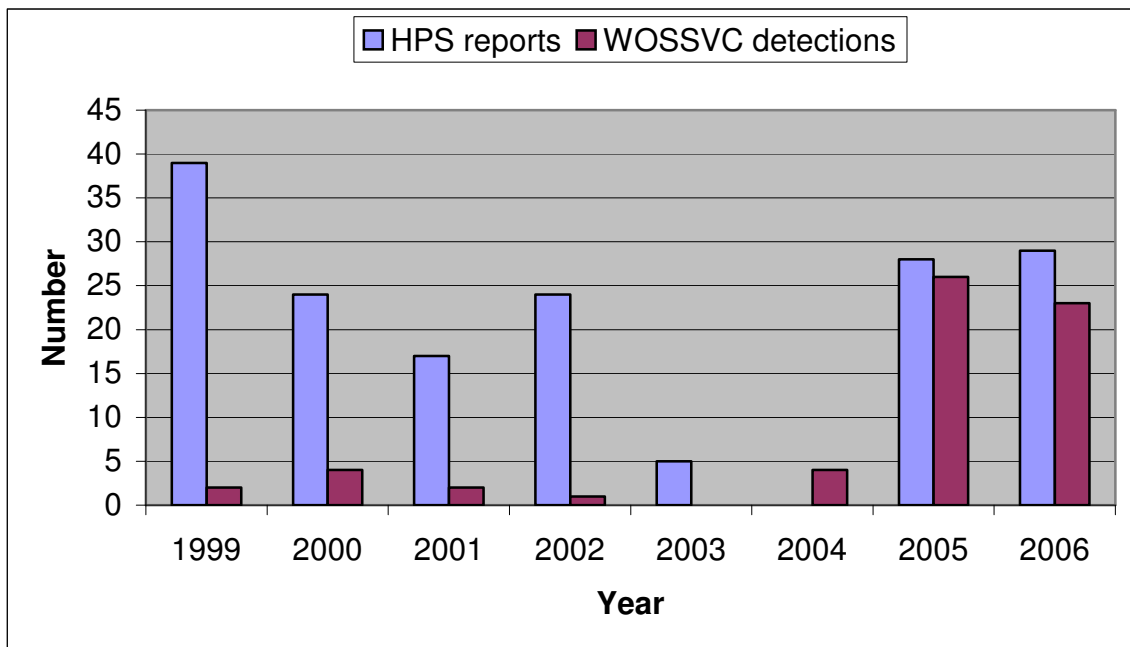
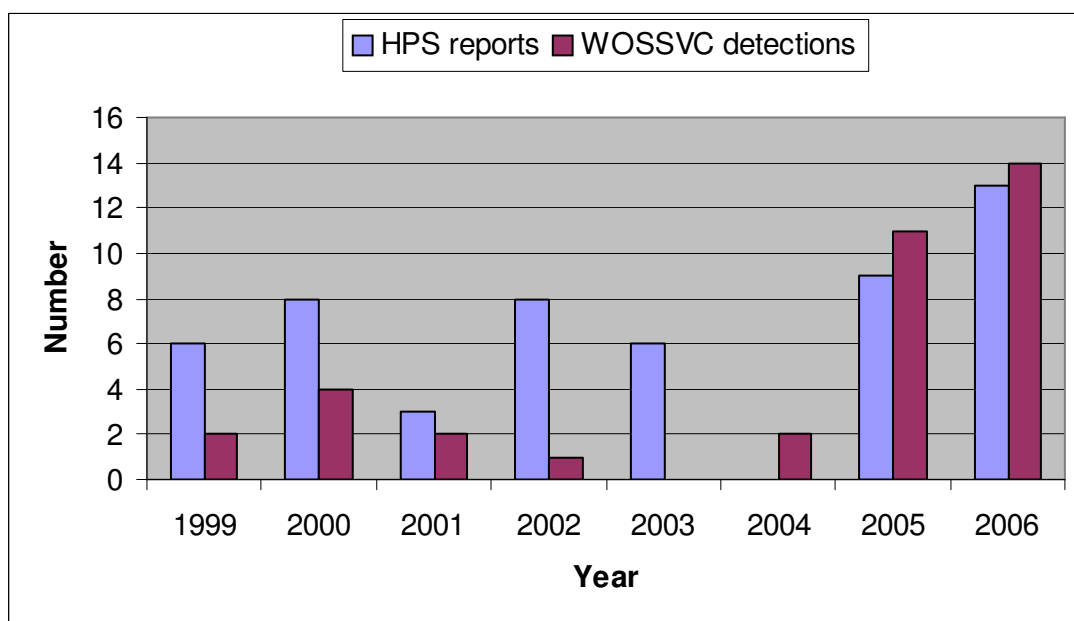


Figure 29: Number of sapovirus detections made at the WOSSVC vs total reported by the HPS surveillance system (1999-2006).



The national surveillance scheme for general outbreaks of IID is another voluntary system operated by the HPS that employs laboratory data. It aims specifically to provide information useful for formulating infection control and prevention policies. Under this scheme, all preliminary reports of suspected ‘general outbreaks’ of IID from laboratories, Consultants in Public Health Medicine (CPHMs), Environmental Health Officers (EHOs) and other sources (for example, news reports, members of the public, hospitals etc.) are investigated. A general outbreak of IID is defined in this context as “*affecting members of more than one private residence, or residents of an institution*” and is considered distinct from family outbreaks. Following the report of a suspected outbreak the HPS sends out a standardised questionnaire to the appropriate lead investigator. The data requested in the questionnaire include the causal pathogen (laboratory-confirmed, suspected or unknown), the vehicle of transmission and source, number of persons involved in the outbreak, number of cases confirmed using laboratory methods, and the NHS board of origin. Once complete, the form can be submitted by mail, fax or email.

Unlike the national surveillance scheme for laboratory confirmed infections, in this system the recording and reporting of IID outbreak activity is not dependent on laboratory results. Outbreaks of gastroenteritis can be included in the final collected data regardless of whether or not any patient samples have been submitted to a laboratory for testing. As a result, the new molecular services implemented at the

WOSSVC as part of this research are likely to have less impact on the outbreak data collected by this surveillance system than on the previous scheme described. However, laboratory confirmation (or otherwise) of the viral cause of the outbreak remains a component in the system and this is dependent on the quality of the laboratory tests employed. As a result, the increased sensitivity of the PCR-based service for the detection of norovirus (the principle cause of outbreaks of IID) is likely to help increase the number of outbreaks proven to be caused by norovirus, thereby improving the quality of the collected data. This will help contribute to a more accurate measure of the role, epidemiology and public health effect of norovirus outbreaks in Scotland, which in turn will provide more accurate information on which to base infection control and prevention policies.

The availability of a rapid, sensitive diagnostic service for norovirus such as that developed in this research could also be used as the basis for new surveillance systems for viral IID. One possible system would be to recruit a small number of GPs throughout Scotland to act as sentinel sites (Clothier *et al*, 2005). Each GP would be paid to submit all or a defined number of IID samples each week to their nearest Specialist Virology Centre (or a central facility) for testing. Results would then be returned both to the GPs and the HPS. Thus, instead of relying on individual clinicians' awareness of the tests available for IID to ensure that samples are submitted for testing, surveillance of community acquired viral IID in Scotland would be based on regular, systematic collection of data from a representative set of geographic locations. While it is possible that such a system could be implemented based on tests other than PCR, the use of molecular methods means that the data are more likely to accurately represent the level of infection in the community and the times at which it occurs, thus increasing the likelihood of the necessary investment in the system. A similar PCR-based system is already in place for the surveillance of viral respiratory infections in Scotland³. Other possible sentinel sites could include microbiological laboratories, schools or accident and emergency departments (Takahashi *et al*, 2001).

³ <http://www.hps.scot.nhs.uk/resp/influenzaseason.aspx#background>

6.6. Future Work

There are a number of areas in which the molecular service for IID developed as described in this thesis would benefit from further research.

Some of this research is already underway. For example, as mentioned in Chapter 5, the current WOSSVC molecular service for IID does not include an internal control, largely because of concerns relating to the increased cost and complexity of using such a system at the time. Research is now underway, however, to investigate the use of an internal control system, based on the recent advent of specialised commercial kits for multiplex real-time PCR (see below).

Research is currently underway at the WOSSVC on a laboratory-wide internal control system that uses murine cytomegalovirus (mCMV) for DNA tests and the plant virus brome mosaic virus (BMV) for RNA tests. The aim is to add these viruses to the sample lysis buffer, which will then be automatically added to each sample during the process of nucleic acid extraction. This method is intended to detect the presence of PCR inhibitors in the sample and will also aid the detection of post-extraction technical errors. It will not, however, detect cases where the original sample was not added to the bio-robot lysis plate. The main breakthrough that has favoured this development is the advent of new commercial kits specially designed for multiplex real-time PCR (in this case the Qiagen Quantitect multiplex kit). These kits contain synthetic factors that increase the concentration of primers at the target site and stabilise the bound primers. As a result, the process of multiplexing an internal control PCR assay to the existing assays is made easier since these kits reduce the need for complex optimisation procedures, and reduce test competition and primer interaction.

The availability of such kits also favours the implementation of multiplex real-time PCR tests. This has led to the development of a novel dual-labelled probe-based multiplex RT-PCR for adenovirus, astrovirus, rotavirus and sapovirus (no such assay is currently available in the published literature) partly based on the results of the research described in this thesis. The multiplex test includes a new in-house astrovirus RT-PCR that targets the 3' NCR region and can theoretically detect all serotypes of astrovirus, and a sapovirus real-time RT-PCR that can detect all 5 genotypes (Oka *et al.*, 2006). The assays for adenovirus and rotavirus, however, are those outlined in this thesis. To date, the new multiplex assay has been shown to be as sensitive as the

equivalent singleton assays using dilution series of typed samples. The inclusion of an RT step, moreover, has been shown to have no effect on the sensitivity of the adenovirus PCR. However, the sensitivity of the multiplex assay is still to be compared with that of the current service on clinical samples. If found to have comparable or improved sensitivity the test is planned for implementation in late 2007, with the aim of further improving the efficiency of platform use within the laboratory, and further reducing turn-around-times and test costs.

Another extension of the research carried out here that is currently being pursued is the development of nucleotide sequencing protocols for viral causes of IID. Nucleotide sequencing is a powerful technique, allowing the determination of the precise order of the nucleotides in a DNA sequence (or RNA molecule following reverse transcription). The sequence can then be compared with a library of known sequences for epidemiological and clinical purposes. If implemented successfully, a sequencing service for the 5 main viral causes of IID will compliment the current molecular diagnostic service as it can be used to provide data on the circulating types, subtypes and variants of each pathogen, to investigate outbreak transmission routes and sources, and to link cases previously thought unrelated. A system of this type has already been published that sequences the RdRp and ORF2 regions of norovirus (Gallimore *et al*, 2007). This method was used to show the emergence of a new variant of norovirus (G2 v4.4) in Scotland in January 2006. The new variant became the predominant strain by April of that year and, like the variant that emerged in 2002, was associated with a significant increase in norovirus detections, outbreaks and sample submissions. The new variant was also found in England and Wales, where it also resulted in an increase in disease activity (Gallimore *et al*, 2007). Following the early success of this system the possibility of offering a typing service for every norovirus outbreak in Scotland is now being considered. The aim would be to use this facility to monitor the emergence of new variants and to investigate large outbreaks (potentially identifying their sources and transmission routes, and any previously unrecognised links between outbreaks). A similar service for rotavirus is also possible if the new vaccination program is implemented in Scotland (see chapter 2). This could be used to monitor the types of rotavirus circulating in the community before and after introduction of the vaccine in order to help determine its effectiveness (Mazick *et al*, 2007). A rotavirus typing service would also be useful in the laboratory for

monitoring the PCR target sites used in the current tests, to ensure that variations do not occur which may result in reduced test sensitivity.

Other research under consideration includes a comparison of alternative sample preparation methods to those employed here. All of the samples tested in the research described in this thesis were processed using the Qiagen Blood kit on the Biorobot 9604. However, in recent years several other kits and extraction platforms have been made available, including some specifically designed for the extraction of viral pathogens from stool (e.g. the Qiagen DNA stool mini kit) or for viruses in general (e.g. the Qiagen Virus kit), together with some kits that concentrate the eluted nucleic acid further than is achievable with the current system (e.g. the Nuclisens™ EasyMag kit). These kits and platforms have the potential to offer improved nucleic acid extraction compared to the method employed in this research, which in turn could lead to improved pathogen detection rates. In future, therefore, a comparison of these methods would be useful.

Similarly, the number of real-time PCR methods published for norovirus has increased significantly in recent years (Houde *et al*, 2006; Jolkihumar *et al*, 2005; Pang *et al*, 2004; Schmid *et al*, 2004; Hoehne *et al*, 2006; Simpson *et al*, 2004; Richards *et al*, 2004), although detailed sensitivity comparisons between these methods remain rare. The dual-labelled probe-based RT-PCR assay investigated in the research described in Chapter 4 and subsequently implemented (Kageyama *et al*, 2003) was one of the first of its type to be published. It is possible that one or more of the assays published subsequently may offer increased sensitivity in comparison. A comparative evaluation of the sensitivity of a number of these published real-time PCR assays, similar to the study carried out by Vinje *et al* for conventional PCR methods (Vinje *et al*, 2003), would therefore be of considerable interest. For the sporadic service a comparison of the latest published methods for the detection of non-norovirus causes of IID would also be useful, although here the choice of real-time assays remains limited to a small number of SYBR green and dual-labelled probe-based RT-PCR tests for astrovirus and sapovirus.

6.7. Thesis Conclusion.

The thesis expounded in this research is that the implementation of the molecular method Polymerase Chain Reaction (PCR) for the diagnosis of the viral

causes of outbreaks and sporadic cases of gastroenteritis can bring significant benefits to a routine laboratory service. The data presented here confirm that this is in fact the case. Since the introduction of the adapted molecular tests at the WOSSVC for the detection of viral pathogens in outbreaks and sporadic cases of gastroenteritis the number of samples tested by the routine diagnostic service has risen steadily. Moreover, the rate of positive detections has improved and this has been achieved at the same time as a substantial reduction in sample turn-around-times. This is a significant improvement over the previous EM-based service.

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