MOLECULAR EPIDEMIOLOGY OF DIARRHOEAL VIRUS INFECTION IN CHILDREN IN SAUDI ARABIA

Hamsa T. Tayeb

A thesis submitted in partial fulfillment of the requirements of the University of Surrey for the degree of Doctor of Philosophy

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Mohammed N. Al-Ahdal, PhD
To my father's soul (may you rest in peace), I love you and I miss you very much.

To my mother, thank you is not enough. But I can't find any other word to describe my feelings to you. I hope I made you proud of me.

To my husband Mohab, my love and my life, my present and future thanks. I hope you like the harvest of our plants.

To my children (Mahmoud, Al-Juhara, Taher, Abd alaziz and Mohammed), I am very happy to have you in my life and I hope you feel the same. Sorry for all the times I missed being with you, and thank you for your understanding and support every time I was away.

To my brothers Mamdouh and Mohammed, for their unconditional love, concern and support. Moreover, I will not forget to thank my special friends and sisters, Hala and Najla.

Hala I am very glad and proud to have a sister like you in my life. Furthermore, Najla (may you rest in peace), I just simply lost a very important and beautiful person in my life when you left our world to a better place.
Acknowledgements

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I would like also to thank all the members of the molecular virology lab in research center especially; Dr. Al-Qahtani, Dr. abdelhamed and Damian, for their support and for their general technical help during my work.

Special thanks to my friends; Halima, Nouf, Safiah, Hanan and Nada for their friendship which help them to support me, and understanding my changeable mode lately and for the good time they provide it to me which really help me and encourage me a lot during my study.

Finally, I would like to thanks the statistical department, and for Mr. Hakkem from purchasing department, and Mr. Essa from genetic department, in RC for there help and support.
Abstract

The etiology of viral diarrhoea in children in the Kingdom of Saudi Arabia (KSA) is incompletely characterized. Available data suggest that the causative agents are found at approximately similar frequencies here as elsewhere. However KSA is not a typical country; the lack of rivers and lakes may affect the paths open for virus spread in the Kingdom and the relatively high year-round temperature may limit virus survival in the environment. Sewage is disposed of to sea after treatment, and although virus is found in seawater, exposure via recreational use is limited those living along the coast. Virus is also found in seafood which may aid transmission inland but drinking water is supplied by desalination, a process that would effectively inactivate most viruses. Thus the bulk of viral transmission must be presumably via person to person or food-borne spread inland. Secondly, KSA plays host to many millions of visitors each year who flock to the country from all over the world in a short time period; the Hajj or annual pilgrimage to Makkah. This influx of persons offers opportunity both for the introduction of new strains of viruses and also for person to person transmission in these crowded venues. Consequently, there may be subtle differences in the manner of circulation of these viruses in KSA.

This project set out to study the epidemiology of diarrhea viruses in pediatric populations. The study addressed initially rotavirus, enteric adenovirus and astrovirus but was later expanded to include norovirus. Viruses were sought in faecal specimens and characterized for genotype using molecular methods for the first time in KSA.

The survey focused on three locations; Jeddah, Makkah and Riyadh. During the Hajj the chief population fluxes are via Jeddah to Makkah. One thousand samples were
obtained from children (aged six years or less) presenting with diarrhoea and thus representing community acquired rather than nosocomial infections.

Human rotavirus (HRV) group A was detected in 6% (60/1000). By RT-PCRG1 was the predominant VP7 genotype (36/58, 62%), followed by the unique G9 (19/58, 33%). The other HRV genotypes found, G2 and G3, were less common at 1.2% (1/58) and 3.4% (2/58), respectively. P-type determination was also performed by RT-PCR and P[8] was clearly the most common (45/56, 80.3%). Overall G1P[8] was the most common, accounting for 60.7% of total samples positive for both genotypes. Rarer types G9P[4] (2/56, 3.57%) and G9P[6] (6/56, 10.7%) were identified for the first time in KSA.

Enteric adenovirus (EAdV) was evident in 14/1000 pediatric stool samples (1.4%) by ELISA and all were also positive by RT-PCR. Enteric types 40 and 41 were distinguished using RT-PCR and RFLP; five samples were positive for EAdV-40 and 7 for EAdV-41. One sample showed a mixed infection of both 40 and 41. A single sample was eventually typed as type 31 by Sequencing.

Human astrovirus (HAstV) was found in 1.9% (19/1000 samples). All but one was identified as type 8. This was a surprising finding since these infections were not nosocomial but independent, community-acquired cases. The remaining sample could not be amplified.

Human caliciviruses are among the most common causes of gastrointestinal and there are no data for these viruses in Saudi Arabia. A smaller panel of 253 stool samples was tested for norovirus by ELISA and 9/253 were found positive (3.5%). Overall, most infections with rotavirus were detected in children of 1 year of age or less (P=0.047), and in children of 3 year of age or less with astrovirus likewise, in adenovirus
most of the infections were detected in children of 2 year of age or less with P-Value of = 0.05, and 0.01 respectively. All viruses were distributed equally between males and females.

HRV was the most common diarrhoeal virus detected followed by norovirus, although the rotavirus incidence found here was lower than that reported in previous studies. Infections showed a peak shortly after the Hajj during the study period. Novel HRV were found, with the first detection of the emergent G9 serotype and some combinations of G and P types new in KSA. Adenovirus 31 was reported for the first time and we found an unexpectedly high incidence of astrovirus serotype 8.
1.6 Current Knowledge of These Agents in Saudi Arabia

1.6.1 Special Features of KSA that May Affect Virus Transmission

Drinking Water
Sewage Disposal
Pilgrimage

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List of Abbreviations:

AMV  Avian Myeloma Virus
BPB  Bromophenol Blue
CaCo-2 Human Colonic Carcinoma Cells
CDC Centers for Disease Control and Prevention
cDNA DNA Complementary to RNA
DLPs Double-Layer Particles
DMSO Dimethylsulfoxide
dNTP Deoxyribo Nucleoside Tri- Phosphates
core RIs Core-Like Replication Intermediates
ds RNA Double Strand RNA
EAdV Enteric Adenovirus
ELISA Enzyme-Linked Immunosorbent Assay
EM Electron Microscopy
FSA Food Standards Agency
GGI Genogroups I
GGII Genogroups II
HAV Hepatitis A Virus
HAstV Human Astrovirus
HEK Human Embryonic Kidney Cells
HRV Human Rotavirus
HuCV Human Calicivirus
IID Infectious Intestinal Disease
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>IEM</td>
<td>Immune Electron Microscopy</td>
</tr>
<tr>
<td>ISVP</td>
<td>Infectious Sub Viral Particle</td>
</tr>
<tr>
<td>KFSH&amp;RC</td>
<td>King Faisal Specialist Hospital &amp; Research Centre</td>
</tr>
<tr>
<td>KSA</td>
<td>Kingdom of Saudi Arabia</td>
</tr>
<tr>
<td>LMP</td>
<td>Low Melting Point</td>
</tr>
<tr>
<td>mA</td>
<td>milli Ampere</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MSF</td>
<td>Multi- Stage Flash Distillation Desalination</td>
</tr>
<tr>
<td>MWs</td>
<td>Molecular Weights</td>
</tr>
<tr>
<td>NoV</td>
<td>Noroviruses</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frames</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Enzyme Length Polymorphism</td>
</tr>
<tr>
<td>RRV-TV</td>
<td>Tetravalent Rotavirus Vaccine</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>SaV</td>
<td>Sapoviruses</td>
</tr>
<tr>
<td>T</td>
<td>Triangulation Number</td>
</tr>
<tr>
<td>TLPs</td>
<td>Triple-Layered Particles</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom of England</td>
</tr>
<tr>
<td>USA</td>
<td>United Stat of America</td>
</tr>
<tr>
<td>VLPs</td>
<td>Virus-Like Particles</td>
</tr>
</tbody>
</table>
WHO  World Health Organization
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1. Introduction

1.1 Viral Gastroenteritis

Viruses are amongst the most common causes of severe gastroenteritis in both the developed and developing world [Middleton et al., 1977; Khuri-Bulso et al., 2006] (Figure. 1). All of these viruses can be transmitted either directly or via contaminated food and water; it is believed that most childhood infections are acquired by direct transmission [Fankhauser et al., 1998; Timbury, 1997]. Overall incidence of these agents is similar in both developed and developing world although age at infection may be delayed and the consequences less severe in the West. In developing countries, mortality associated with diarrhoea is estimated at 2.4-2.8 million deaths every year and largely restricted to children below 5 years [Bern, 1992; Murray, 1997]. About half of these cases are believed to be viral in origin and although deaths are much fewer, viruses are also a significant cause of morbidity in children in developed countries [Glass et al., 2001].

Infections are commonly characterized by watery diarrhoea leading to dehydration in infants and young children and sometimes accompanied by nausea, vomiting, abdominal cramps, headache and fever [Timbury, 1997]. Management is largely limited to oral rehydration [Christensen, 1989; de Quadros et al., 2004] no antivirals are currently available for treatment and although rotavirus vaccines are in trial, there have been problems. Rotashield for instance was potentially associated with intussusceptions [de Zoysa and Feachem, 1985; Miller and McCann, 2000; Glass et al.,
This vaccine is now in trials again and several other candidates are under evaluation (see Table 3).

The costs of enteric virus infection are considerable: in the UK viral gastrointestinal infections costs app. £176 per case for medical treatment 2 persons in every 1000 will make visit a clinic each year [FSA 2000]. A single outbreak of Hepatitis A virus (HAV) exposed up to 5,000 persons in Colorado in USA cost $50,000 for medical treatment. Neither of these two estimates includes the economic costs of days of work lost.

The significance of these infections has prompted the study of these agents and the development of diagnostic tests for virus identification. Across the world it has been found that the same viruses induce diarrhoea, although the frequency of each and the outcomes of infection may vary.

The main viruses concerned are the human rotaviruses (HRV), adenoviruses group F (serotypes 40 and 41), human astroviruses (HAstV) and the human caliciviruses: norovirus (NoV) and sapovirus (SaV) [Christensen, 1989; Clark and McKendrick, 2004; Clark et al., 2004]. Most of these viruses were identified originally by electron microscopy (EM): rotavirus in 1973 [Bishop et al., 1973], Enteric adenoviruses (EAdv) which comprise Group F viruses (serotypes 40 and 41) in 1975 [Wadell et al., 1987], astroviruses (HAstV) in 1975 [Madeley et al., 1975], and human caliciviruses in 1978 [Chiba et al., 2000]. The group F adenoviruses observed by electron microscopy were found to be difficult to culture in conventional cell lines, for this reason they have been termed “fastidious adenoviruses. However the group F viruses are not the only adenoviruses capable of replication in the gut, other types such as Group A may also do
so. Group A viruses however, do not induce clinical signs of gut infection, and are associated primarily with respiratory or pharyngeal symptoms. They are also shed in much lower numbers than group F viruses and are not usually observed by EM examination of faeces. Since Group A strains are readily cultivable, it is these viruses that comprise the bulk of the adenoviruses routinely cultured from stool, but for the reasons described above these may not be considered true enteric pathogens. For this reason the term “enteric adenoviruses” will be used in this thesis to refer only to group F viruses.

Unlike the other agents, the noroviruses have usually been thought of as symptomatic pathogens in young adults/adults rather than in children, although recent data suggests that this may not always be true and seroconversion to noroviruses is observed early in life [Cubitt, 1990; Umesh et al., 2004]. These viruses are illustrated below (Figure 2) and the basic properties of each virus and the type of illness induced are given in Table 1. Of these viruses, rotaviruses are the most significant pathogen for children, and a recent European report estimated 23 million children (<5 y) are infected annually with rotavirus (likely the old children) [PROTECT, 2006].

In spite of the enormous health burden of diarrhoeal virus-associated disease, particularly in infants and young children, these viruses remain little studied in the Middle East in general and in Saudi Arabia in particular. Therefore, this project set out to increase knowledge of these agents by determining which viruses are circulating in different locations, what strains are involved and the relative contributions of each. However, before this can be addressed it is necessary to consider the features and current knowledge of each virus and these are set out in greater detail below.
Figure 1. Causes of severe acute gastroenteritis among children less than 5 years. Viruses are amongst the most common causes of severe gastroenteritis in both the developed and developing world.

(Source: http://www.uct.ac.za/microbiology/cann/335/Diarrhoea.html).
Figure 2. Electron microscope images of viral agents of gastroenteritis. Panels are labeled above. Bar = 100nm. These pictures are taken from the following addresses

http://upload.wikimedia.org/wikipedia/commons/thumb/f/f7/Rotavirus.jpg/250px-Rotavirus.jpg
[Carter and Willcocks, 1996].
http://pathmicro.med.sc.edu/virol/norwalk.jpg
<table>
<thead>
<tr>
<th>Virus</th>
<th>Family</th>
<th>Characterization</th>
<th>EM appearance</th>
<th>Nucleic Acid</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotavirus Group A</td>
<td>Reoviridae</td>
<td>Multiple serotypes based on variation in VP4 and VP7</td>
<td>Double shelled-outer layer resembles spokes of a wheel</td>
<td>ds RNA segmented</td>
<td>70nm</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>Adenoviridae</td>
<td>47 types; group F (40 and 41) are enteric. Also group A (12, 18 and 31) may occasionally induce symptoms of GI infection.</td>
<td>Icosahedral</td>
<td>ds DNA</td>
<td>80nm</td>
</tr>
<tr>
<td>Astrovirus</td>
<td>Astroviridae</td>
<td>Serotypes 1-8 infect man</td>
<td>5 or 6 pointed star motif visible on some particles</td>
<td>ss (+) RNA</td>
<td>28-30 nm</td>
</tr>
<tr>
<td>Calicivirus</td>
<td>Caliciviridae</td>
<td>Two genera Norovirus and Sapovirus, each with multiple antigenic clusters</td>
<td>Small round structured viruses with calices</td>
<td>ss (+) RNA</td>
<td>28-35 nm</td>
</tr>
</tbody>
</table>

[Charles, 1990; Huq et al., 1987; Monroe et al., 2001; Glass, 2001]
1.2 Human Rotavirus (HRV)

1.2.1 Particle Structure

Rotaviruses form a distinct genus within the family Reoviridae, whose members possess a multilayered capsid approximately 70 nm in diameter. The complete rotavirus particle displays radial structures at its periphery which have been compared to the spokes of a wheel and from which the viruses take their name (rota-a wheel). The mature rotavirus virion is a non-enveloped particle with a segmented, double-stranded RNA genome. This is enclosed by a complex triple-layered protein capsid, the outer capsid is made of proteins VP4 and VP7, the inner capsid from protein VP6 and the core from proteins VP1, VP2 and VP3 [Crawford et al., 1994] (Figure 3). The whole structure is relatively robust and human rotaviruses (HRV) have been shown to be resistant to chloroform, ether, other fluorocarbons, cesium chloride, non-ionic detergents pH 3-9, although they are inactivated by ethanol, phenol, chlorine (hypochlorite solution) and formaldehyde [Estes, 1990].

The particle weight of the virion is $129.5 \times 10^6$ Daltons, and $52.3 \times 10^6$ Dalton for the core. The particle weight of the virion includes $109 \times 10^6$ Daltons of protein and $15 \times 10^6$ Daltons of RNA. The cores contain $37.3 \times 10^6$ Daltons of protein and $15 \times 10^6$ Daltons of RNA. About 85% of the virion consists of protein and 15% of RNA [Schiff et al., 1990]. The relation between optical density (at 260 nm) and viral protein has been analyzed by spectrophotometer and resulted in: $1 \text{ OD unit} = 2.1 \times 10^{12}$ virus particles = $185 \mu\text{g}$ protein [Schiff et al., 1990].
Figure 3. The mature rotavirus virion is a non-enveloped particle with a segmented (11 segments) double-stranded RNA genome. The genome is enclosed by a triple layered protein capsid, consisting of outer capsid proteins VP4 and VP7 and inner capsid proteins VP6 and the core protein VP1, VP2 and VP3. Genome segments and proteins are illustrated following electrophoretic separation. Coding specificities and protein location in the virion are indicated.

(Source:http://www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/images/04rotavirusfigure.jpg)
1.2.2 Protein Variation and Virus Typing

Rotaviruses are widely distributed in nature as indicated by their recovery from the newborn of many animal species [Ashley et al., 1978] and are often associated with diarrhoea in these hosts e.g. in calves, piglets, rhesus monkeys, lambs, and puppies [Eugster et al., 1978; Snodgrass et al., 1977; Gouvea et al., 1986; Bell et al., 1987]. In China, a human rotavirus type B was shown to induce a severe diarrhoea illness in a non-human primate [Pang et al., 1983].

The internal proteins of the virus show least variation and protein VP6 (located in the inner shell) has been used to cluster the rotaviruses from different hosts into seven groups termed (A-G). Rotaviruses that infect humans belong to groups A, B and C. Group A rotaviruses are the most common in man, and are detected in up to 70% of all patients hospitalized with diarrhoea worldwide [Cook et al., 1990; Kapikian et al., 1996]. Most symptomatic illness caused by group A viruses occurs in children although the elderly are also susceptible. Group B rotaviruses, also known as adult diarrhoea rotaviruses, have been associated with epidemic outbreaks of waterborne diarrhoeal disease among adults. They were reported first in China [Hung et al., 1987] and more recently in India [Krishnan et al., 1999]. Group C rotaviruses have been detected worldwide [Riepenhoff-Talty et al., 1997; Schnagl et al., 2004] and are primarily associated with sporadic cases of diarrhoea among both children and adults. Recent detection of group C in patients with enteric infection have been reported in Slovenia [Steyer et al., 2006].

The surface proteins VP4 and Vp7 are the main inducers of neutralizing immune responses [Hoshino et al., 1985]. Each of these exist in a limited number of antigenic variants, thus we can recognize distinct “types” of both VP4 and VP7. These are
termed G types (for glycoprotein) relating to VP7 and P types (protease sensitive) relating to VP4 [Christensen, 1989].

14 G serotypes (G1-14) and 11 P (P1-11) serotypes have been described for group A viruses, many are capable of infecting both animals and man [Hoshino and Kapikian, 1996]. Ten G serotypes and 7 P serotypes have been identified in human infections. The genes encoding VP7 and VP4 and thus determining G type and P type are highly polymorphic both G and P types can be distinguished by serology and RT-PCR [Green et al., 1987; Green et al., 1988; Gouvea et al., 1990].

The segmented nature of the genome permits easy reassortment from a mixed infection. Thus, each genome segment and thus the protein(s) it encodes may be inherited independently. Seventy different group strains of rotavirus could result from various combinations of the 10 G and 7 P serotypes known in human infections [Flores et al., 1990; Gentsch et al., 1992]. The most prevalent type varies considerably from one geographic area to another. Types G1-G4 are the most common G-types in man globally [Gentsch et al., 1996, Delfina Urbina et al., 2004]. Types G5, G8 and G10, G9 commonly circulate in farm animals; these could be introduced to humans either by recombination and segment exchange in a mixed infection or by zoonotic of a whole virus [Timenetsky et al., 1997; Sanchez-Fauquier et al., 2006]. Recently viruses of this type (especially G9P[8]) have been increasingly reported in humans [Santos et al., 1998; Ramachandran et al., 1996; Cunliffe et al., 1999; Martella et al., 2003; Sanchez-Fauquier et al., 2004].

The most common P type is P[8] followed by the less common types P[4] and P[6]. Combinations of G1, 3 and 4 commonly associated with P type [8], whereas G2 is more often associated with P[4] [Hoshino and Kapikian, 1996].
However, unusual strains may predominate in some developing countries, such as the P[6] containing strains found in India (G1 P3, G2 P3, G9 P3, G4 P3) and the atypical G9P[6] strain [Ramachandran et al., 1996]. Furthermore, Analysis showed atypical strains of rotavirus are circulating in the human population throughout the world; G9P[6], G9P[8] in UK [Iturriza-Gómez et al., 2000; Cubitt et al., 2000] G9P[6], G3P[6] in Spain [Sanchez-Fauquier et al., 2006], G8P[6] in Africa [Santos et al., 2005]. So far these have not been reported in KSA.
Table 2. Description of Human Rotavirus RNA Genome Segments, Gene Products and Location.

<table>
<thead>
<tr>
<th>Genome segment</th>
<th>Gene product</th>
<th>Location</th>
<th>Protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VP1</td>
<td>Inner capsid</td>
<td>Contributes to negative-strand RNA synthesis caps protein</td>
</tr>
<tr>
<td>2</td>
<td>VP2</td>
<td>Inner capsid</td>
<td>As above</td>
</tr>
<tr>
<td>3</td>
<td>VP3</td>
<td>Inner capsid</td>
<td>As above</td>
</tr>
<tr>
<td>4</td>
<td>VP4, VP5, VP8</td>
<td>Outer capsid spikes</td>
<td>Assist in attachment to host cell membranes</td>
</tr>
<tr>
<td>5</td>
<td>NSp1</td>
<td>Non-structural</td>
<td>Contributes to RNA packaging</td>
</tr>
<tr>
<td>6</td>
<td>VP6</td>
<td>Middle capsid</td>
<td>Rotavirus group antigen specificity</td>
</tr>
<tr>
<td>7</td>
<td>NSp3</td>
<td>Non-structural</td>
<td>Facilitates mRNA packaging</td>
</tr>
<tr>
<td>8</td>
<td>NSp2</td>
<td>Non-structural</td>
<td>Contributes to RNA packaging</td>
</tr>
<tr>
<td>9</td>
<td>VP7</td>
<td>Outer capsid glycoprotein</td>
<td>Serotype specificity of group A. Neutralize antibody response</td>
</tr>
<tr>
<td>10</td>
<td>NSp4</td>
<td>Non-structural</td>
<td>Assists in virus release enterotoxin</td>
</tr>
<tr>
<td>11</td>
<td>NSp5</td>
<td>Non-structural</td>
<td>Contribute to translation processes</td>
</tr>
</tbody>
</table>
1.2.3 Rotavirus Prevalence

Among the diarrhoeal viruses, rotavirus remains by far the most important cause of infantile gastroenteritis and mortality worldwide and has been the focus of intensive investigations [Middleton et al., 1977; Szlagatys-Sidorkiewicz et al., 2006]. It is estimated that in developing countries severe dehydrating diarrhoea caused by HRV results in an estimated 500,000 to 870,000 childhood deaths annually [de Zoysa and Feachem., 1985; Miller and McCann., 2000] and even in the developed world may account for over one million cases of diarrhoea each year [Ho et al., 1988; Parashar et al., 1998; PROTECT, 2006]. However hard evidence of rotavirus-induced mortality is difficult to obtain.

1.2.4 Epidemiology of HRV Infection

The epidemiological studies from both the developed and developing countries show that rotaviruses are the major etiologic agents of serious diarrhoeal illness in infants and young children less than 2 years of age [Sung et al., 2004]. Generally, two patterns of disease are noted, endemic and epidemic diarrhoea. Typically, children suffer serial bouts of infection by strains inducing endemic diarrhoea in their communities. Greater than 90% of children have developed antibody to group A rotavirus by age 3 [Riepenhoff-talty et al., 1997] and all have had at least one infection by age 5 [Nguyen et al., 2004; Lorgelly et al., 2007]. Sequential acquisition of strains leads to increased immunity and the frequency of infection declines with age. Consequently, symptomatic infections by HRV are unusual in adults although the elderly are vulnerable. Sub clinical infections may occur throughout life, providing another means for maintaining the virus as an endemic
infection within the community and boosting immunity within the individual. Occasionally, these infections can cause illness in parents of children with rotavirus diarrhoea, exhibiting gastrointestinal symptoms such as diarrhoea or abdominal cramps. In temperate countries, rotavirus shows seasonal preference for Winter/Spring with a peak in March, whilst in tropical and developing countries diarrhoea occurs all year round, with a peak in summer. In Saudi Arabia, infection with rotavirus occurs the year around with no significant seasonal peak [Arif et al., 2005].

Superimposed on this pattern are the epidemic strains, which typically include the more unusual viruses of groups B and C. These outbreaks often result from a contaminated food or water source [Heffernan, 2004; Timbury, 1997]. Adults have usually no history of exposure to these viruses and thus little protective immunity. Several large outbreaks of group B rotaviruses have occurred in adults. [Anderson and Weber., 2004; Sanekata et al., 2003]: In China, 12,000 to 20,000 adults developed cholera-like, watery diarrhoea and some elderly patients died [Chen et al., 1985; Hung et al., 1983; Hung et al., 1984].

1.2.5 Control of Rotavirus Infection

Attempts to develop vaccines have concentrated on endemic strains. Analysis of strains collected worldwide showed that the most common combination of rotavirus genotypes are G1[P8], G2[P4], G3[P8], and G4[P8] [Gentsch et al., 1996]. Since serotypes G1-G4 are the most common globally, accounting for almost all endemic rotavirus gastroenteritis [Gentsch et al., 1996; Urbina et al., 2004]. These four serotypes were, incorporated into Rotashield, the tetravalent rhesus monkey rotavirus-HRV reassortant vaccine [CDC, 1998; Boudville et al., 2006]. Tetravalent
rotavirus vaccine (RRV-TV) had been developed to protect against the four epidemiological rotavirus serotypes. It was estimated that 1.5 million doses had been given. Because of a number of intussusception cases were reported that prompted further investigation, Therefore, the Centers for Disease Control and Prevention (CDC) in 1999 withdrew its recommendation for RRV-TV [CDC 1999a; CDC 1999b].

However, other G serotypes have now been found to be common in several other regions of the world, serotypes G5, G8 and G10 in Brazil [Santos et al., 1998], G8 in Malawi [Cunliffe et al., 1999], G9 in India [Ramachandran et al., 1996] (see pages 26-27 and page 148 in the discussion section) and, G12 in Brazil [Pietruchinski et al., 2006] and this implies that modifications to the strains used in vaccine preparation will be necessary for each region.

Since rotaviruses are the most common cause of severe diarrhoea in infants and children worldwide. The vaccines development program continues to receive attention. There are a variety of vaccines currently in development around the world (see Table 3).
Table 3. Overview of rotavirus vaccines

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Serotype</th>
<th>Concept</th>
<th>Status</th>
<th>Company/Inventor</th>
</tr>
</thead>
<tbody>
<tr>
<td>RotaTeq</td>
<td>G1, 2, 3, 4</td>
<td>Pentavalent vaccine, modified WC3-QV to also contain VP7 gene from human serotype G4</td>
<td>Phase III</td>
<td>Merck/HF Clark [Clark et al., 2003; Orellana et al., 2003].</td>
</tr>
<tr>
<td></td>
<td>P1A[8], 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WC3-QV</td>
<td>G1, 2, 3</td>
<td>Quadrivalent vaccine, human-bovine re-assortants; bovine parent strain (WC3) with 3 VP7 and 1 VP4 genes from human strains.</td>
<td>Phase III</td>
<td>Merck/HF Clark</td>
</tr>
<tr>
<td></td>
<td>P1A[8], 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rotarix</td>
<td>G1</td>
<td>Monovalent vaccine, symptomatic human rotavirus strain 89-12.</td>
<td>Phase III</td>
<td>GSK/RL Ward and DI Berstein [Vesikari et al., 2004; Clemens et al., 2004].</td>
</tr>
<tr>
<td></td>
<td>P1A[8]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LLR</td>
<td>G10</td>
<td>Monovalent vaccine, lamb rotavirus.</td>
<td>Licenced, China, 2000</td>
<td>Lanshou Institute of Biological Products, China/Z-S Bai [Kirkwood et al., 2003].</td>
</tr>
<tr>
<td></td>
<td>P[12]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV3</td>
<td>G3</td>
<td>Monovalent vaccine, human neonatal strain</td>
<td>Phase II</td>
<td>Biofarm Indonesia/RF Bishop and GL Barnes</td>
</tr>
<tr>
<td></td>
<td>P2[6]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>116E</td>
<td>G9</td>
<td>Monovalent vaccine, human neonatal strain</td>
<td>Phase I</td>
<td>Bharat Biotech India/BK Das and RI Glass</td>
</tr>
<tr>
<td></td>
<td>P[11]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I321</td>
<td>G10</td>
<td>Monovalent vaccine natural human/bovine reassortant</td>
<td>Phase I</td>
<td>Bharat Biotech India/BK Das and RI Glass</td>
</tr>
<tr>
<td></td>
<td>P[11]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP7</td>
<td>G1, 2, 3, 4 P7[5]</td>
<td>Monovalent and Quadrivalent vaccine human-bovine reassortants: bovine parent strain (UK) with VP7 genes from human G1, 2, 3 and 4 strains.</td>
<td>Phase I</td>
<td>AZ Kapikian</td>
</tr>
<tr>
<td></td>
<td>G2 P1a[8]</td>
<td>Monovalent reassortant vaccine: two human genes encoding VP4 and VP7, in a background of bovine strain UK.</td>
<td>Phase I</td>
<td>AZ Kapikian</td>
</tr>
</tbody>
</table>

1.2.6 Overview of Rotavirus Replication Cycle

Of the six groups, only group A can be propagated in cell cultures and most strains may be successfully grown in MA104 cells [Ward et al., 1984; Bosch et al., 2004]. Replication of rotaviruses is illustrated in (see Figure 4).

The process by which rotaviruses enter the cell is not completely clear. Only the double-layer particles (DLPs) attach to the cells [Petrie et al., 1981]. It is thought that attachment of the VP4 protein to cellular receptors integrins, is essential for the attachment and entry of rotavirus [Zarate et al., 2004; Halasz et al., 2005]. This leads to uptake via receptor mediated endocytosis [Petrie et al., 1981; Quan et al., 1983]. Engulfed virions then enter the cytoplasm when the endosome interior is acidified and the capsid acquires the ability to penetrate the membrane. However, since rotavirus is an enteric infection, some viruses are activated extracellularly by gut acids. These form an activated structure termed an infectious sub viral particle (ISVP), which may penetrate directly at the plasma membrane [Estes, 1990].

Rotavirus replication is entirely cytoplasmic; internalized viruses are stripped of the outer layer of capsid and act as transcription centers, exporting 11 mRNAs transcribed from the 11 dsRNA segments in the core to the cytoplasm [Patton, 1996; Wentz et al., 1996]. The mRNA is translated to form all the virus proteins (six structural and six non structural proteins).

The new virions are made firstly as fresh cores that enclose single stranded mRNA (core RIs). VP1 (RNA polymerase), VP2 and VP3 (capping enzyme) interact with mRNA to form the core RIs. At this stage non-structural proteins NSP2, NSP3 and NSP5 are also present, but these are removed during maturation [Gallegos et al., 1989].
These are located close to the nucleus and are the sites at which progeny virus is assembled. This is then copied forming dsRNA within the core. Interaction of VP6 with RIs generates the DLPs. Cores acquire their outer protein layer by budding through the endoplasmic reticulum using the protein NSp4 as an adaptor forming triple-layered particles (TLPs) [Estes, 1996]. Maturing rotaviruses thus have a transitory membrane, which is lost before the particles are released by cell lysis [Altenburg et al., 1980; Chen et al., 1989].

HRV replication is shown diagrammatically in Figure 4. One unusual feature of this replication is that the cores never uncoat fully, presumably this prevents exposure of dsRNA within the cell, as this is a powerful inducer of interferon. Secondly, replication of the dsRNA is conservative not semi-conservative as each new duplex consists of 2 new strands of RNA.
Figure 4. Schematic representation of rotavirus life cycle. Rotavirus replication is entirely cytoplasmic. Viruses enter the cell by attachment of the VP4 protein to cellular receptors and penetrate the membrane by receptor mediated endocytosis. Internalized viruses are stripped of the outer layer of capsid and act as transcription centers, exporting mRNA to the cytoplasm. This is translated to form all the virus proteins. These are located close to the nucleus where progeny virus is assembled. The new virions are made firstly as fresh cores that enclose single stranded mRNA. This is then copied forming dsRNA within the core. Maturing rotaviruses thus have a transitory membrane which is lost before the particles are released by cell lysis. (Source: http://www.stanford.edu/group/virus/1999/caryn/replication.html)
1.3 Human Adenoviruses (HAdV)

Adenoviruses belong to the family *Adenoviridae*, which is divided into two genera, Mastadenovirus and Avidenovirus [Norrby *et al.*, 1976]. The genus Mastadenovirus includes the human viruses. There are some 51 types; numbers 42 and above have only been reported in AIDS and immunocompromised patients [De Jong *et al.*, 1999; Jalal *et al.*, 2005]. All 51 types can be divided into six groups, A to F, based on various biological criteria [Shenk, 1996]. Most adenoviruses have a propensity to replicate in the gut and can often be recovered from faeces even if the major site of symptomatic infection in the body lies elsewhere, e.g. respiratory or ocular, but types 40 and 41 (group F) are true enteric pathogens.

1.3.1 Particle Structure

Adenoviruses are non-enveloped, regular icosahedral particles displaying 20 triangular faces and 12 vertices. Proteins making up the adenovirus particle are identified by roman numerals in figure 5 which also indicates their location in the virion. A fiber (IV in fig 5) projects from each of the vertices and differs antigenically between viruses thus conferring serotype specificity [Norrby, 1966; Norrby *et al.*, 1976] (Figure 5). The virus capsid is composed of 252 morphological units, 240 of these are hexon protein (II in fig 5). These are distributed across the virus capsid in groups of 6. Twelve capsomers (located at the vertices of the icosahedron) are constructed from pentomers of a different protein termed penton (III in fig 5) and are located at each vertex of the icosahedron [Ginsberg *et al.*, 1966]. The capsid encloses the virus genome which is double-stranded, linear DNA with a size of 30-38 kbp. The terminal sequences of each strand are inverted repeats, hence a denatured single
strand can form “panhandle” structures, a 55kD protein is covalently attached to the 5’ end of each strand (Figure 6) [Horwitz, 1990].

The map at Figure 7 shows the position of all characterized open reading frames larger than 200 codons [Howitt et al., 2003], intron locations are not shown. The virus has no membrane or lipids and is, therefore, stable to solvents such as ether and ethanol [Majhen et al., 2006].
Figure 5. Diagrammatic representation of an adenovirus particle. Adenoviruses are non-enveloped, regular icosahedral particles displaying 20 triangular faces and 12 vertices. A fiber projects from each of the vertices and differs with serotype. The capsid encloses the virus genome which is double-stranded, linear DNA with a size of 30-38 kbp. (IV: Fiber protein, II: Hexon protein, III: Penton protein).

(Source: http://pathmicro.med.sc.edu/mhunt/adoeno-diag.jpg).
Figure 6. Structure of adenovirus DNA. Adenovirus consists of a linear double-strand DNA with a size of 30-38 kbp. Both ends consist of a repetitive nucleotide sequence, inverted terminal repeat [Horwitz, 1990].
Figure 7. Adenovirus genome [Horwitz, 1990].
1.3.2 Enteric HAdV (EAdV)

Although, many types of adenovirus can replicate in the intestine and are shed in the stool, types other than 40 and 41 do not generally cause gut-associated illness. The enteric adenoviruses, types 40 and 41 together comprise group F. These are true enteric viruses for whom the gut is both the portal of entry and the target tissue. Symptoms, when they occur, are those of gastroenteritis. These viruses are shed from the gut in large numbers and so were easily seen in the EM. However these could not be grown in normal cell cultures and thus, prior to their serological and genomic characterization, these viruses were termed “fastidious adenoviruses” and could be cultured only in human embryonic kidney cells (HEK). This distinguished them from the non-fastidious (all other types) adenoviruses [Kidd and Madeley, 1981; Green and Pina, 1963, Choo et al., 2006]. Non fastidious viruses were shed in lower numbers, rarely seen in the EM but grew very well in culture and were thus easily isolated. Later the fastidious viruses were found to consist of two distinct types of viruses differing serologically and in sequence or restriction end nuclease digestion products. These were designated types Ad40 and Ad41 [de Jong et al., 1983; Li et al., 1999].

1.3.3 The Adenovirus Genome

The total length of the DNA was estimated to be 34.0 kb for Ad40 and 34.7 kb for Ad41. DNA homology studies have shown that Ad40 and Ad41 genomes have 62-69% identity [van der Avoort et al., 1989; van Loon et al., 1985]. Nucleic acid hybridization and PCR conditions were developed to distinguish between types 40 and 41 [Scott-Taylor et al., 1993; Kidd et al., 1996; Fukuda et al., 2006; Jothikumar et al., 2005]. A finer analysis, using direct sequencing of viral genome after cloning in
bacteriophage M 13 was able to identify 11 Ad40 DNA variants (D1-D11) and 28 Ad41 DNA variants (D1-D28) [Davison et al., 1993].

1.3.4 Epidemiology of Group F adenovirus Infection

EAdV types 40 and 41 appear to be endemic in most countries [Brandt et al., 1984; de Jong et al., 1983; Kidd et al., 1983; Kidd et al., 1986; Shinozaki et al., 1987; Uhnno et al., 1984; Glass et al., 2001; Leclerc et al., 2002] and cause 5-17% of cases of gastroenteritis in infants and preschool children [Albert, 1986; Horwitz, 1990; Fodha et al., 2006]. Peak incidence is among children under 2 years of age [Kidd et al., 1986; Uhnno et al., 1984; Lin et al., 2000; Saderi et al., 2002]. Infections occur throughout the year with no clear seasonal peak. Adult contacts are infrequently affected with or without symptoms, although some cases have been observed in the elderly [Charles, 1990; Chikhi-Brachet et al., 2002]. Adenoviruses have never been shown to be transmitted by food but many cases of waterborne transmission have been documented [Martone et al., 1980; Leclerc et al., 2002]. The bulk of enteric adenovirus infection is believed to be contracted through a person-to-person route [reviewed by Carter, 2005]. Furthermore, In an IID investigation in the UK, adenoviruses formed 12% of all viruses identified, and were mostly detected in children less than 5 years old [FSA, 2000].

1.3.5 Adenovirus Replication

The replication process of all adenoviruses is similar and involves a nuclear stage [Shenk, 1996]. The virus replication cycle takes place in two phases, which are separated by DNA replication. Early events (first phase) include attachment to the cell receptors in a two stage process; firstly the fiber interacts with a range of cellular
receptors. The penton base then binds to the integrin family allowing internalization via receptor-mediated endocytosis.

Adenovirus penetrates by phagocytosis, the penton protein assists exit of the phagocytic vacuole. The partially uncoated core is released into the cytoplasm [Seth et al., 1984]. The viral DNA then enters the nucleus through nuclear pores and associates with host cell histones.

This is followed by transcription of early mRNA from the input DNA and manufacture of proteins E1A, E1B, E2B, E3, and E4 before the onset of viral DNA replication. Many transcripts are spliced [Sharp, 1977]. Newly replicated DNA is used for late gene transcription and/or packaging into new virions. Assembly of progeny virions takes place in the nucleus. Lysis of infected cells, releases the virus particles from the nucleus. This type of mechanism is known as lytic infections [Horwitz, 1990]. Replication is shown diagrammatically in Figure 8.
Figure 8. Adenovirus replication. The virus replication cycle takes place in two phases, which are separated by DNA replication. Early events (first phase) include adsorption, penetration, and passage of the viral DNA into the nucleus. The late events (second phase) commence after DNA replication and constitute the expression of late viral genes and assembly of progeny virions. These picture is taken from the following address [Lonberg-Holm et al., 1969].
1.4 Human Astroviruses (HAstV)

Human astroviruses are small, round non-enveloped RNA viruses that were first detected by electron microscopy (EM) in 1975 in stool specimens from infants with diarrhoea [Appleton and Higgins, 1975; Madeley and Cosgrove, 1975]. HAstV are a common cause of sporadic cases and outbreaks of viral diarrhoea among young children [Lew et al., 1991; Gabbay et al., 2006] and the elderly [Marshall et al., 2007; Rittwika et al., 2006]. Food borne transmission is uncommon, although an outbreak of food borne astrovirus infection was reported in Japan in 1991 and associated with astrovirus type 6 [Oishi et al., 1994; Sakon et al., 2000]. Moreover, astrovirus are common infections in the young of many animals including cats, dogs, lambs, deer, mice, and cows [Tzipori et al., 1981; Rice et al., 1993; Vieler et al., 1995] and the possibility of cross-species transmission deserves consideration.

1.4.1 Particle Structure

Astroviruses are small, non-enveloped particles, 28-30 nm in diameter. They were initially characterized as having a smooth margin, but it is now clear that they actually have surface projections, which are sometimes ill defined. The viruses often display a 5 or 6 pointed star-like motif on their surfaces, although this too is often not clear (Fig 2). These features lead to marked variation in particle appearance and astroviruses may be misidentified by EM as either caliciviruses or paroviruses [Carter, 1994]. The capsid is composed of three proteins, which are derived by proteolysis from the single capsid protein precursor [Mendez et al., 2002]. This
proteolysis involves both intracellular and extracellular stages, caspases are thought responsible for the intracellular stage [Mendez et al., 2004] and an enteric enzyme (possibly trypsin) for the extracellular cleavage. [Willcocks et al., 1990; Bass et al., 2000]. In culture extracellular trypsin is essential to ensure that released particles are infectious [Willcocks et al., 1995]. This process generates a capsid consisting of three overlapping peptides, corresponding to some 70% (50,000 Daltons of protein) of the information in the capsid protein gene. The fate of the missing 30% (or 20,000 Daltons of protein) is currently unknown.

1.4.2 Astroivirus Variation and Prevalence

The development of detection methods that are not reliant on particle structure has improved the reliability of astrovirus diagnosis in faeces. Eight serotypes of HAstV have been identified, according to the reactivity of the capsid proteins with polyclonal sera and monoclonal antibodies, but type 7 is extremely rare [Lee et al., 1994; Noel and Cubitt 1994; Noel et al., 1995]. Methods such as ELISA, RT-PCR, and real time PCR have revealed that astroviruses are a common cause of viral gastroenteritis in children worldwide [Glass et al., 1996; Sakon et al., 2000; Sakamoto et al., 2000; Grimm et al., 2004]. Estimates of astrovirus incidence vary in different locations: Studies have shown a remarkably wide variation in astrovirus prevalence in cases of symptomatic diarrhoea: Astroviruses accounted for 8.6% of such cases in Thailand [Herrmann et al., 1991], 4.2% in Melbourne [Palombo and Bishop, 1996], 61% in Chiapas, Mexico [Maldonado et al., 1998], 4.9% in Spain [Guix et al., 2002] and 52% in India in 2006 [Bhattacharya et al., 2006]. In Guatemala, Cruz and his colleagues have found astrovirus infection frequently associated with gastroenteritis among young children living in poor areas, and the positivity of astrovirus infection was 28/830 (3.4%) [Cruz et al., 1992]. Regardless of these
findings it is clear that astrovirus infections are very common everywhere and sero-surveys show very high seroprevalences: in the UK 64% of children had antibodies by 4 years of age and 87% by age 10 [Kurtz and Lee1978]. Most of children are infected in the first 2 years of life producing early immunity to astroviruses [Bhattacharya et al., 2006; Cruz et al., 1992].

However astrovirus type 1 is not the most prevalent everywhere, astrovirus type 2 was the most prevalent in Mexico City (35%) and type 1 was relatively rare (4%) [Guerrero et al., 1998]. However, in any one location this situation may not be stable and the emergence of more cases of astrovirus type 4 in the UK has been reported [Willcocks et al., 1995].

1.4.3 Genome Structure HAstV

The virus genome consists of positive sense, single-stranded RNA, approximately 6,800 nucleotides in length. The RNA contains three open reading frames (Figure 9). ORF 1a (approximately 2,842 nucleotides long) overlaps with ORF 1b (approximately 1,557 nucleotides long) by 70 nucleotides. The length of ORF2 varies according to strain, but is between 2,358 and 2,388 nucleotides. ORFs 1a and 1b are expressed from the full length genomic RNA (Figure 9), and encode the non structural proteins of the virus. These have been identified by seeking sequence motifs indicative of function. ORF 1a is thought to encode a viral protease and, ORF 1b an RNA dependent RNA polymerase, ORF 2 encodes the structural proteins [Carter, 1994; Carter and Willcocks, 1996]. Unlike ORFs 1a and 2, ORF 1b has no start codon at the opening of the frame. This is because ORF1b is expressed by ribosomal frame shifting at the end of the ORF1a. This means that a proportion of ribosomes translating ORF 1a slip back one nucleotide and then proceed into ORF 1b. The
determinants of this slippage have been reported [Marczinke, 1994] although the frequency of slippage may be higher in vivo than in vitro [Matsui, 2001]. ORF 2 is expressed from a subgenomic message transcribed from the genome. This arrangement is reminiscent of the caliciviruses and is thought to allow independent adjustment of the levels of structural and non-structural proteins at the transcriptional level.

Interestingly, the region of the capsid gene (ORF2) represented in the astrovirus particle is similar in size to that which forms the calicivirus capsid. In these viruses extra information is form of in the additional ORF encoding a small basic protein, similar in size to the missing portion of the astrovirus ORF2. Recently, this small protein has been identified as a minor structural component of the calicivirus particle that may help assembly [Guo et al., 2001]. Interestingly, the C terminal section of the astrovirus ORF2 is also basic [Carter, 1996] and might serve a similar role.

1.4.4 Astrovirus Replication

HAstV from faeces can be grown in a continuous human colonic carcinoma cell line (CaCo-2) [Willcocks et al., 1990 and Noel et al., 1995], allowing for routine isolations of strains. The full details of the replication cycle are not known, but it is suspected that replication occurs primarily in the cytoplasm although there is a possible nuclear involvement [Carter and Willcocks, 1996]. RNA and protein nsp1a are found associated with the endoplasmic reticulum. Nonstructural protein nsp1a is probably involved in the RNA replication process in endoplasmic reticulum-derived intracellular membrane complexes [Guix et al., 2004].
Figure 9. Genome structure HAstV The genome is composed of three open reading frames which encode both a full genomic and a subgenomic RNA. ORF 1a is thought to encode a viral protease and, ORF 1b an RNA dependent RNA polymerase, ORF 2 encodes the structural proteins. VPg is, small virus-encoded protein at the 5' terminus, and it is suggest to be required to initiate translation. Stem–loop structure driving the frameshifting expression of ORF 1b indicated at the overlap of ORFs 1a and 1b. [Carter, 1994; Carter and Willcocks, 1996].
1.4.5 Epidemiology of HAst Infection

HAstV cause infectious diarrhoea worldwide and account for 2-8% of cases of diarrhoea in infants and young children [Vernacchio et al., 2006; Guix et al., 2002]. Astrovirus infections are detected mostly in winter [Glass et al., 1996; Guix et al., 2002]. Symptomatic virus shedding has been noted in the newborn, although asymptomatic infections are frequent. Symptomatic illness occurs rarely in adults but when it does it is often associated with the less common serotypes to which adults may not have been exposed as children. [Maldonado et al., 1998; Oishi et al., 1994, Pager et al., 2002; Maunula et al., 2004]. However, in Australia, Marshall and his colleagues reported an astrovirus serotype 1 outbreak among the elderly population even though these persons had very likely been previously exposed to this very common serotype [Marshall et al., 2007]. In addition there have been several outbreaks in the elderly in UK [Lewis et al., 1989; Gray et al 1987].

1.5 Human Caliciviruses (HuCV)

Caliciviruses are single-stranded RNA viruses belong to family Caliciviride which are divided into 4 genera (Vesivirus, Lagovirus, Norovirus and Sapovirus) [Clarke et al., 1997]. Within the Caliciviride the Human strains, comprise two agents each found in a separate genus within the family. The noroviruses (formerly the small round structured viruses and Norwalk-like viruses), and the sapoviruses, (formerly the Human caliciviruses and Sapporo-like viruses). This family includes the most common cause of non-bacterial gastroenteritis amongst adults [Chiba et al.1979; Chiba, 1980].
1.5.1 Norovirus and Sapovirus

In 1968 an outbreak of acute gastroenteritis occurred amongst students and teachers in a school in Norwalk, Ohio [Adler et al., 1969]. The infection was characterized by vomiting and diarrhoea and showed a high attack rate and short incubation period. Attempts to propagate the agent in cell, and organ culture failed [Blacklow et al., 1972; Dolin et al., 1972]. In 1972, Kapikian used immune electron microscopy (IEM) to identify viral particles, which became known as the Norwalk agent [Kapikian et al., 1972]. Initially these were reported as 27nm but this was later revised upwards to 34nm. The particles were fuzzy and indistinct in outline but showed indications of surface structure reminiscent of the caliciviruses then known in animals. The characteristic cup-like depressions were however almost always unclear. Similar studies later demonstrated that other caliciviruses (now termed sapoviruses) were associated with a gastroenteritis outbreak in infants in Sapporo, Japan in 1977 [Chiba et al., 2000]. These particles resembled animal strains much more closely and did display the cup-like depression on the surface. These were termed human caliciviruses and for some while it was not clear whether the noroviruses and sapoviruses were actually distinct types of virus. This situation has now been resolved using molecular data to resolve these viruses into distinct genera by sequence comparison analysis. More details regarding the differentiation between NoV and SaV genome are placed in the calicivirus genome structure section.

However, progress in the characterization of the human caliciviruses has been severely hampered by the lack of a cell culture system, the low number of viruses
often shed in stool during the infection and the absence of a reliable animal model. Recent developments using molecular biology especially RT-PCR and sequencing have increased our knowledge and understanding of this group of viruses [Atmar et al., 2001], and the first successful culture in cells has now been reported [Straub et al., 2007].

1.5.2 Particle Structure

A classification scheme described by Caul and Appleton in 1982, drew a morphological distinction between NoV and SaV. The NoV have an amorphous structure with a ragged outer edge and SaV display the true cup-shaped structures from which the calicivirus family derives its name. Both particles were approximately 30-35nm in diameter.

The virion is composed of a single type of capsid protein. Expression of the capsid protein using the baculovirus system results in the self assembly of virus-like particles (VLPs) [Prasad et al., 1994]. The structure of these VLPs has been resolved by cryoelectron microscopy [Prasad et al., 1999]. The major structural protein folds into 90 dimers arranged in a T = 3 icosahedral symmetry. The points of 5 and 6-fold symmetry are ringed with arch like projections that rise from the surface of the particle and create the 32 cup-shaped depressions.

Both NoV and SaV have been subdivided by cluster analysis into genogroups, these in turn have been divided into genotypes (see Figure 10). NoV with genogroups I and II, have been genotyped so far into 15 genotypes and SaV have been divided into 2 genogroups with 4 genotypes [Ando et al., 2000; Green et al., 2000; Vinijé et al., 2000; Jiang et al., 1997]. For NoV genogroups I (GGI), includes Norwalk, Southampton, Desert shield, Queens arms, and Winchester viruses. NoV genogroups
II (GGII) includes Hawaii, Mexico, Lordsdale, Melksham, Hillingdon, Grimsby and others [Dingle et al., 1995; Lew et al., 1994]. From the epidemiological studies, the outbreak with GGII strains is relatively more common than GGI strains. Strains circulating in the community vary with time [Vinje and Koopmans, 1996; Vinje et al., 1997]. For example in winter season of 1995/1996 in Netherland a large epidemic scale was observed due to a Lordsale-like virus, in 1994, a small epidemic was associated with a Mexico-like virus, and from September through December of 1996 outbreaks were caused by the Leeds genotype were observed [Koopmans et al., 2001; Ando et al., 1995]. Since 2002 there has been a global emergence of a new type of norovirus commonly associated with outbreaks in nursing home, termed genogroup 2, genotype 4 (GII4) viruses. These show increased severity of infection and have displaced strains that were circulating previously to become the dominant strains worldwide; UK, United States, France, Japan and Thailand, [Gallimore et al., 2004; Fankhauser et al., 2002; Bon et al., 2004; Ozawa et al., 2007; Ramirez et al., 2007; Khamrin et al., 2007]. In addition recombination between caliciviruses appears frequent and takes place primarily at the ORF1 and ORF2 boundary [Phan et al., 2007; Lindesmith et al., 2008]. However, for sapovirus GGI (Saporo (Houston/86), Houston/90, Stockholm) genus Saporo were the most common strain detected in SaV cases in Netherland from 1996-1998 [Koopmans et al., 2001]. London strains which belong to GGII, were the most commonly detected strains of sapoviruses infection in Sweden and UK [Vinje et al., 2000]. As well as, in recent study of genetic characterization of calicivirus among children with acute gastroenteritis in the United States at 2005, half of the positive samples of sapovirus grouped with London strain [Gallimore et al., 2004]. In Hungary at 2002, all the positive samples for sapovirus in infant and children was belong to London strain [Reuter et al., 2002].
Figure 10. Norovirus, and sapovirus genus. Both NoV and SaV have been subdivided by Cluster analysis into genogroups, these in turn have been divided into genotypes. NoV with genogroups I and II, have been genotyped so far into 15 genotypes and SaV have been divided into 2 genogroups with 4 genotypes. Accession numbers of calicivirus are as follows: Houston SLV (U95643), Houston 90 SLV (U95644), Stockholm (AF194182), London/92 SLV (U95645), Southampton NLV (L07418), Norwalk virus (M87661), Desert Shield NLV (U04464), Lordsdale NLV (X86557), Hawaii NLV (U07611), Mexico/89 NLV (U22498), Melksham/89/UK NLV (X81879), Hillingdon/90/UK, NLV (AJ277607), Grimsby/95/UK, NLV (AJ004864), Winchester/94/UK, NLV (AJ277609).
1.5.2 Genome Structure HuCV

Caliciviruses possess a single-stranded, positive-sense RNA genome approximately 7400-7800 nucleotides length not including the poly A tail. The genome has a characteristic arrangement of ORFs that distinguishes them from the *Picornaviridae* [Clarke and Lambden, 1997].

The genome of NoVs is organized in three ORFs. The first ORF at the 5' end encodes a large polyprotein of 1738 amino acids with molecular weight of 193.5K. The 5' end codes for precursor of the nonstructural proteins. ORF2 encodes 530 amino acids capsid protein of molecular weight of 56.6K. The ORF3 at the 3' end of the genome encodes a small basic protein of 212 amino acids with molecular weight of 22.5K [Hardy *et al.*, 1996; Jiang *et al.*, 1993]. In Feline calicivirus this forms a minor component of the virion and is known as VP2 [Sosnovtsev *et al.*, 2005]. It is assumed that the ORF3 protein from NoV performs a similar function.

The genome of SaV is slightly different. The major difference between the genome of SaV and the NoV genome (Figure 11), is that the capsid structural protein gene is in the same frame as ORF1 [Liu *et al.*, 1995; Liu *et al.*, 1997]. The second ORF then encodes the small basic and presumed minor capsid protein which shows no sequence homology to other viral proteins in the database [Liu *et al.*, 1995].
Figure 11. Genome organization of caliciviruses (NoV and SaV). The first ORF encodes a large polyprotein, molecular weight of 193.5K. The 5' end codes for precursor of the nonstructural proteins. ORF2 encodes capsid protein, molecular weight of 56.6K. The ORF3 at the 3' end of the genome is encodes for a small basic protein, molecular weight of 22.5K [Hardy et al., 1996]. The different between the genome of SaV and NoV, is that the capsid structural protein gene of SaV is in the same frame as ORF1 [Liu et al., 1995]. Genome organization of NoV (MV 24 (Torontovirus), GenBank accession number M87661) [Lew et al., 1994], genome organization of SaV (pJG- Sap01, GenBank accession number AY694184) [Fullerton et al., 2007].
1.5.3 Calicivirus Replication

The understanding of calicivirus replication is not yet clear because of the lack of cell culture system for virus propagation, all attempts to do so have failed [Green, 1997]. Therefore, the studies of viral replication and structure have relied on the expression of the recombinant virus proteins in heterologous systems. The expressed norovirus capsid assembles to form virus like- particles (VLPs) [Jang et al., 1995] and expression of the polyprotein and virus proteases has allowed the proteolytic processing patterns of the viruses to be mapped [Belliot et al., 2003].

1.5.3 Epidemiology of HuCV Infection

Caliciviruses are among the most common cause of acute non bacterial gastroenteritis outbreaks in all age groups in industrial countries [Fankhauser et al., 2002; Lopman et al., 2002] [Tompkins et al., 1999; Hale et al., 2000; de Wit et al., 2001]. Their significance as a cause of gastroenteritis outbreaks in developing countries is not clear [Berg et al., 2000; Gallimore et al., 2004]. Transmission of these viruses is associated with food and waterborne contamination and also person to person spread [Fankhauser et al., 1998; Koopmans et al., 2000]. Several studies have found human caliciviruses second only to rotaviruses as a cause of gastroenteritis in young children [Bon et al., 1999; Kirkwood et al., 2001].

Epidemiological studies were conducted in various locations worldwide; for example a molecular epidemiological study in Spain reported that NoVs are the most common cause of gastroenteritis outbreaks. They were detected in 25 cases out of 44 (56%) cases positive for caliciviruses [Buesa et al., 2002]. A similar study was conducted in France between December 1998 to February 2004 and it was reported
that 172 cases of caliciviruses have been detected (93% NoV and 7% for SaV), most of the positive cases (91%) were detected in winter [Bon et al., 2005].

The incidence of NoV in an epidemiological study of infectious intestinal disease (IID) in Netherlands from 1996-1999, was 5.1% and it was significantly higher in young children, but remained at around 3-5% for all ages [de Wit et al., 2001]. In England 1% of children 1 year of age and less were infected with NoV [FSA, 2000].

In Netherland, NoVs were associated with more than 80% of reported outbreaks of gastroenteritis from 1994-1999. NoV and SaV were detected from community in 16.5%, 6.3% respectively by using RT-PCR and 5.1%, 2.4% from patients visiting their physician with acute gastroenteritis [Koopmans et al., 2000].

In Sweden at 2005 Johansson et al have first reported the nosocomial outbreaks with sapovirus infection among adults and as a result, it was recommended to include the diagnostic test for sapoviruses in investigation of gastroenteritis in adults. Sapovirus infection of adults was also recognized in the UK in 1985 when HuCV infection (the older term for SaV) was noted in this host [Cubitt and Barrett, 1985]. Moreover studies of calicivirus prevalence in the USA [Zintz et al., 2005] have concluded that gastroenteritis was frequently caused by these viruses in children; leading to hospitalization in 7.1% for NoV and 1.4% for SaV infections. Incidence of SaV has also been estimated. In the Netherlands SaV was found in 2.4% of samples, almost exclusively from children [de Wit et al., 2001], a similar incidence was found in England [FSA, 2000].
1.6 Current Knowledge of These Agents in Saudi Arabia

Since rotavirus is the most important cause of non-bacterial gastroenteritis in children, epidemiological studies performed to date in the Saudi Arabia have focused on this agent. Even so, the available data are limited in scope and detail. They are restricted largely to prevalence of rotavirus infection in children (6 years of age and less) attending out-patient clinic or admitted to hospital with gastroenteritis symptoms [Akhter et al., 1994a; Akhter et al., 1994b; el Assouli et al., 1992].

HRV reported in 14% (8135/58,110) to 42% (520/1,242) of cases of diarrhoea overall in KSA [Akhter et al., 1994a; Mohammed et al., 1994]. This is a high value and recent studies suggest that the incidence may be falling. Ghazi et al., (2005) found that the incidence of rotavirus infections had decreased in the city of Makkah in 2005 [Ghazi et al., 2005]. Despite any apparent decrease in HRV incidence, Kheyami and his colleagues (2006) made a comparison study for the incidence in Saudi Arabia by reviewing of 22 studies published between 1982 to 2003 and concluded that rotavirus remains the most common cause of diarrhoeal infection in infant and young children in Saudi Arabia [Kheyami et al., 2006].

Prevalence is similar in different locations rates of 34.6% (199/576) to 42.2% (524/1,242) were reported in Jeddah [el-Sheikh and el-Assouli, 2001; el Assouli et al., 1992; Mohammed et al., 1994], 39.6% (59/150) in Dammam [Huq et al., 1987], and 43% (150/349) in Taif [elAssouli et al., 1996].

Similarly detailed investigation of the viruses concerned is limited to determining the distribution of RNA electrophoretic variants and basic typing of
antigenic strains in Jeddah and Taif [el Assouli et al., 1995; elAssouli et al., 1996]. Both long and short profiles, and the four most common VP7-related serotypes (G1-4) were identified [Milaat and el Assouli., 1995]. The serotypes exhibited genomic RNA polymorphism similar to other global isolates. However there is currently no knowledge of the distribution of rotavirus genotypes across the country or concerning their association with the major G and P antigenic groups in Saudi Arabia. Knowledge of the diversity and distribution of rotavirus strains circulating in communities of different global regions is clearly needed.

Further there are even fewer data on other agents, such as EAdV, HAstV and caliciviruses (NoV, SaV) and no thorough epidemiological study has ever been carried out in Saudi Arabia. Akhter et al., [1995a] have shown that 8.5% of diarrhoeal cases in Saudi Arabia were due to adenoviruses but obtained no data on distribution or age related and there are no clear data on astroviruses in Saudi Arabia at all.

### 1.6.1 Special Features of KSA that May Affect Virus Transmission

#### Drinking Water

Saudi Arabia is now the world’s largest producer of desalinated water. Since 2000, there has been a growth in desalination facilities in the Kingdom and this source now supplies 70% of the country’s drinking water requirement; supplying urban and industrial centers through a network of pipes that run for more than 2,300 miles. Desalination is achieved by using the multi-stage flash distillation desalination process (MSF), a high temperature treatment raising the water temperature 100°C. This is sufficient to inactivate most intestinal viruses [reviewed in Carter, 2005]. The majority of the remainder of the country’s need for drinking water is supplied by bottled water that meets standards set by Saudi Arabia and World Health Organization (WHO).
**Sewage Disposal**

Sewage networks now cover more than 85% of the houses in urban areas. Sewage is disposed of to the sea after treatment by six public-sector secondary-level treatment plants and chlorination.

**Pilgrimage**

The Hajj and Umrah: each year, the kingdom hosts with honour more than two million Muslims to perform Hajj, as well as several million who visit the holy Makkah to perform Umrah. As a result the kingdom is in challenged to improve its the services and health measures. Therefore, Ministry of Health in Saudi Arabia took some precautionary procedures to minimize transmission and import of diseases to the Kingdom. They required all pilgrims (adults and children) must be vaccinated against the circulating infections; Meningococcal meningitis, Polio, Hepatitis B, Influenza, Vectorborne diseases: Yellow fever, Malaria [Eurosurveillance editorial office, 2006]. Also, all kinds of goods are not allowed to be imported into the country. Figure 12 shows how Hajj is very crowded with people from all over the world with all ages.

Few studies have been carried out across the Middle East, but those that have been reported suffer similar limitations to those performed in Saudi Arabia, and were restricted to investigating RNA electropherotypic variation of HRV [Aithala et al., 1996; Dutta et al., 1990; Radwan et al., 1997; Sethi et al., 1988]. Rarely there have been studies employing molecular methods [Fodha et al., 2006]. However, detection of rotavirus G9 serotype by the polymerase chain reaction (RT-PCR) has been reported from both Israel [Shif et al., 1994] and Libya [Cunliffe et al., 2001] and a single study has addressed HASTV occurrence in Egypt [Monroe et al., 2001].
Figure 12. Hajj and Umra are very crowded season. Several million of Muslims of all ages from all over the world, specially the third world country visit the kingdom to perform Hajj and Umra. These pictures are taken from the following addresses

http://alislam.org/gallery/photos/hajrasw2.gif,
http://butterflysoup.com/imagesBlog/HajjaSanaa06/HajjahSanaa.jpg,
http://www.foxnews.com/photoessay/photoessay_1319_images/1229060926_M_122906_Hajj4.jpg,
http://static.filefront.com/images/personal/d/DanishAlHyderab/63212/izjgurbjwj.jpg,
http://www.flickr.com/photos/41535457@N00/382311963

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In conclusion it seems clear that health measures and awareness in Saudi Arabia have been improving gradually in recent years. Major improvements have been ongoing in terms of water provision and sewage disposal in recent years and a history of outbreaks associated with pilgrimage have cause the ministry of health to take these matters seriously and introduce improvement in facilities for al Hajj and Umra seasons. Therefore our hypothesis is that the incidence of water related infections will be lower in this study compared to those conducted prior 2002.

1.7. Aims and Objectives

The above discussion summarizes the main viruses associated with diarrhoeal infections, the available data concerning their incidence in KSA and the features of KSA that might contribute towards a unique and changing pattern of virus circulation in this country. This study has therefore the following objectives

1. To gather improved and extended data concerning the incidence of diarrhoeal viruses in patients reporting with community acquired diarrhoea in KSA.

2. To conduct molecular characterization of the viruses in circulation.

3. To determine whether the viruses circulating now match or differ from those detected in the past (where such data are available).

4. To compare viruses circulating in KSA with those found elsewhere and seek new viruses in KSA that may reflect exposure to emergent viruses worldwide.

5. To investigate whether diarrhoeal viruses might show seasonal distribution or be associated temporally with periods of pilgrimage most notably the Hajj.
To address these requirements, 1000 diarrhoeal stool specimens were collected and analysed over a year-time period from children of six years of age or less. Presenting with community acquired diarrhea.
2. Materials and Methods

2.1 Subjects and Study Areas

Previous studies estimated HRV as responsible for 14%-40% collected stool of the gastroenteritis cases in infant and young children overall in the country [Akhter et al., 1994a; Mohammed et al., 1994]. When the equation was applied, we found out that the sample number should have been 865. We opted to increase it to 1000 samples.

Between September 1st, 2002 and August 31st, 2003, a total of 1000 stool specimens were collected from children aged 6 years or less, with acute diarrhoea and attending out-patient clinic or admitted for diarrhoea to a pediatric ward in the main hospitals of the following cities in Saudi Arabia. Jeddah (a major coastal city and the commercial capital with hot, humid climate), Makkah (a major city and religious capital with hot, dry climate), Riyadh (the largest city and political capital with colder, dry climate), Jeddah have a population of 3.4 million, and Makkah have a population of 3.4 million, while Riyadh has a population of over 4.5 million. Figure 13 shows their relative geographical locations in the country. These locations were selected because they represent the largest urban areas of Saudi Arabia and span the diversity in topography, climate and population. In addition, these cities are subject to heavy traffic of millions of Pilgrims during Hajj and Umrah times of the year which during the study period, occurred in February 13 and ended at March 14. The approximate number of Moslems arriving in Makkah for Hajj and Umra is around 2.5 million each year [Fadaak et al., 2002].
2.2 Collection of Specimens

Faecal specimens were collected in sterilised containers (Jar 1 oz. 30 ml) from hospitals in these three cities and sent to our laboratory on ice by courier to be received within 24 hours of shipping. In the laboratory, they were immediately stored at -70°C until examined.

2.3 Preparation of Stool Extracts

In order to analyze the samples, approximately 100 mg of each of the frozen specimen was thawed and diluted with 1 ml of Dulbecco’s phosphate-buffered saline (PBS, pH 7.0) (ICN Biomedicals Inc., Ohio, USA), mixed gently in micro centrifuge tubes using minishaker, and clarified by centrifugation at 250 x g for 10 min at 4°C. One ml of the supernatant was recovered and divided into 250 µl aliquots, which were analyzed immediately or stored at -70°C until examined.

2.4 Enzyme Immunoassay for the Detection of Virus Antigens

One hundred µl of each extracted sample was tested for viral antigens using commercial ELISA kits (IDEA for rotaviruses, IDEA for adenoviruses, IDEA for astroviruses, and IDEA for Noroviruses) from DAKO (Cambridgeshire, UK), and Oxoid (Ely) Ltd, Denmark House, Cambridgeshire, UK) according to the manufacturers’ instructions. Briefly, the tests use antibody in a solid-phase sandwich enzyme immunoassay format to detect a cross-reactive generic epitope of each virus.

In the case of astroviruses, the kit uses a polyclonal antibody and dextran polymer conjugate with a high incorporation of enzyme and antibody molecules [Tanaka et al., 2000; Chernesky et al., 2001] recognizes a conserved epitope on the
capsid Protein. For rotaviruses the kit uses a polyclonal antibody to detect a group A-specific antigen [Grauballe et al., 1981; Coulson et al., 1984]. For adenoviruses the kit uses a genus specific monoclonal antibody to detect an epitope of the adenovirus hexon antigen, present in all human adenoviruses [Cepko et al., 1983]. Finally, for norovirus the kit detects Genogroup 1 and 2 viruses using specific monoclonal and polyclonal antibodies recognizing each [Antony et al., 2000]. Performance of the test was similar in each case: in brief: the specimen was added to the microwells pre-coated with the viral capture antibody. Non adherent material was removed by washing and a detector antibody conjugated to horseradish peroxidase (100 µl) was added. The mixture was incubated for 60 minutes at room temperature. The microwells were washed and 100 µl of chromogenic substrate was added and incubated for 10 minutes at room temperature. Positive samples developed a blue color. Acidic "stop solution" was added (100 µl), turning the color yellow. Absorbance was read spectrophotometrically at 450 nm in a Titratek Multiskan® MCC/ 340. Samples were designated positive if they developed an absorbance of 0.10 units over background control levels.
Figure 13. Map of Saudi Arabia showing the relative location of the three large urban cities (Makkah, Jeddah and Riyadh) from which the project’s samples have been collected from. Jeddah (pop 3.4m), Makkah (pop 1.7m) Riyadh (pop 4.5 m).
(http://www.euroHajmision.org/images/map_1_saudi_arabia.gif).
2.5 Nucleic Acid Extraction

2.5.1 Extraction of DNA from Adenovirus-Positive Stool Samples.

DNA was extracted using standard molecular biology techniques (Sambrook Fritsch Maniatis, 1989). One hundred μl of each stool extract, 10 μl proteinase K (10mg/ml) (Life Technologies, Gaithersburg, MD, USA), and 90 μl of TE/SDS buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% SDS) were mixed in a microcentrifuge tube and incubated for 1 h at 50°C. At the end of incubation, 100 μl of buffer saturated phenol and chloroform (1:1) solution (Sigma, St. Louis, MO, USA) were added. The mixture was vortexed and centrifuged at 16,500g for 15 min at 4°C to separate the phases. To remove residual phenol, the upper aqueous phase was recovered in a separate tube and mixed with an equal volume (about 300 μl) of a 24:1 chloroform and iso-amyl alcohol solution (Sigma), vortexed briefly, and centrifuged at 16,500g.

The aqueous phase was placed in a fresh tube, to which 15 μl of 3 M sodium acetate and 2 μl of t-RNA (5 mg/ml) (Life Technology) were added. The nucleic acids were precipitated by the addition of 500 μl of 95% cold ethanol (Sigma), mixed briefly. To insure the precipitation the mixture was left at -20°C overnight and centrifuged at 16,500g for 30 min at 4°C. The supernatant was discarded. The pellet was washed once with 500 μl of 70% cold ethanol. The supernatant was discarded and the pellet was dried under reduced pressure. The nucleic acid pellet was finally dissolved in 50 μl of TE buffer and stored at -20°C until used.
2.5.2 Extraction of RNA from Rotavirus- and Astrovirus-Positive Stool Samples

One-hundred μl of each stool extract was mixed with 400 μl of TRI RNA/DNA and protein isolation reagent. This product is, a mixture of guanidine, thiocyanate and phenol in a mono-phase solution (Sigma). The mixture was vortexed briefly and allowed to stand at room temperature for 10 min. 200 μl of chloroform were added, the mixture was centrifuged at 16,500 x g for 15 min at 4°C. The upper, aqueous phase containing the RNA was transferred to a separate microcentrifuge tube. 1 μl of glycogen (20 μg/μl) were added and mixed. RNA was precipitated with 0.5 ml isopropanol and allowed to stand at room temperature for 30 min, RNA then were pelleted by centrifugation at 16,500 x g for 15 min at 4°C. The pellet was washed once with 75% ethanol, dried under vacuum, resuspended in 50 μl of nuclease-free water containing 40 units ofRNasin (Ribonuclease inhibitor, Promega, Madison, WI, USA), and stored at -70°C until used.

2.6 Spectral determination of Nucleic Acid Concentration

Samples were diluted 20 fold (5 μl of sample + 95 μl sterile water) and their OD was read at 260nm using the GeneQuant (Amersham Biosciences., Piscataway NJ, USA). This machine outputs concentration directly using the relationship 1 OD=50μg/ml (for any dsDNA) X The dilution factor X The visual reading, and 40μg/ml for any ss DNA or RNA (Sambrook Fritsch Maniatis,1989).

2.7 Gel Analysis [Agarose and staining procedures]

1.5% Agarose gels were prepared by dissolving agarose (Sigma) in 1x electrophoresis buffer TBE [0.9M Tris, 0.9M borate, 20mM EDTA]. Agarose was dissolved by boiling, allowed to cool and then poured as 100 ml gels in the flatbed
apparatus (Bio-Rad Laboratories, Inc. Hercules CA, USA.). When the gel was set, the comb was removed and gels were assembled in the running tanks and covered with 1X TBE running buffer. Meanwhile samples were prepared by mixing with an equal volume of 2X TBE containing 20% glycerol and marker dye (Bromophenol Blue (BPB), final concentration 2 mg/ml) before they were loaded onto the gel and electrophoresed at 120 V for 1-2 hr or until the marker dye had traveled to the end of the gel. Negative controls (the reaction mixture with no DNA template) have been run in all gels. After electrophoresis, the gels were stained with ethidium bromide (1μg/ml) for 30 minutes and photographed under UV light on Polaroid film type 667.

2.8 Reverse Transcription

Reverse transcription-polymerase chain reaction (RT-PCR) was used to determine G and P genotypes of positive rotavirus samples and to type astroviruses. Adenoviruses were identified by PCR without prior RT treatment. For RT-PCR, extracted RNA (5μl -10μl) was mixed with DMSO (7%, final concentration) and heated at 90°C for 5 min and chilled quickly on ice. cDNA synthesis was performed using 12μl of RNA mixture (above) in a 30μl reaction containing 20μg of random hexamer primers (Promega ), 1 mM each dNTP (Life Technologies), 0.1 M Tris-HCl, pH 8.3, 50 mM KCl, 10 mM MgCl₂, 10 mM dithiothreitol . 0.5 μl of RNasin (40U/μl) (Life Technologies ), were added. The reaction was started by addition of 5 units of avian myeloma virus (AMV) reverse transcriptase (Life Technologies) and the mixture was incubated at 42°C for 1 h, before heating at 95°C for 5 min to inactivate the AMV-RT enzyme. Then, the cDNA could be amplified by addition of appropriate primer pairs and PCR buffer as follows.
2.9 G-Typing of HRV by Nested RT-PCR Amplification

Gene 9 of the virus (specifying VP7 and carrying G-type specificity) was identified using a nested RT-PCR procedure [Gouvea et al., 1990]. First and second round primer sequences and locations of their binding sites on the gene 9 sequence are listed in Table 4. All primer sequences were derived from Gouvea et al., 1990 and were synthesized in the core facility of King Faisal Specialist Hospital & Research Center. The full length gene 9 (1.1 kb) was amplified from 15 μl of the cDNA preparation in the first round using 100 pmole/μl of both the upstream primer (Beg 9), and the downstream primer (End 9). Amplification used 2U of VentR (exo-) DNA Polymerase (New England BioLabs, Beverly, MA, USA). Thirty cycles of amplification were performed with the following parameters; denaturation at 94°C for 60 sec, annealing at 42°C for 90 sec, and extension at 72°C for 60 sec per cycle, followed by a 7 min extension step for the last round at 72°C to complete formation of duplex molecules. Fifteen μl of the RT-PCR product was analyzed by agarose gel electrophoresis as described above to confirm samples were positive by this technique.

G-typing was performed using 2 μl of DNA first round amplification product prepared above combined with 100 pmol/μl of each of the downstream primer (RVG9) combined with G-type-specific primers, G1 to G4, G8 and G9 in a multiplex RT-PCR. The cycling parameters were the same, except that cycle number was reduced to 25. A diagram of rotavirus gene 9 showing locations and directions of synthesis is given as Figure 14.
Table 4. Oligonucleotide primers for RT-PCR amplification of group A rotavirus gene segment 9 and (G) types

<table>
<thead>
<tr>
<th>Primer</th>
<th>Serotype</th>
<th>Sequence (5' – 3')</th>
<th>Polarity</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beg 9</td>
<td>All group A</td>
<td>GGCTTTAAAAGAGAGAGAAATCCGTCTGG</td>
<td>+</td>
<td>1-28&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>End 9</td>
<td>All group A</td>
<td>GGTCACTCATACAATTC TAATCTAAG</td>
<td>-</td>
<td>1062-1036&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ABT1</td>
<td>G1</td>
<td>CAAGTACTCAAATCAATGATGG</td>
<td>+</td>
<td>314-335&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>ACT2</td>
<td>G2</td>
<td>CAATGATATTACACATTTTCTGTG</td>
<td>+</td>
<td>411-435&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>aET3</td>
<td>G3</td>
<td>CGTTTGAAAGAAGTTGCAACAG</td>
<td>+</td>
<td>689-709&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>aDT4</td>
<td>G4</td>
<td>CGTTTCTGGTGGAGGATGG</td>
<td>+</td>
<td>480-498&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>aAT8</td>
<td>G8</td>
<td>GTCACACCATTGTAATTCG</td>
<td>+</td>
<td>178-198&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>AFT9</td>
<td>G9</td>
<td>CTAGATGTAACTACAACCTAC</td>
<td>+</td>
<td>757-776&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>RVG9</td>
<td>All group A</td>
<td>GGTCACTCATACAATTCT</td>
<td>-</td>
<td>1062-1044&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup><sup>b</sup> are primer pairs for the amplification of the whole segment that do not distinguish between types.
<sup>c</sup> are sense primers.
<sup>d</sup> the antisense primer that determines the different genotypes.

[Gouvea et al., 1990].
Figure 14. Diagram of Rotavirus gene 9 showing locations of variable regions, directions of synthesis and RT-PCR primers with their expected genotype group, and length of the amplified products. A full length VP7 gene (1.1 kb) was amplified in the first round using 100 pmole/μl of each of the upstream primer, Beg 9, and the downstream primer, End 9. G-typing was performed in the second round using the downstream primer (RVG9) combined with G-type-specific primers, G1 to G4, G8 and G9 in a multiplex RT-PCR. This figure was obtained from Gouvea et al., 1990.
2.10 P- Typing of HRV by Semi-Nested RT-PCR Amplification

Semi-nested RT-PCR was used to determine P genotypes as described by Gentsch et al [1992]. Primer sequences derived from this report and their sites of annealing in the gene 4 sequence are listed in Table 5. The RT-PCR conditions described above were followed including the cycling parameters for both rounds. The first round RT-PCR was performed to amplify 876 bp fragment of gene segment 4 of group A rotaviruses using upstream (con3) and downstream (con2) consensus primers. The product from the first round was then amplified as a template in the second round using the upstream primer (con3) in combination with individual primers specific for each genogroup. P genogroups were assigned by identification of RT-PCR products of appropriate size defined by the type-specific primer. A diagram of HRV gene 4 showing the locations and directions of synthesis is located at Figure 15.
Table 5. Oligonucleotide primers for semi-nested RT-PCR amplification of gene segment 4 and (P) types of group A rotaviruses.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Serotype</th>
<th>Sequence (5′ – 3′)</th>
<th>Polarity</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con3</td>
<td>All group A</td>
<td>TGGCTTCGCCATTTTATAGACA</td>
<td>+</td>
<td>11-32a</td>
</tr>
<tr>
<td>Con2</td>
<td>All group A</td>
<td>ATTTCCGACCATTTATAACC</td>
<td>-</td>
<td>356-339b</td>
</tr>
<tr>
<td>1T-1</td>
<td>P[8]</td>
<td>TCTACTTGGATAACGTGC</td>
<td>-</td>
<td>339-356c</td>
</tr>
<tr>
<td>2T-1</td>
<td>P[4]</td>
<td>CTATTGTTAGAGGTTAGAGTC</td>
<td>-</td>
<td>474-494c</td>
</tr>
<tr>
<td>3T-1</td>
<td>P[6]</td>
<td>TGTTGATTAGTTGGATCAA</td>
<td>-</td>
<td>259-278c</td>
</tr>
<tr>
<td>4T-1</td>
<td>P[9]</td>
<td>TGAGACATGCAATTGGAC</td>
<td>-</td>
<td>385-402c</td>
</tr>
<tr>
<td>5T-1</td>
<td>P[10]</td>
<td>ATCATAGTTAGTATGTCGG</td>
<td>-</td>
<td>575-594c</td>
</tr>
</tbody>
</table>

[175x314] Sequence (5′ – 3′)

[TGGCTTCGCCATTTTATAGACA]

[ATTCCGACCATTTATAACC]

[TCTACTTGGATAACGTGC]

[CTATTGTTAGAGGTTAGAGTC]

[TGTTGATTAGTTGGATCAA]

[TGAGACATGCAATTGGAC]

[ATCATAGTTAGTATGTCGG]

[Gentsch et al 1992].

^a,b^ are primer pairs for the amplification of part of segment 4 that do not distinguish between types.

^c^ are antisense primers that are used to determine the different genotypes.
Figure 15. Diagram of Rotavirus gene 4 showing locations of variable regions, directions of synthesis and RT-PCR primers with there expected length of the amplified products from each genotype. An 876-bp product from the first round was amplified as a template in the second round using upstream primer (con3) in combination with individual primers specific (3T-1, 1T-1, 4T1, 2T-1 and 5T-1) for each genetic group. The figure drives from Gentsch et al., 1992.
2.11 PCR Amplification of Adenovirus

PCR amplification of a segment of the hexon gene (300bp) was used to confirm the results obtained from enzyme immunoassays. All primers for nested PCR amplification and the sites at which they anneal are listed in Table 5 and were derived from [Pring-Akerblom., et al 1994; Allard et al., 1990]. DNA extracted from positive samples (10 μl) was used as a template and amplified by nested PCR using 100 pmo/l/μl of each of the general primers hexAA1885 and hexAA1913 which amplify the hexon gene from all human adenovirus types. Reactions were carried out using 2.5 U of Ampli Taq DNA Polymerase (Roche Molecular Systems, inc., Branch Burg, NJ, USA.). Thirty cycles of amplification were performed with the following parameters; denaturation at 91°C for 1 min, annealing at 50°C for 30 sec, and extension at 72°C for 45 sec per cycle, followed by a 7 min extension step for the last round at 72°C to complete formation of duplex molecules with size of 300bp [Allard et al., 1990]. EAdV 40, EAdV 41 and Ad 31 were differentiated using specific primers for each genotype as described by Pring-Akerblom et al (1994). Amplification was carried in 50 μl reaction mixture containing 10 μl DNA extract from ELISA-positive samples, 2.5 U of Ampli Taq DNA Polymerase. The cycling parameters were the same, as above. When required Ad 12 and Ad 18 were investigated using specific primers for each genotype with the same conditions and parameters for EAd40, 41 and Ad31. Specific primers for genotypes Ad12 and Ad18 (Table 6) were designed using Oligo 6 Program and synthesized in the core facility of King Faisal Specialist Hospital & Research Center.
2.12 Restriction Fragment Length Polymorphism (RFLP) Analysis of Adenovirus Isolates

RFLP experiments were conducted on all positive samples for adenovirus in order to confirm our PCR genotyping results, to genotype the remaining isolates that were not typable by PCR, and to examine the possibility of co-infection with more than one adenovirus genotypes. These experiments used the restriction enzymes Rsa I and Hha I (New England BioLabs), which have been reported to produce a distinctive RFLP pattern of EAd 40 and EAd 41.

The hexon gene PCR products (approximately 1 µg) generated from positive samples were digested with 10 units of Hha I or Rsa I in a total volume of 30 µl. The mixture was incubated at 37 °C overnight. For a better intensity, samples were loaded onto a composite gel (3 % agarose low melting point (LMP) (Life Technologies ) + 1% agarose gel (Sigma), and run at 100 Volts for 2 hr in 1X TBE buffer. Cleavage sites and fragments expected following Rsal and Hhal digestion of the 300bp product are shown in Table 7. Taken from [Allard et al., 1994].
Table 6. Oligonucleotide primers for nested PCR amplification of adenovirus.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Genotype</th>
<th>Sequence (5' – 3')</th>
<th>Polarity</th>
<th>Position</th>
<th>Length of Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>hexAA1885</td>
<td>Hexon gene</td>
<td>GCCGCAGTGGTCTTACATGCACAT</td>
<td>+</td>
<td>18,858-18,883</td>
<td>300 bp</td>
</tr>
<tr>
<td>hexAA1913</td>
<td>Hexon gene</td>
<td>CAGCACGCCGCGATGTCAAAGT</td>
<td>-</td>
<td>19,158-19,136</td>
<td></td>
</tr>
<tr>
<td>H1*</td>
<td>Ad40 and Ad41</td>
<td>TTGACATCCGCGGGTGCT</td>
<td>+</td>
<td>302-321 / 14-33</td>
<td>939 bp</td>
</tr>
<tr>
<td>H40</td>
<td>Ad40</td>
<td>TATTCTGAGACCAGTTAGTT</td>
<td>-</td>
<td>1240-1221</td>
<td></td>
</tr>
<tr>
<td>H41</td>
<td>Ad41</td>
<td>CTGCAGTCCAGTGGTCCGCA</td>
<td>-</td>
<td>955-936</td>
<td>942 bp</td>
</tr>
<tr>
<td>31H1</td>
<td>Ad31</td>
<td>TTGATATAGGAGGTGCTG</td>
<td>+</td>
<td>2-21</td>
<td>932 bp</td>
</tr>
<tr>
<td>H31</td>
<td>Ad31</td>
<td>TTTACCATCCATTTCCGT</td>
<td>-</td>
<td>933-914</td>
<td></td>
</tr>
<tr>
<td>Ad12-U**</td>
<td>Ad12</td>
<td>ATATATGGCCCATCGATAACCT</td>
<td>+</td>
<td>61-84</td>
<td>270 bp</td>
</tr>
<tr>
<td>Ad12-L</td>
<td>Ad12</td>
<td>CCAAAATGTCACTGCTG</td>
<td>-</td>
<td>313-331</td>
<td></td>
</tr>
<tr>
<td>Ad18-U</td>
<td>Ad18</td>
<td>TACTTTGTGTACTCGGGAACCA</td>
<td>+</td>
<td>209-231</td>
<td>693 bp</td>
</tr>
<tr>
<td>Ad18-L</td>
<td>Ad18</td>
<td>AATCCGCACACCACGTGCAA</td>
<td>-</td>
<td>884-902</td>
<td></td>
</tr>
</tbody>
</table>

*H1 General primer for Ad40 and Ad41.
**U: Upper Primer, L: Lower Primer
Table 7. Cleavage sites and size of RsaI and HhaI on the 300bp hexon product.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Recognition Sequence</th>
<th>Amplifier</th>
<th>No. of Cleavage Sites</th>
<th>Position</th>
<th>Fragment Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hha I</td>
<td>gcg/c</td>
<td>Ad40</td>
<td>2</td>
<td>180-228</td>
<td>180-75-48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ad41</td>
<td>2</td>
<td>80-228</td>
<td>148-80-75</td>
</tr>
<tr>
<td>Rsa I</td>
<td>gt/ac</td>
<td>Ad40</td>
<td>1</td>
<td>46</td>
<td>256-45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ad41</td>
<td>1</td>
<td>91</td>
<td>211-90</td>
</tr>
</tbody>
</table>

[Allard et al., 1994].
2.13 Typing of HAstV by Semi-Nested Multiplex RT-PCR Amplification

Astroviruses were identified by seminested multiplex RT-PCR targetting the capsid protein gene. All antigenic types can be amplified using the primer set End (-) and For (+) in RT-PCR [Matsui et al., 1998; Sakamoto et al., 2000] Semi-nested RT-PCR was then performed on this product as described above for rotavirus. First round was achieved by the following parameters; denaturation at 94°C for 1 min, annealing at 45°C for 2 min, and extension at 72°C for 2 min per cycle, followed by a 7 min extension step for the last round at 72°C. The RT-PCR product from this round was used as a template with downstream primer (End-) in combination with individual type-specific primers for each genotype. RT-PCR conditions and cycling parameters were the same as the first round, except that cycle number was reduced to 25. Primer sequences obtained from the reference above are listed in Table 8.
Table 8. Oligonucleotide primers for semi-nested RT-PCR amplification of Astrovirus.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Genotype</th>
<th>Sequence (5’ – 3’)</th>
<th>Polarity</th>
<th>Position</th>
<th>Length of Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>For</td>
<td>First Round Product</td>
<td>AAAGAAGTGTGATGGCTAGCA</td>
<td>+</td>
<td>1-21</td>
<td>2376 bp</td>
</tr>
<tr>
<td>End</td>
<td></td>
<td>TCCTACCGCCGGCCGC</td>
<td>-</td>
<td>2377-2359</td>
<td></td>
</tr>
<tr>
<td>AST-1</td>
<td>HAST-1</td>
<td>AACCAAGGAATGACATGAC</td>
<td>+</td>
<td>2166-2185</td>
<td>212 bp</td>
</tr>
<tr>
<td>AST-2</td>
<td>HAST-2</td>
<td>ACCTGCGCTGAGAAACTG</td>
<td>+</td>
<td>2247-2264</td>
<td>158 bp</td>
</tr>
<tr>
<td>AST-3</td>
<td>HAST-3</td>
<td>CTGCTTGCATCTGGTTTCA</td>
<td>+</td>
<td>2283-2303</td>
<td>119 bp</td>
</tr>
<tr>
<td>AST-4</td>
<td>HAST-4</td>
<td>TGATGATGAAGAAGACTCTAATAC</td>
<td>+</td>
<td>2071-2091</td>
<td>258 bp</td>
</tr>
<tr>
<td>AST-5</td>
<td>HAST-5</td>
<td>TAGTAACTTATGATAGCC</td>
<td>+</td>
<td>2014-2031</td>
<td>388 bp</td>
</tr>
<tr>
<td>AST-6</td>
<td>HAST-6</td>
<td>TGGCCACCCTTGTTCTGAGA</td>
<td>+</td>
<td>1951-1971</td>
<td>427 bp</td>
</tr>
<tr>
<td>AST-7</td>
<td>HAST-7</td>
<td>CTAGACAAACACACCCCG</td>
<td>+</td>
<td>1842-1859</td>
<td>548 bp</td>
</tr>
<tr>
<td>AST-8</td>
<td>HAST-8</td>
<td>GGTAAGTGTTACCTGCTAATAG</td>
<td>+</td>
<td>1753-1775</td>
<td>599 bp</td>
</tr>
</tbody>
</table>

[Matsui et al., 1998; Sakamoto et al., 2000]
Figure 16. Diagram of astrovirus RT-PCR amplification. Astroviruses were identified by seminested multiplex RT-PCR targeting the capsid protein gene. All antigenic types can be amplified using the primer set End (-) and For (+) in RT-PCR. Semi-nested RT-PCR was then performed on this product as described by [Matsui et al., 1998; Sakamoto et al., 2000]. The figure drives from [Matsui et al., 1998].
2.14 DNA Sequencing Using 3130XI Genetic Analyzer

Amplicons for sequencing were purified using DyeEx TM 2.0 Spin Kit (QIAGEN). The samples then were propose to the Research Center Genetic department in King Faisal Specialist Hospital, to determined the nucleotide sequence using 31 30XI Genetic Analyzer (Applied Biosystems).

Sequencing reactions were set up according to the manufacturer’s instructions (ABI PRISM®BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Victoria, Australia): a 20µl reaction mix containing 20 ng PCR products, 4µl termination reaction mix, 2 µl Sequencing Buffer (5X), 1.6 µM single primer (either forward or reverse primer specific for the target sequences) and the volume was brought to 20 µl with ddH2O. The reaction was performed in cycling at 96 °C for 1 minute, 50 °C for 5 seconds and 60 °C for 4 minutes for 25 cycles. Each nucleotide sequence was compared to those of reference strains by using the BLAST program in order to assign each genotype. DNASTAR Megalign software was used for DNA sequence alignments.

2.15 Statistical Methods

The sample size proportion test was carried out to calculate the significance sample size for our study using the Confidence Intervals with the following equation;

\[ n = \frac{Z^2_{1,\alpha/2} p(1-p)}{D^2} = \frac{(1.96)^2 * (0.1) * (0.9)}{0.02^2} = 865 \]

\( n; \) is the samples size, \( p; \) the sample proportion, \( \alpha; \) level of significance.

Our estimation for the proportion 0.1± 0.02, then
Moreover, the chi-square test, $\alpha$ level of significance and Fisher Exact test were used when appropriate to analyze the season of infection, geographical location, age distribution and the prevalence of rotavirus, enteric adenovirus and astrovirus infection among Saudi and non-Saudi patients of positive samples among children from the cities covered in this study. P-values less than 0.05 were considered significant.

$D = 0.02, p = 0.1, and \infty = 0.05$ so $Z_{1-\alpha/2} = Z_{0.975} = 1.96$ (from tables).
3. RESULTS

During the period of September 1\textsuperscript{st}, 2002 to August 31\textsuperscript{st}, 2003, a total of 1000 fecal samples were collected from children (6 years of age or below) either attending outpatient clinic or admitted to a pediatric ward in Hospitals in Jeddah, Makkah and Riyadh for diarrhoea. Ethical permissions were obtained from each hospital and clinical details were obtained from patients' data files (age, sex, hospitalization and nationality).

Stool samples were analysed using ELISA and different molecular techniques, for the presence of rotavirus, adenovirus, astrovirus, and norovirus. Where possible the sero/genotype of the virus present was also identified.

3.1 Incidence of Viruses in the Samples

In this study we hypothesized that the incidence of viruses causing diarrhoea may be reduced by recent developments in public health in Saudi Arabia. Therefore, we started by investigating the presence of these viruses in the collected stool samples using different serological and molecular methods. Rotavirus has been recognized as the major cause of diarrhoea in previous studies in Saudi Arabia [el Assouli et al., 1992; Mohammed et al., 1994] and world wide [Cook et al., 1990; Kapikian et al., 1996] and thus this virus was investigated first.

3.1.1 Prevalence of Rotavirus

The presence of rotavirus group A antigen was investigated in all stool samples. The percent of rotavirus-positive stool samples was 6.8\% (14/205 samples) in Jeddah,
4.6% (19/405 samples) in Makkah and 6.9% (27/390 samples) in Riyadh. Overall the total percent of positive stool samples in the 3 cities was 6% (60/1000 samples); these results are summarized in Table 9. Of these 60 rotavirus ELISA-positive samples, one sample was weakly ELISA-positive and RT-PCR-negative, and one was strongly ELISA-positive but could not be processed further because of lack of sample material. These two samples were both derived from Jeddah, and thus only 58 of the 60 rotavirus ELISA-positive samples were used for further investigation which included 12 samples from Jeddah. An analysis for the age of children positive for rotavirus showed a significant increase in infection among the children who are 1 year of age or less (61.6%) compared with those who are above 1 year of age (38.3%) (P=0.047) (Figure 17). However, the difference between males (55%) and females (45%) in terms of rotavirus positivity was not statistically significant.
Table 9. Description of fecal samples collected and tested for rotavirus antigen by ELISA from September 1st, 2002 to August 31st, 2003.

<table>
<thead>
<tr>
<th>City</th>
<th>Total Samples</th>
<th>Samples Tested</th>
<th>Rotavirus Positive&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jeddah</td>
<td>205</td>
<td>205</td>
<td>14</td>
<td>6.8</td>
</tr>
<tr>
<td>Makkah</td>
<td>405</td>
<td>405</td>
<td>19</td>
<td>4.6</td>
</tr>
<tr>
<td>Riyadh</td>
<td>390</td>
<td>390</td>
<td>27</td>
<td>6.9</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
<td>1000</td>
<td>60</td>
<td>6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Rotaviruses were screened by an ELISA kit from DAKO [IDEIA™, Cat# k6020]
Figure 17. Age distribution of rotavirus positive samples among children from three cities in Saudi Arabia. This figure shows that the number of children infected with rotavirus, who are 1 year of age or less, is more than those who are over 1 year of age. Statistical analysis supports this finding with P-Value of =0.047.
3.1.1.1 Molecular Analysis of Rotavirus different Strains in Positive Samples

We wished to compare the incidence and genotype of rotaviruses in circulation nowadays to those reported previously in Saudi Arabia from children (6 years of age and less) attending out-patient clinic or admitted to hospital with gastroenteritis symptoms [el-Assouli et al., 1992; Mohammed et al., 1994; el-Sheikh and el-Assouli, 2001]. RT-PCR was used to characterize G and P types of rotavirus in present in these ELISA-positive stool samples for the first time in Saudi Arabia.

Distribution of G & P Types of Human Rotavirus in Saudi Arabia

G Types

All 58 rotavirus-positive samples were analysed to determine the genotype of each rotavirus Group-A isolate from stool extracts. We analysed VP7 (segment 9) to identify the most common genotypes G1-G4, G8, and the unusual emergent type G9 which usually infects animals but has recently been found in humans [Iturriza-Gomara et al., 2000; Sanchez-Fauquier et al., 2006].

Nested multiplex RT-PCR was performed, in which first round RT-PCR produced positive results in all 58 samples. Figure 18 presents gels of a RT-PCR product of first and second round amplification for rotavirus gene 9 and the data obtained are given in table 10. Our results indicate that G1 was the most common genotype in this panel (36/58, 62%), followed by the novel G type G9 (19/58, 33%). This pattern was reflected in each city. In fact G1 was significantly more common than the other genotypes in each
location. The other genotypes found, G2 and G3, were much less frequently identified at 1.2% (1/58) and 3.4% (2/58), respectively. Types G4 and G8 were not detected at all.
Figure 18. Representative gel for the first round RT-PCR amplification product of VP7 full length gene (1.1 kb) and the arrow sizes are the sizes of HaeIII marker. Amplification was performed using upstream primer Beg 9 and downstream primer End 9 (A). The product was used as a template for the second round RT-PCR reaction. In the second round (B) downstream primer was combined with G type specific primers in a multiplex RT-PCR using 2 μl of amplicons obtained from the first round as templates to identify the G types. B: all lanes are representative of various samples. In lanes 2, 3, 4, 5, 6, 7, and 11 are G9 genotype (size of RT-PCR product is 306bp) and lanes 8, 9, 10, 12, 13 are G1 genotype (749bp). M is the ΦX174/ HaeIII size marker, and N is the negative control.
Table 10. Correlation between the incidence of VP7 genotypes and serotypes of human rotaviruses Group- A determined in 58 samples found among children in Saudi Arabia using reference strains.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of Samples (%)</th>
<th>Jeddah</th>
<th>Makkah</th>
<th>Riyadh</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td></td>
<td>4</td>
<td>33</td>
<td>10</td>
<td>53</td>
</tr>
<tr>
<td>G2</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G3</td>
<td></td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>G4</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G8</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G9</td>
<td></td>
<td>8</td>
<td>67</td>
<td>7</td>
<td>37</td>
</tr>
</tbody>
</table>

*Stats to compare distribution of rotaviruses- is preference for G1 in Riyadh and G9 in Jeddah, were significant.*
P Types

We examined the distribution of VP4 types by investigating segment 4 for the most common genotypes that are found in humans (P[8], P[4], P[6], P[9], P[10]). First round RT-PCR, for the VP4 region, produced positive results for all 58 samples as evident by 876-bp RT-PCR product.

Of the 58 samples, 2 samples did not produce any genotyping results, although were positive in the first round. Consequently a total of 56 samples could be typed by this technique. Representative gels for the first and second round of VP4 amplification using RT-multiplex PCR are showing in (Figure 19). Genotype P[8] was the most common (45/56, 80.3%), followed by P[6] (8/56, 14.2 %) and P[4] (3/56, 5.3%). P[9] and P[10] were not detected in this study.

Distributions of VP4 genotypes of human rotaviruses Group-A found among 56 stool samples from children in Saudi Arabia are summarized in Table 11. There were no statistical differences between the incidences of the P types from the three cities.

However, out of the 8 positive samples for P[6] 2 samples were from newborn babies from Jeddah and Makkah. This finding has been reported in several studies from different regions in the world; in Brazil [Linhares et al., 2002] and from South African [Mphahlele et al., 1995].

Overall we obtained P and G type designations for 56 samples (Figure 20). G1P[8] was the most common genotype combination accounting for 60.7% of total samples positive for both genotypes (34/56). This has also been reported as the most common genotype combination elsewhere [Gentsch et al., 1992; Gentsch et al., 1996].
The second most common combination was between G9P[8] accounting for 16% of the total designations. Moreover, we also observed a rare combination of type G9 as G9P[4], and G9P[6]. This has been reported occasionally elsewhere [Ramachandran et al., 1996; Santos et al., 2005; Sanchez-Fauquier et al., 2006].
Figure 19. Representative gels for P genotypes of rotavirus using Semi-nested RT-PCR (the arrow sizes on the Right are the sizes of HaeIII marker, and the arrow sizes on the Left are the sizes of the expected products). A: first round RT-PCR was performed to amplify an 876 bp fragment of gene segment 4 of group A rotaviruses using upstream primer (con3) and downstream primer (con2). B: this is the results obtained in the second round RT-PCR. The amplicon (2 µl) from the first round was amplified as a template using upstream primer (con3) in combination with genotype-specific primers. P genotypes were assigned by identification of RT-PCR product of appropriate size defined by the type-specific primer. All lanes in B are representatives of genotype P1 (size of RT-PCR product is 345bp). M is the φX174/ Hae III size marker, and N is the negative control.
Table 11. The incidence of VP4 genotypes of human rotaviruses Group-A found among 56 stool samples from patients in Saudi Arabia.- percentage distributions are relatively even between the cities.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of Samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Jeddah</td>
</tr>
<tr>
<td>P[8]</td>
<td>9</td>
</tr>
<tr>
<td>P[4]</td>
<td>0</td>
</tr>
<tr>
<td>P[6]</td>
<td>2</td>
</tr>
<tr>
<td>P[9]</td>
<td>0</td>
</tr>
<tr>
<td>P[10]</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 20. Association between G and P genotypes found in the 56 samples that were genotyped for both gene regions (VP7 and VP4). All other combinations were not detected in this study. G1P[8] was the most common genotype accounting for 60.7% of total samples positive for both genotypes (34/56). G9 strain, was found in this study with both type combinations G9P[4], and G9P[6].
In conclusion, this the first determination of G and P types for rotaviruses in Saudi Arabia. G1 was the most common genotype. Surprisingly the second most common genotype was the emergent type G9. This result shows that the G9 serotype recently detected in Saudi Arabia in 2004 [Kheyami et al., 2008] was already present in 2002-2003.
3.1.2 Prevalence of Adenoviruses

Enteric adenoviruses (EAdV) are reported to cause 5-17% of cases of gastroenteritis in infants and preschool children in both developed and developing countries [Albert, 1986; Horwitz, 1990; Fodha et al., 2006]. Moreover, there are some studies that describe adenovirus as the second largest cause of gastroenteritis in infants and young children after rotaviruses [Zlateva et al., 2005]. However there is a lack of data on the occurrence of this virus in Saudi Arabia. Investigating this point was one of the main aims in this study. The prevalence of adenovirus in pediatric stool samples was investigated using ELISA which detects all types of human adenovirus. Types were subsequently identified by PCR and RFLP. The total number of positive samples was 14 out of 1000 (1.4%). Table 12 shows that the prevalence of positive samples is 0 out of 205 (0.0%) in Jeddah, 9 out of 405 (2.2%) in Makkah, and 5 out of 390 (1.2%) in Riyadh. In view of the low numbers identified these differences were not statistically significant.

EAdV infection was found mainly in children below the age of 2, (85.7 %), even given these small numbers this was a significant association. The ratio of infected males to females was equal. Results are shown in Figure 21.
Table 12. Results of pediatric fecal samples screened for the presence of adenovirus by ELISA

<table>
<thead>
<tr>
<th>City</th>
<th>Total Samples</th>
<th>Samples Tested</th>
<th>Adenovirus Positive(^b)</th>
<th>Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jeddah</td>
<td>205</td>
<td>205</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Makkah</td>
<td>405</td>
<td>405</td>
<td>9</td>
<td>2.2</td>
</tr>
<tr>
<td>Riyadh</td>
<td>390</td>
<td>390</td>
<td>5</td>
<td>1.2</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
<td>1000</td>
<td>14</td>
<td>1.4</td>
</tr>
</tbody>
</table>

\(^b\) Adenoviruses were screened by an ELISA kit from DAKO [IDEIA™, Cat# k6021]. This test scores all human adenoviruses as positive.
Figure 21. Age distribution of adenovirus infections. Number of children infected with adenovirus who are 2 year of age or less (12/14, 85.7 %), was relatively more than those who are over 2 year of age (2/14, 14.2 %). Statistically significant with P-Value of = 0.01.
3.1.2.1 Distribution of Adenovirus Types in Saudi Arabia

Since it was also our aim to type adenovirus positive samples, we established a type specific PCR by direct amplification of adenovirus hexon gene using hexAA1885 and hexAA1913 primers (Table 5). This used the method of [Allard et al., 1990], and was validated using a sequenced sample of the adenovirus hexon gene amplified and cloned from a clinical sample as a known control.

All ELISA-positive samples were also PCR positive. The expected PCR product of 300 bp was evident in all samples tested (Figure 22). The viruses were further analysed using type-specific primers aiming to amplify only EAdV 40 or 41. Of the 14 PCR-positive samples, 4 were PCR-typed as EAdV-40 (Figure 23 A) and 3 were PCR typed as EAdV-41 (Figure 23 B). One sample was positive for both EAdV 40 and 41 by PCR. This left 6 samples that were positive for human adenovirus but negative for both types 40 and 41. These samples were further analysed using type-specific primers to detect types Ad-12, Ad-18 and Ad-31 since these have been reported as associated with symptomatic enteric infection [Hierholzer, 1992]. Using these specific primers, we found only one sample to be positive and this was typed as Ad-18 (Figure 23 C). None of the samples tested positive for Ad-12 or Ad-31.
Figure 22. Ethidium bromide-stained agarose gel showing PCR product from the hexon gene of adenovirus (300 bp). The arrow sizes on the Right are the sizes of HaeIII marker, and the arrow sizes on the Left are the sizes of the hexon expected product. Using hexAA1885 and hexAA1913 primers, 300 bp adenovirus hexon gene was successfully amplified from all ELISA positive samples. P is the positive control for adenovirus hexon gene, lanes 1-10 are the PCR products from 10 EAdV ELISA positive samples. M is the ΦX174/ Hae III size marker, and N is the negative control.
Figure 23. A, B and C are representative gels of the positive samples for adenovirus. PCR was performed using specific primers for (EAdV) 40, 41 and Ad18 (the arrow sizes on the Right are the sizes of HaeIII marker and the one on the Left are the products sizes). Panels A, B lanes 1-6 contain experimental samples; panel C lanes 1-9 contain experimental samples. Of the 14 PCR-positive samples, 4 were EAdv-40 (panel A lanes 2A-5A; 932bp) and 3 were EAdV-41 (panel B show 2/3 positive samples for EAdV-41, lanes 4B and 6B; 942bp), and 1 was positive for Ad-18 (panel C, lane 8C; 693bp). M is the ΦX174/Hae III size marker, and N is the negative control. All the 14 ELISA-positive samples for adenovirus have been amplified with each specific primer using in this study for typing adenovirus. The numbers of samples in (A, B, and C) are in order but the samples are not necessary the same.
Since we could not type all isolates by type-specific amplification we also typed them by RFLP analysis of the initial hexon gene PCR product. This analysis would also identify any mixed infections. RFLP experiments were conducted on all 14 positive samples using the restriction enzymes Rsa I and Hha I, which have been previously reported to distinguish between genotypes 40 and 41 [Allard et al., 1994]. Figure 24 shows the expected RFLP pattern corresponding to the different EAdV types derived using Rsa I and Hha I as described by Allard et al., 1994. This is combined with panel C which depicts some of the results obtained here.

Results showed that 7 samples were EAdV-41 and 5 samples were EAdV-40. This included all samples identified as 40 and 41 by type-specific PCR. One sample was positive for both EAdV-40 and 41 RFLP in agreement with our findings from PCR. Moreover, one sample (sample number 7) was not typed by RFLP and was identified as Ad-18 by PCR. The result are summarized in Table 13.

However this analysis also raised a discrepancy related to one of the initially untyped samples. Isolate number 7 failed to amplify with type 40 or 41-specific primers but did react with primers specific for type 18 by PCR. However, RFLP analysis of this sample did not gave a pattern indicative of type 40 or 41 (Table 13). Therefore, this isolate was subjected to sequencing to confirm it's identity. Sequence information indicated that this sample was most similar to adenovirus type 31 of adenovirus. The alignment results of the sequence of this PCR product and adenovirus type 31 (GenBank accession number Y17253) yields 91.5% similarity (Figure 25). Further investigation revealed an explanation for this result (chapter 5).
Figure 24. RFLP pattern of EAdV using HhaI and Rsal restriction enzymes (the arrow sizes in (A) are the sizes of PUC18/ Msp I molecular weight marker). (A) is a drawing that explains the bands that result after digestion of the hexon gene PCR product with the specified enzymes. (B) Shows RFLP pattern of EAd-41 and EAd-40 (expected products sizes for EAd-41; 148 bp, 80 bp, 75 bp, and EAd-40; 180, 75bp) using HhaI enzymes. (C) is a representative gel that shows RFLP pattern of EAd-41 and EAd-40 using Rsal restriction enzymes (expected products sizes for EAd-41 are; 211 bp, 90 bp, and for EAd-40 are; 256, 45). (D) is a representative gel that shows RFLP pattern of EAd-41 using Rsal enzyme. The arrows in panels B,C, and D indicate the sizes of the expected digestion products. The first products corresponding to arrow size 300 bp in (B) and (C), are the remaining undigested product. M is the PUC18/ Msp I molecular weight marker.
Table 13. Summary of adenovirus genotyping investigation.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>PCR of hexon gene segment</th>
<th>Nested PCR with specific primers for</th>
<th>RFLP Rsal /Hha</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
<td>Ad-12 Ad-18 Ad-31 Ad-40 Ad-41</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>- - - +</td>
<td>Ad41/-</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>- - - -</td>
<td>Ad41/-</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>- - - -</td>
<td>Ad41/-</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>- - - +</td>
<td>-/Ad-40</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>- - - +</td>
<td>-/Ad41</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>- - - +</td>
<td>-/Ad40</td>
</tr>
<tr>
<td>7d</td>
<td>+</td>
<td>- + - -</td>
<td>-</td>
</tr>
<tr>
<td>8e</td>
<td>+</td>
<td>- - - +</td>
<td>Ad41/Ad40</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>- - - -</td>
<td>Ad41/-</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>- - - +</td>
<td>Ad40/Ad40</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>- - - -</td>
<td>Ad41/Ad41</td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>- - - -</td>
<td>Ad41/Ad41</td>
</tr>
<tr>
<td>13</td>
<td>+</td>
<td>- - - -</td>
<td>Ad41/Ad41</td>
</tr>
<tr>
<td>14</td>
<td>+</td>
<td>- - - +</td>
<td>Ad40/Ad40</td>
</tr>
</tbody>
</table>

a Confirmation of the ELISA-positive adenovirus samples was performed by direct PCR of adenovirus hexon gene.

b The virus was further analysed with specific primers for enteric adenovirus 40 and 41, and for Ad-31, -12, and -18.

c RFLP experiments were conducted on all 14 positive samples for adenovirus, using the restriction enzymes Rsal and Hha I.

d Isolates 7 gave results in PCR and was not confirmed by RFLP. Therefore, these isolates was subjected to sequencing.

e Isolates 8 was positive for both EAdV-40 and 41 by PCR and RFLP.
<table>
<thead>
<tr>
<th>Similarity Index</th>
<th>Length</th>
<th>Gap Length</th>
<th>Consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 25.** Alignment results of sequence derived from sample 7 with adenovirus type 31. The alignment between sample 7 adenovirus sequence and the sequence of Ad31 (GenBank accession number Y17253) show 91.5% similarity DNASTAR Megalign software was used for DNA sequence alignments. * Alignment coloring: match color = black, mismatch color = red, consensus color = green.
In conclusion, the incidence of adenovirus in infants and children in Saudi Arabia is lower than that of rotavirus with only 1.4% of the samples in this survey scoring for adenovirus. The infection was significantly more common in children below the age of 2 years. Using molecular method adenovirus group F, types 40 and 41 were the main cause of enteric adenovirus infection. Moreover, adenovirus group A type 31 was detected in one of the adenovirus ELISA positive samples. Ad-31 has been reported in association with gastroenteritis symptoms [Hierholzer, 1992] but we cannot conclude that Ad31 was responsible for the symptoms in this case. Although this sample contained no other viruses sought in this study, we do not have information on bacterial/other pathogens that may have been present in this stool.
3.1.3 Prevalence of Astrovirus

There are no extensive data concerning the distribution of this virus in the kingdom, a single study dates from 1995 and used RT-PCR to detect astrovirus serotype 1. This examined astrovirus detection in 130 pediatric stools collected from age-matched children in Saudi Arabia and Leicester, UK and reported the presence of astrovirus-1 in 1.5% of Saudi Arabian samples and 4.6% of those from the UK [Akhter et al., 1995b]. This difference was not statistically significant although, the difference does prompt further investigation. Using our larger sample panel and also to seek the presence of other serotypes.

Prevalence of astrovirus in pediatric stool samples was examined. The positivity rate was 1/205 (0.5%) in Jeddah, 8/405 (2%) in Makkah, and 10/390 (2.6%) in Riyadh. The differences in isolation rates between the cities were not significant A total positivity of 19/1000 (1.9%) was found overall in the survey a value very close to that reported by Akhter and colleagues [Ahkter et al., 1995b]. Results are given in (Table 14). Astrovirus infection was significantly more common in children of 3 years of age or less (84.2 %) compared with those over 3 year of age (15.7 %) (Figure. 26). Positivity ratio for male and female was equal.
Table 14. Description of fecal samples collected and tested for the presence of astrovirus using ELISA kit from September 1\textsuperscript{st}, 2002 to August 31\textsuperscript{st}, 2003.

<table>
<thead>
<tr>
<th>City</th>
<th>Total Samples</th>
<th>Samples Tested</th>
<th>Astrovirus Positive(^c)</th>
<th>Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jeddah</td>
<td>205</td>
<td>205</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>Makkah</td>
<td>405</td>
<td>405</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Riyadh</td>
<td>390</td>
<td>390</td>
<td>10</td>
<td>2.9</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
<td>1000</td>
<td>19</td>
<td>1.9</td>
</tr>
</tbody>
</table>

\(^c\) Astroviruses were screened by an ELISA kit from DAKO [IDEIA\textsuperscript{™}, Cat# k6042]
Figure 26. Age distribution of ELISA-positive samples among children from three cities in Saudi Arabia (Jeddah, Makkah and Riyadh). The figure shows that the number of children infected with astrovirus who are 3 year of age or less (16/19, 84.2%) is more than those who are over 3 year of age (3/19, 15.7%). Statistically significant difference was distinguished, with P-Value of = 0.05.
3.1.3.1 Distribution of Astrovirus Types in Saudi Arabia

Astrovirus, ELISA positive samples were further investigated using RT-PCR in order to determine the type of the virus present. The approach for this typing was developed by Matsui and extended to include astrovirus type 8 by Sakamoto [Matsui et al., 1998; Sakamoto et al., 2000]. Of the 19 ELISA-positive samples, 18 were also found to be positive in multiplex RT-PCR. In each case the amplicons size corresponded with that expected for Hast-8. This was a surprising finding and in order to confirm the type-specific amplification observed we re-examined each sample using mono-specific RT-PCRs for each serotype. Once more positive results were obtained using only those primers directed towards serotype 8. (Figure 27A and Figure 27B). One sample was negative by RT-PCR and could not therefore be typed.

This distribution was a surprising finding since Astrovirus type 8 is often a relatively uncommon serotype [Guix et al., 2002; Méndez-Toss et al., 2004; Noel et al., 1995; Mustafa et al., 2000] Astrovirus type 8 has also been obtained from Pretoria in South African [Taylor et al., 2001].

The identification of type 8 astrovirus in different locations in the kingdom deserves further investigation. However there was insufficient time remaining during this study for further analysis. This topic will form the subject of a separate investigation in KSA for which funding is currently being sought.
Figure 27. Multiplex RT-PCR detection of HAstV. (A) is a representative gel for the Multiplex RT-PCR product, all samples were positive to HAstV-8. Positive results were confirmed by repeating the nested RT-PCR for that specific genotype 8 in a single reaction, (B) is a representative gel for this confirmation reaction. First lane in (A&B) is the ΦX174 (RF DNA-Hae III Digest) molecular weight marker and the arrow sizes on the Right of the gels are the sizes of HaeIII marker, and the one on the Left are the sizes of the products. N is the negative control. The strong band in (B) lanes 12 and 13, does not correspond to any of the expected astrovirus product sizes.
Figure 28. Representative gel for repeated RT-PCR (the arrow sizes on the Right are the sizes of HaeIII marker, and and the one on the Left are the sizes of the products.). The numbers of samples in Figure 27 and 28 are in order but the samples are not necessary the same. All the 19 positive samples were repeated individually, each on a separate day, they all produced positive results for astrovirus type 8 (599bp). First lane is the ΦX174 (RF DNA-Hae III Digest) molecular weight marker, and N is the negative control.
In conclusion we found that at this stage infection with astroviruses in Saudi Arabia was marginally more common than that with adenoviruses but second in importance after the rotaviruses. Astrovirus infection appeared to be more common in children of 3 years or less. Distributions of astrovirus types in the kingdom were investigated using RT-PCR; all samples reacted positively with primers specific for type 8 and this aspect deserves further investigation.

At this stage in the study new ELISA kits became available for noroviruses and it was necessary to decide between the need to follow up the findings above and examine the occurrence of astrovirus type 8 in greater detail, or to extend the survey to include this new virus. Since relatively few samples had at this stage been scored as positive for any viruses it was decided that an examination of the incidence of norovirus in this panel would allow us to draw a more complete picture of virus distribution in KSA than a more detailed investigation of the astroviruses. Accordingly therefore we sought to use remaining samples to determine the likely incidence of Nov in this panel and decided to test any remaining stool samples for the presence of noroviruses.
3.1.4 Prevalence of Norovirus

Human caliciviruses are frequent causative agents of diarrhoea and vomiting. Human pathogens are found in two genera of the family; the noroviruses and sapoviruses [Chiba et al., 1979; Chiba, 1980]. At present there are no data at all on the occurrence of these agents in Saudi Arabia. These viruses were not originally included in the design of the survey because there were no convenient tests for their detection and this survey was predicated on first stage screening by ELISA followed by subsequent molecular analysis. This prevented the inclusion of norovirus and sapovirus in the study. However, during the currency of the study such a test became available for norovirus, and although there is still no simple test for the sapoviruses, we decided to include norovirus in view of the large number of samples in which no viruses had been detected.

The distribution of norovirus was investigated using an ELISA kit that detects both genogroup 1 and 2 viruses but which cannot distinguish between them. Our sample panel consisted of 253 samples for which material remained, the others having been used up in previous analyses or discarded during storage under the KFSH & RC policy on sample storage and freezer maintenance which discourages long term storage of diagnostic samples. This panel was derived from the cities of Jeddah 46; Makkah 81 and Riyadh 126. A total positivity of 9/253 (3.5%) was found and within each city positivity was 2/46 (4.3%) in Jeddah, 5/81 (6.1%) in Makkah, and 2/126 (1.6%) in Riyadh. These differences were not significant. Results are shown in (Table 15). Age group analysis showed that although infections were most common in those below the age of three, this
was not statistically significant and in contrast to the other viruses we have examined infection appeared to be relatively equally spread across the age groups (Figure 28). Again this virus showed no significant difference in sex ratio of patients with 44.4% of infections in males and 55.5% in females.
Table 15. Results of pediatric fecal samples screened for the presence of norovirus by ELISA.

<table>
<thead>
<tr>
<th>City</th>
<th>Total Samples</th>
<th>Samples Tested</th>
<th>Norovirus Positive(^d)</th>
<th>Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jeddah</td>
<td>205</td>
<td>46</td>
<td>2</td>
<td>4.3</td>
</tr>
<tr>
<td>Makkah</td>
<td>405</td>
<td>81</td>
<td>5</td>
<td>6.1</td>
</tr>
<tr>
<td>Riyadh</td>
<td>390</td>
<td>126</td>
<td>2</td>
<td>1.6</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
<td>235</td>
<td>9</td>
<td>3.5</td>
</tr>
</tbody>
</table>

\(^d\) Noroviruses were screened by an ELISA kit from Oxoid [IDEIA™, Cat# k6044]
**Figure 29.** Age distribution of ELISA-positive samples among children from three cities in Saudi Arabia (Jeddah, Makkah and Riyadh). The figure shows that the infection is relatively more common in children below 1 year of age but infections are also frequently observed in older children. Number of children infected with norovirus who are 1 year of age or less (4/9, 44.4%) is near to those who are over 1 year of age (5/9, 55.5%). Although, no statistically significant difference was notable between the numbers of infected children with all age group.
In conclusion, it seems that the incidence of noroviruses (3.5%) in this restricted panel was greater than both that of astroviruses and adenoviruses. If this panel is reflective of the sample panel overall then this would suggest that noroviruses are the second most common cause of gastroenteritis in KSA after the rotaviruses. Furthermore, there were no significant differences between the numbers of infections detected in all age groups (1 years and less to 6 years old).
3.2 Comparison between the Three Centres

The centres chosen represent the diversity of geographic region, topography, climate, year-round global visitors and different ethnic population in Saudi Arabia. We gave details earlier in the material and methods about these differences and we will talk about them further in the discussion section.

As can be seen from Figure 30, the results show differences in the prevalence of the ELISA-positive samples collected from Jeddah, Makkah and Riyadh. Riyadh, showed a total of 44 (11.2 %) virus-positive samples out of the 390 samples collected from this city and values from the other centers were; 41/405 (10.1 %) from Makkah, and 16/205 (7.8 %) from Jeddah.

Looking at the viruses individually the prevalence of rotavirus (in symptomatic diarrhoeas serious enough to require medical attention) were; 6.9% (27/390 samples) in Riyadh, 6.8% (14/205 samples) in Jeddah, and 4.6% (19/405 samples) in Makkah. The prevalence rates for adenovirus in symptomatic diarrhoeal cases was highest in Makkah, 9 out of 405 collected samples (2.2%) followed by Riyadh, 5 out of 390 (1.2 %), and adenovirus were not detected in this study from Jeddah. Astrovirus positive cases were relatively higher in Riyadh, 10 out of 390 (2.5%) than Jeddah, 1 out of 205 (0.5 %) and Makkah, 8 out of 405 (1.9 %). Finally infection with noroviruses detected in symptomatic cases record the highest peak in Makkah with positivity rate of 5/81 (6.2%), followed by Jeddah (2/46, 4.3%), and Riyadh (2/120, 1.5%). We found that there was no significant difference between the incidences of viruses per city or of individual virus types between
these centres. Although, we had hypothesized that these locations might differ in virus distribution due to the differences in traffic, location or climate, this hypothesis was not borne out by this study. A larger panel would be needed to analyse any such effects in greater detail.

However, we did observe differences concerning the distribution of individual rotavirus genotype combinations. Although G1 was found to be overall the most common G type of rotavirus in the survey, this was not true in all locations. G1 was indeed the most common identification in Riyadh (22/27, 81.4%) a finding that is comparable with a previous survey [Mohammed et al., 1994], but this was not true in Jeddah where G1 was markedly reduced (4/12, 33.3%) and G9 was the most common type detected (8/12, 66.6%). Makkah showed an intermediate pattern with incidence of G1 (10/19, 52.6%) lower than that in Riyadh but higher than that in Jeddah, and incidence of G9 (7/19, 36.8%) lower than in Jeddah but higher than that observed in Riyadh (4/27, 15%). Most G9s were identified in Makkah and Jeddah (Table. 10, above), these differences were statistically significant with P-Value = 0.005. In contrast type G1 viruses were more commonly detected in Riyadh than Jeddah and Makkah, and again although the numbers of positive samples were small, these results were statistically significant with a P-Value of = 0.009.

In contrast P type distributions appeared more evenly spread with P[8] detected at similar frequencies in the three centres regardless of the G types with which it was paired. G1P[8] was more common in Riyadh (20 out of the 26 samples typed for both genotype G and P, which is 76.9%) [P-Value of = 0.007], whilst G9P[8] was more common in Makkah (3/19, 15.7%) and Jeddah (5/11, 45.4%) [P-Value of = 0.006]. This indicates that
although infection by a rotavirus may be just as likely in each location, there may be a real difference between the types of rotaviruses circulating in these locations, and thus in the types most likely to cause infection detectable in this survey. Table 16 shows the distribution of G[P] strains from the three cities.

None of the other virus types sought showed significant differences between locations.

In conclusion, with the exception of the detection of G9 viruses there were no significant differences between the infections in the three cites. However emergent type of rotavirus G9 was detected significantly more frequently in Makkah and Jeddah. This was an interesting observation because these two locations are closely related to the traffic of international pilgrims coming to the country and who might have introduced these novel strains from overseas. Since the bulk of the travelers arrive at relatively restricted times of year (during the annual Hajj) it may be possible to test this suggestion by examining the time of year at which infections are detected. Therefore, we have to track the season of infection plus the nationality of infected patient with these viruses in the Kingdom.
Figure 30. Distribution of virus-positive stool specimens from pediatric patients with diarrhoea in the three cities tested: Jeddah, Makkah and Riyadh. Viruses identified are indicated: rotavirus (Group-A), enteric adenovirus, astrovirus and norovirus. The peak of astrovirus infections was noted in Riyadh while the peak of adenovirus and norovirus was in Makkah. Rotavirus has almost the same prevalence in Riyadh and Jeddah. These differences were not statistically significant. Total samples collected from Jeddah were; 205 samples, and 405 samples from Makkah, and 390 samples from Riyadh. Norovirus were screened out of 46 samples from Jeddah, 81 samples from Makkah and 120 samples from Riyadh.
Table 16. The distribution of 56 samples positive for both rotavirus genotypes (G&P) in the Jeddah Makkah and Riyadh.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of Samples (%)</th>
<th>Jeddah</th>
<th>Makkah</th>
<th>Riyadh</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>G1P[8]</td>
<td></td>
<td>4</td>
<td>7.1</td>
<td>10</td>
<td>17.8</td>
</tr>
<tr>
<td>G1P[6]*</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>G2P[4]</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>G3P[8]</td>
<td></td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>3.5</td>
</tr>
<tr>
<td>G9P[8]</td>
<td></td>
<td>5</td>
<td>8.9</td>
<td>3</td>
<td>5.3</td>
</tr>
<tr>
<td>G9P[4]*</td>
<td></td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td>G9P[6]*</td>
<td></td>
<td>2</td>
<td>3.5</td>
<td>3</td>
<td>5.3</td>
</tr>
</tbody>
</table>

*Unusual combination
3.3 The Incidence of Infection between Hospitalization patients and there Nationality (Saudi and non-Saudi)

If infections are being introduced to the country by pilgrims during the Hajj then we might see infections preferentially in the non Saudis at that time, followed by emergence of infection in the resident population. The population in Saudi Arabia is about 83% Saudis and 17% non-Saudis. The composition of the panel of samples used in this study reflected this population mix. Therefore, we kept in mind that if infections are distributed equally between the two populations we would expect the split of samples from Saudi and non-Saudi patients to reflect this population difference to yield a ratio of 4:1 (Saudis versus non-Saudis).

The incidence of infection between Saudi and non Saudi infected with rotavirus, adenovirus, astrovirus, and norovirus are summarized in Table 17. Overall rotavirus was higher in the non-Saudi group than in the Saudis and G9 detections were mainly identified in the non-Saudis However, none of the differences in the incidence of infection in the two groups were statistically significant. Table 18 summarize the incidence of G9 strains in Saudi and non-Saudi patients.
Table 17. Prevalence of infections among Saudi and non–Saudi

<table>
<thead>
<tr>
<th>Nationality</th>
<th>Rotavirus Positive %</th>
<th>Adenovirus Positive %</th>
<th>Astrovirus Positive %</th>
<th>Norovirus Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saudi</td>
<td>45</td>
<td>5.4</td>
<td>12</td>
<td>19</td>
</tr>
<tr>
<td>Non-Saudi</td>
<td>15</td>
<td>9.1</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

*836 stool samples were collected in this study from Saudi patients, and 164 samples were taken from non-Saudi patients. Both were tested for the presence of rotavirus, adenovirus, and astrovirus antigens. For norovirus only 211 stool samples were taken from Saudi patients, and 42 samples from non-Saudi patients. Both were tested for norovirus antigens.
Table 18. The differences in the distribution of G9 strains detected in this study with Saudi and non-Saudi patients, (%).

<table>
<thead>
<tr>
<th>G9 strains</th>
<th>Number of Samples / (%)</th>
<th>Jeddah</th>
<th>Makkah</th>
<th>Riyadh</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arabic</td>
<td>Saudi</td>
<td>Non-</td>
<td>Saudi</td>
<td>Non-</td>
</tr>
<tr>
<td></td>
<td>Strains</td>
<td>(T)%</td>
<td>Saudi</td>
<td>(T)%</td>
<td>Saudi</td>
</tr>
<tr>
<td>G9P[8]</td>
<td></td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>G9P[4]</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G9P[6]</td>
<td></td>
<td>1</td>
<td>50</td>
<td>1</td>
<td>50</td>
</tr>
</tbody>
</table>

* Occurrence of each strain by nationality of patient as percentage of the total detections of that strain in each city.

* T: standing for the total number of the positive samples.
We analyzed the data of the positive samples to see if there any relation between the viruses identified and the likelihood of hospitalization for the condition. We found a significant association only for the noroviruses (P-Value of = 0.02). Figure 31 review the analyzed data for rotavirus hospitalized cases.
20 (33.3%) positive samples from hospitalized patients

8/20, 40% samples from non-Saudi
6/8, 75% samples from Makkah
2/8, 25% samples from Riyadh

12/20, 60% samples from Saudi
7/12, 58.3% samples from Makkah
5/12, 41.6% samples from Riyadh

**Figure 31.** This chart depicts the distribution of rotavirus hospitalized cases, by nationality (Saudi or not Saudi) and location. Most of the hospitalized patients were from Makkah regardless of nationality. No hospitalized cases were detected from Jeddah. It is not known whether this reflects difference in the severity of the viruses circulating or more likely, differences in the likelihood of hospitalization following presentation according to practice in the different locations.
3.4 Season of Infection

Virus diarrhoea outbreaks might be associated temporally with periods of pilgrimage, most notably the Hajj. This is one of the questions we planned to answer in our study.

In order to do that, we analyzed seasonality of infection with these viruses. As can be seen from Table 19 and Figure 32, main feature of this analysis was a dramatic increase in rotavirus incidence in April of the study year, the statistical analysis is significant with P-Value of $= 0.0001)$. Moreover, most of the positive samples for the unusual G9, were collected in April just after the Hajj season (11 out of 19, 57.8%), In fact April was the peak month for adenovirus and astrovirus detections as well. Furthermore, as table 19 shows, the bulk of this effect was accounted by an increase in infections in Makkah and Jeddah the two locations most closely associated with the Hajj. Surprisingly no clear seasonal peak occurs throughout the year with norovirus infection.
Table 19. Incidence (%) of positive sample during the year of collection (September 1st, 2002 to August 31st, 2003)

<table>
<thead>
<tr>
<th>Region</th>
<th>Positive Viruses</th>
<th>SEP</th>
<th>OCT</th>
<th>NOV</th>
<th>DEC</th>
<th>JAN</th>
<th>FEB</th>
<th>MAR</th>
<th>APR</th>
<th>MAY</th>
<th>JUN</th>
<th>JUL</th>
<th>AUG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jeddah</td>
<td>Rotavirus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6.6</td>
<td>0</td>
<td>13.3</td>
<td>3.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Adenovirus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Astrovirus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Norovirus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>22.2</td>
</tr>
<tr>
<td>Makkah</td>
<td>Rotavirus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.6</td>
<td>1.6</td>
<td>0</td>
<td>20</td>
<td>1.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>Adenovirus</td>
<td>0</td>
<td>0</td>
<td>14.2</td>
<td>14.2</td>
<td>0</td>
<td>0</td>
<td>35.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Astrovirus</td>
<td>0</td>
<td>0</td>
<td>5.2</td>
<td>10.5</td>
<td>0</td>
<td>0</td>
<td>26.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Norovirus</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>22.2</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Riyadh</td>
<td>Rotavirus</td>
<td>3.3</td>
<td>0</td>
<td>0</td>
<td>6.6</td>
<td>0</td>
<td>0</td>
<td>6.6</td>
<td>1.6</td>
<td>6.6</td>
<td>0</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adenovirus</td>
<td>7.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7.1</td>
<td>0</td>
<td>0</td>
<td>7.1</td>
<td>14.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Astrovirus</td>
<td>10.5</td>
<td>5.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10.5</td>
<td>0</td>
<td>0</td>
<td>26.3</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Norovirus</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
Figure 32. Monthly distribution of positive samples during the year of collection (September 1st, 2002 to August 31st, 2003) for rotavirus (Group A), adenovirus, astrovirus, and norovirus. Infections in the three viruses (adenovirus, astrovirus, and norovirus) occur throughout the year with no significant seasonal peaks. Increase in rotavirus incidence in April of the study year, was statistically significant with P-Value of = 0.0001.
To conclude, this study has revealed some unexpected results;

- morbidity caused by rotavirus in the kingdom is lower than reported previously and suggests that the trend towards lower incidence is continuing
- Rotavirus infections showed a marked peak soon after Haj
- G9 strains were detected in 2003; the earliest detection in the country.
- Norovirus was second only to rotavirus as a cause of viral gastroenteritis in children in Saudi Arabia.
- Astrovirus type 8 appears common in the kingdom
- Adenovirus type 31 was found associated with enteric symptoms for the first time in KSA.
4. Discussion

Viral gastroenteritis is the most common cause of diarrhoeal disease among infants and young children [Middleton et al., 1977; Khuri-Bulso et al., 2006] and remains a major cause of mortality in developing countries [Bern, 1992; Murray, 1997]. Among the several groups of viruses implicated in the etiology of gastroenteritis are rotavirus, enteric adenovirus (serotypes 40 and 41), astrovirus and caliciviruses, both norovirus and Saprovirus [Middleton et al., 1977; Khuri-Bulso et al., 2006].

Central to our understanding of the health problem presented by these viruses is to study the epidemiological data regarding prevalence, distribution and variation of serotypes as well as genotypes worldwide. In Saudi Arabia, such studies are of particular importance for the following reasons:

1. Saudi Arabia is a country that hosts two of the three most important Islamic Shrines that are well-visited year round. The potential of virus strain migration from various parts of the world is of concern.

2. There is no study on the genoprevalence of these viruses in important urban areas, such as Jeddah, Makkah and Riyadh.

3. The improvement of hygienic conditions and the emergence of public health awareness necessitate continuous survey for these viruses to monitor progress.

4. Saudi Arabia is a large country, lacking rivers and occupying two-thirds or more of the Arabian Peninsula, with scattered foci of urban and sub-urban areas.
Further study is needed in order to assess the extent of influence that each factor may have in the determination of virus introduction and circulation within the country and this would be best addressed by ongoing surveillance. This should be aimed especially at the detection of novel or unusual strains that may be emerging in the KSA. Such epidemiological data will help to provide valuable insights into antigenic or genetic identities and possible sources of virus strains involved not only in individual acute pediatric gastroenteritis but also possible focal outbreaks in the community. In addition, the long term goal of developing a broadly effective rotavirus vaccine requires not only epidemiological data on the distribution of serotypes and genotypes worldwide, but also information on the strain variation both within and between communities. This extremely important information was not hitherto available for Saudi Arabia.

Many episodes of acute gastrointestinal illnesses of viral etiology may be due to other agents such as enteric adenovirus, astrovirus and calicivirus (norovirus or sapovirus). Data for astrovirus, adenovirus and calicivirus prevalence in Saudi Arabia are sparse. For instance, a few studies have described the detection of astroviruses [Akhter et al., 1995b; Arif et al., 2005; Mamdoh et al., 2007] and enteric adenoviruses [Akhter et al., 1995a; Akhter et al., 1994b] in Saudi Arabia. As such, the epidemiological data on these two groups of diarrhoeal viruses in Saudi Arabia are not adequate and further studies are needed.

The three cities, from which samples were collected, represent the diversity of geographic elevation and topography, climate, year-round global visitors and different ethnic population in Saudi Arabia. The bulk of the population are also resident in
these cities (>10m combined) and the major healthcare facilities are located here on
which this study depended. Jeddah is a major coastal city and the commercial capital
with hot, humid climate. It is the gateway to Holy Islamic Shrines in the country.
Makkah is a major city and religious capital with hot, dry climate. It is of high
importance in the Muslim World as it is the place where millions of pilgrims gather
every year form all over the world for Hajj and Umrah, with obvious implications on
public health. During peak times, the population may triple or quadruple and a high
incidence of both viral and bacterial respiratory infections have been reported [Balkhy
et al., 2004; Lingappa et al., 2003].

Riyadh is the largest city and the political capital with colder, dry climate. It is
the main location for government and subject to heavy traffic of people. Jeddah and
Makkah have a population of over 5.5 million combined, while Riyadh has a
population of over 4.5 millions. Therefore, samples from this city represent subjects
coming from different regions of the world. It is a valley with a tropical weather,
surrounded by arid mountains. Riyadh is the capital city and represents a totally
different climate (desert) and habit of nutritional style. It includes the major health
institutions in the country with referral medical centers where patients are sent from
different parts of the Kingdom.

Our survey of these four groups of viruses in 1000 paediatric stool samples
collected from three major cities in Saudi Arabia showed that rotavirus infection
ranked first (6.3%), followed by norovirus [smaller panel size] (3.5 %), astroviruses
(1.9 %), and enteric adenoviruses (1.4%). These frequencies are similar to those
reported all over the world [Chen et al., 2007; Moyo et al., 2007]. However, our
findings are at variance with previously published reports of rotavirus infection in
Saudi Arabia. For example, al-Bwardy et al. [1988] reported rotavirus as a major
cause of gastroenteritis in children in Riyadh with a prevalence of 44.3%, [al-Bwardy et al., 1988]. While Al-Ahdal et al. [1991] reported 31.6% [Al-Ahdal et al., 1991] and el-shikh et al., 2001 reported rotavirus in 34.6% of hospitalized patients and 5.9% of outpatient specimens. However, these studies were performed over a decade ago and at that time there public health measures were relatively poorly advanced and the effect of these improvements has yet to be systematically investigated. However, the incidence of rotavirus infection in children has been observed to decrease year by year [Arif et al., 2005; Ghazi et al., 2005] which may be partially explained by improvements in public health introduced in recent years [Quresh et al., 1996; Alyousuf et al., 2002]. Improved supply of clean water for washing and better disposal of sewage have both been introduced into the survey areas: Sewage networks now cover more than 85% of the homes in urban areas. Water quality has also improved; Saudi Arabia is now the world's largest producer of desalinated water and supplies 70% of the country’s drinking water by this route. Desalination in KSA is achieved mainly by distillation as opposed to reverse osmosis technology and this function via high temperature vaporization (70-120°C); a process that should destroy intestinal viruses [Carter, 2005]. Our study reveals that this trend towards decreased incidence of rotavirus in particular has continued and the incidence we found amongst symptomatic children was again lower.

Saudi Arabia occupies most of the Arabian Peninsula. There are no rivers or permanent streams, environmental transmission through water is thus unlikely. Furthermore, numerous studies, addressing a range of enteric viruses, have consistently found elevated temperature to be a major limiting factor in virus survival in the environment (reviewed in Carter 2005). Temperature has also been proposed to reduce the spread of respiratory viruses such as influenza and for both respiratory and
enteric viruses spreading in temperate climates there is a marked peak in Spring/winter [Glass et al., 1996; Sung et al., 2004; Kheyami et al., 2006; Marc et al., 2006] that is not necessarily found in areas with more even and elevated year round temperatures [Cook et al. 1990] – a global study; Doan and colleagues in Vietnam [Doan et al., 2003] and previously in Saudi Arabia [Arif et al. 2005]. Temperature and humidity fluctuation is not extreme in these regions of KSA where “seasons” are generally lacking. Figure 33 shows the variation in temperature and humidity in each location. Increasing humidity and decreasing temperatures have been reported to stabilize environmental viruses and thus might aid virus transmission (Carter, 2005). However, these effects were not marked at the time of rotavirus peak incidence as observed in this study. Thus we conclude that the peak occurrence of this virus was most likely related to the arrival of travelers and associated close contact between them during Hajj rather than to climatic effects.

Our data show a sustained incidence of rotavirus throughout the year, there is no obvious peak in winter (December, January, February) and the peak in April does not coincide with either temperature minima or increases in rainfall. We attribute this effect to human rather than environmental factors, namely the performance of the Hajj.

In KSA the temperature generally does not fall below 20°C in Makkah and Jeddah, but it may drop below 10°C in Riyadh for a short period in winter. Consequently the elevated temperatures encountered in KSA may reduce environmental survival times and thus oppose virus transmission other than by short range means. We suggest that improvements in sanitation may have more profound effects in KSA than elsewhere; provision of clean water for washing and removal of contaminated sewage from the immediate environment may interrupt short range
transmission processes, whilst longer range effects are naturally opposed by poor virus survival in the environment and lack of rivers for rapid virus dispersal. If this is true then we might expect viruses that do spread successfully in Saudi Arabia to be more thermally resistant than those found elsewhere and this hypothesis is testable experimentally.
Figure 33. This chart shows the deference's in the temperature degree and the humidity between Jeddah, Makkah, and Riyadh during the year 2003 [Kingdom of Saudi Arabia Ministry of Defence & Aviation Presidency of Meteorology & Environment Protection National Meteorology & Environment Center Surface Annual Climatologically Report]. MO: Month, chart number {1} represent Jeddah, {2} represent Makkah, and {3} represent Riyadh.
Our results showed that the major cause of viral diarrhoea was rotavirus, followed by norovirus (3.5 %). This is similar to findings elsewhere which reported human calicivirus as second only to rotavirus as a cause of gastroenteritis in young children [Bon et al., 1999; Kirkwood et al., 2001]. As reported elsewhere, [Christensen, 1989; Clark and McKendrick, 2004; Clark et al., 2004; Giordano et al., 2001], enteric adenovirus and astrovirus infections were found at similar frequencies in our panel (1.4% and 1.9% respectively). The rate of astrovirus identification is relatively similar to that reported from elsewhere [Vernacchio et al., 2006; Guix et al., 2002] but adenoviruses appeared to be lower; accounting for 1.4% of cases in this survey and 5-17% of cases of gastroenteritis in infants and preschool children surveyed elsewhere [Albert, 1986; Horwitz, 1990; Fodha et al., 2006]. Although numbers of both viruses are small it is feasible that adenovirus infection is also being reduced by the same factors that are affecting rotavirus incidence.

However no virus was detected in the majority of samples in our panel even though the initial screening tests used (ELISA) were objective and sensitive. Although we were unable to test for the sapoviruses, estimates of sapovirus incidence made elsewhere do not suggest that these are a major cause of infection [Khamrin et al., 2007; Gomes et al., 2007]. Furthermore, since the frequencies of astrovirus- adenovirus- and rotaviruses reported here are if anything equal or lower to those reported elsewhere it seems likely that sapovirus infections would follow a similar pattern. Thus we suggest that the bulk of the diarrhoeas we have sampled were probably caused by non-viral agents. This is in agreement with earlier data published by [Akhter et al., 1994a; Ghazi et al., 2005].
Many studies have shown the important role of rotavirus as a cause of diarrhoea in children in both developed and developing countries. Most of the cases occur in children less than 5 years of age, sometimes less than two [Sung et al., 2004; Ghazi et al., 2005] and in our case less than 1 year of age.

Since enteric adenovirus infection has been reported mostly from children of 2 years and less [Saderi et al., 2002; Lin et al., 2000], we tested this variability in our study and we observed children infected with adenovirus who are 2 year of age or less (85.7 %), to be relatively more than those who are over 2 year of age (14.2 %). Statistically significant with P-Value of $= 0.01$. Astrovirus infections were likewise statistically more common in children under the age of 3y. P-Value of $= 0.05$. In contrast norovirus infection appeared more evenly spread across the panel. Similar spread has been reported from the Netherlands [de Wit et al., 2001], finally, norovirus this was also the only virus with which a statistically significant association with hospitalization was shown, despite the smaller number of samples used in norovirus testing.

We also presented in this study the G and P typing of HRV strains using molecular methods for the first time in Saudi Arabia. Type G1 was the most common, as is the case globally [Beards et al., 1989; Gerna et al., 1990; Wilhelmi et al., 1999] and locally in Saudi Arabia [Mohammed et al., 1994]. The most common association of G and P genotypes in this study was between G1 and P[8], similar to that reported previously worldwide [Gentsch et al., 1992; Gentsch et al., 1996]. However, this work has also identified the newly emerging strain G9 [Martella et al., 2003; Sanchez-Fauquier et al., 2004; Zhou et al., 2003] for the first time in Saudi Arabia.
and in the Gulf region, although it follows a previous report of its identification in another Arab country, Libya [Cunliffe et al., 2001]. In fact, this novel type was actually the second most frequently detected. Our explanation for the novel finding of a G9 strain in Saudi Arabia is that it may have been imported from abroad by visitors coming for work-related or religious- reasons. Most of the identifications of this virus in our panel follow closely on the Hajj in the year 2003. Some cases were detected before this date so we are unable to show that the virus was actually introduced by the Hajj that year but our data are at least consistent with such a hypothesis with 2003 possibly representing a re-introduction. Because of this global spread of this strain (G9), it seems wise to include it in the rotavirus vaccine production efforts. There was no correlation between G9- positive samples and age of patient and in our study G9 was found most frequently in association with P[8], which is in agreement with other reports [Gentsch et al., 1992]. Our study implies that there has been a change in the viruses in circulation in KSA: types G1P[8], G2P[8], G3P[8] no longer predominate as in the past [Gentsch et al., 1996; Urbina et al., 2000]. Indeed types G1-G4 were formerly the most common types of rotavirus infection in Saudi Arabia [Kheyami et al., 2006; elAssouli et al., 1996]. Although we still found type G1 to be the most common, this was now followed by type G9 with types G2 and G3 being relatively rare; type G4 was not identified at all.

G9 when it is found is usually observed association with [P8]. Occasionally other less common strains have been detected in various countries. Among these, G9P[6], G9P[4] and G1P[4], G2P[4] have been reported [Ramachandran et al., 1996; Banyai et al., 2004]. It has been suggested that G9P[6] maybe the first G9 strain found in human and this is subsequently substituted by G9P8 [Iturriza-Gómara et al., 2000].
In this study we have also made the first observation of G9 association with P[4] and with P[6] in Saudi Arabia (Figure 20).

Therefore, we conclude that there is a genetically diverse and evolving pattern of rotavirus strains circulating in humans in Saudi Arabia. Similar conclusions have been reached in several recent studies: rotavirus G2 and G4 predominated in Spain from 1998-2002, but appear to have been superseded by G9 and G3 [Sánchez et al., 2006].

We investigated and typed enteric adenovirus using a combined approach: Enteric adenovirus was first detected using an ELISA kit reactive to detect the hexon antigen of all known adenoviruses. Typing was performed using a type-specific PCR and the amplicons generated from hexon gene were subjected to RFLP analysis (1) to confirm our PCR genotyping results, (2) to genotype isolates that were not typeable by PCR, and (3) to examine the possibility of co-infection with more than one adenovirus genotype. DNA sequencing was used to analyze the samples that produced genotype only with PCR technique.

Although the results obtained showed that the published molecular methods are indeed suitable for the detection and typing of adenovirus types found in stool in most cases, the combination did yield contradictory data in one instance. Isolate number 7 reacted in type-specific PCR using primers specific for type 18; RFLP of the hexon amplicon was unsuccessful for typing this sample. Finally sequencing this isolate identified it as type 31. These data imply that type specific PCR alone is not always a reliable means of typing; in this case types 18 and 31 are both members of adenovirus group A. Alignment between the hexon genes of these two viruses reveals close similarity with 95% of the residues conserved in the forward primer and 75%
conservation in the reverse. These values are sufficient to permit amplification and thus miss-typing of these two strains. Figure 34 shows the alignment between the forward and the reverse primers of adenovirus type 18 with the sequence of Ad-31 obtained from this study. Surprisingly- despite the bulk of the sequence corresponding to Ad31 the match across the binding sites of the so-called Ad31- specific primers was poor such that this virus would amplify with the Ad18-specific primers but not with the type 31-specific pair. The Co-infection with Ad 40 and Ad 41 found in sample number 8 was unexpected finding in this population and could have been missed if non molecular methods had been used.
Figure 34. Alignment results between the sequence derived from sample 7 (adenovirus type 31) with the forward (+), and the reverse primer of Ad-18. Section (A) show 95.5% similarity between sample 7 adenovirus sequence and the sequence of Ad18 (+). In section (B) the alignment between sample 7 sequence and Ad18(-) show 100% similarity with 13 bp out of the 18 bp size long of Ad18(-) primer. The DNASTAR Megalign software was used for DNA sequence alignments. * Alignment coloring: match color = black, mismatch color = red, consensus color = green.
Out of 19 ELISA positive samples for astrovirus, 18 were confirmed to be positive for astrovirus using molecular methods (semi-nested multiplex RT-PCR). Interestingly, the 18 samples all reacted with primers specific for type 8 (HAstV-8). This is the first description of HAstV 8 in Saudi Arabia population. For confirmation of our unique results, each of the 18 HAstV-8 samples was typed twice, once with multiplex primers for types 1-7 and another with primers for type 8 only. The results remains that 18 positive samples were type 8. Again for further confirmation, experiments (ELISA, RNA extraction, RT-PCR, and typing) on each of the 19-positive samples were repeated, each on a different day, to prevent any chance of contamination and the results remained the same.

Two published works were found on astrovirus occurrence in the country. One showed 0.8% prevalence but the few positive samples identified were not typed [Arif et al., 2005]. The other older study showed serotype 1 infecting 1.5% of the population studied. Our finding which implies that genotype 8 was predominant at the survey time is unexpected and demands further investigation since there may have been a shift in astrovirus serotype/genotype prevalence in the 8-years between our study and the last. Past studies do hint at the possibility for such change: Work from London has determined seroprevalences of astrovirus type 1 and 4 and 6 in age group cohorts. In this study children up to the age of 5 showed a steadily increasing seroconversion towards type 1. However children of 6 years and older display practically half the seroprevalence implying that infection by type 1 astrovirus was significantly less common when these children were infants [Kriston et al., 1996]. Other studies have provided a snapshot as to the most common types at different times:. For example, type 8 was found commonly in Mexico [Mendez-Toss et al., 2004], type 3 in London had apparently replaced type 1 as the most common in
London [Gallimore et al., 2005], and type 2 was common in Brazil [Gabbay et al., 2006]. The development of astrovirus vaccine that protects from more than one serotype will perhaps eventually be needed [Glass et al., 1996; Atreya, 2004].
5. Conclusion

1. Incidence of all viruses was lower than expected and since most samples lacked an identifiable virus it seems likely that most of diarrhoeal cases in this panel were of non-viral origin.

2. From this survey, HRV was the virus most commonly identified (6%) in this study followed by; norovirus (3.5%), astroviruses (1.9%), and enteric adenoviruses (1.4%).

3. Rotavirus infection occurred predominantly in children below 1year; adenoviruses below 2years and astroviruses below 3years. Noroviruses appeared more evenly spread.

4. Incidence of rotaviruses detected (and possibly adenoviruses as well) appears to be lower than reported previously and maybe continuing the downward trend in the incidence of this virus identified by others, if true, we attribute this to recent developments in public health.

5. Rotavirus G9 was detected in this panel and was thus present in the country in 2003. This is the earliest detection of this virus type in KSA.

6. Characterization and identification of HRV G and P types indicates a genotype shift may have occurred in the country. In 2001 types G1-G4 predominated [el-Sheikh et al., 2001]. From this study G1 and G9 were the most common genotypes followed by the less common G2 and G3. G4 was not detected at all in this study.

7. Virus identifications in diarrhoea followed closely periods of pilgrimage, most notably the Hajj. G9 was detected more often after al Hajj season and in the
areas frequented most by pilgrims. This supports the potential for introduction of viruses to the country by pilgrims.

8. Our finding that astrovirus type 8 is the predominant type is unique, as genotype 1 is the predominant type world-wide. It is therefore possible that there has been a shift in astrovirus serotype/genotype prevalence in the 8 years since the last study in KSA.
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