Chapter 4

Chapter 4: Group B rotavirus infections in India


4.1 Material and methods

4.1.1 Patients and specimens

4.1.1.1 Sporadic cases

The study included patients with acute gastroenteritis, admitted at or visiting outpatient department of hospitals from Pune (Maharashtra state), Alappuzha (Kerala state) and Belgaum (Karnataka state) cities of western/southern India. A total of 2101 fecal specimens comprising 1794 from Pune, 110 from Alappuzha and 197 from Belgaum were collected from children (0-10 years, n=1019), adolescents (11-18 years, n=135) and adults (>18 years, n=947) at the two time points, 1994-1995 (n=924) and 2004-2010 (n=1177). The specimens collected during 1994-1995 were available only from Pune. A sporadic case of acute gastroenteritis in the present study was defined as the passage of ≥3 loose or watery stools a day, with or without associated symptoms such as vomiting, fever and abdominal pain occurring singly in a scattered manner. One specimen per patient was collected for the study within 24 hr of hospitalization or immediately after the visit of the patient to the outpatient department in a sterile screw capped container of 10 ml capacity with prior informed consent from the parents/guardians (in the case of children and adolescents) or adult patients. All specimens were stored at +4°C in the respective hospital/clinic and transported on ice to the laboratory.

4.1.1.2 Outbreak cases

Two outbreaks of acute gastroenteritis occurred in southern Mumbai, India in the months of March and October, 2006. Fecal specimens were collected from 72 (30 children ≤10 years, 5 adolescents aged 11-17 years and 37 adults ≥18 years) and 146 (61 children ≤10 years, 6 adolescents aged 11-17 years and 79 adults ≥18 years) patients hospitalized at Gokuldas Tejpal Hospital / Sir Jamshedjee Jeejeebhoy Hospital, respectively, during the outbreak periods.

In February through April, 2010 and July through August, 2011, two outbreaks of acute gastroenteritis occurred in Sholapur, Maharashtra. Fecal specimens were collected from 283 (93 children ≤10 years, 45 adolescents aged 11-17 years and 145 adults ≥18 years) and 57 (42 children ≤10 years, 6...
adolescents aged 11-17 years and 9 adults ≥18 years) patients hospitalized at Dr. Vaishampayan Memorial Government Medical College Hospital.

Fecal specimens collected during the outbreaks of gastroenteritis occurred in Daman \( n=5 \) in 2000, Surat \( n=3 \) in 2004, Sangli \( n=29 \) in 2009 and Bhor \( n=2 \) in 2011 and referred to NIV, Pune for detection of rotavirus were also included in the study. All of these specimens belonged to adolescents and adults aged 16-68 years.

All specimens were stored at +4°C until transported to the laboratory.

**4.1.2 Clinical information and calculation of Vesikari score**

Clinical information about each patient is collected and recorded in the case report form. All patients were examined for fever, number of episodes and duration of vomiting and diarrhea, extent of dehydration and treatment for the assessment of severity of disease by 20-point scale of the Vesikari scoring system (Ruuska and Vesikari, 1990). According to the scores obtained, the disease condition of each patient was categorized as mild (scores 0-5), moderate (scores 6-10), severe (scores 11-15), and very severe (scores 16-20). The information was available mainly for the patients from the 2000s.

**4.1.3 Preparation of fecal suspensions**

Thirty percent suspensions of fecal specimens [liquid feces \( v/v \), semisolid feces \( w/v \)] were prepared by suspending approximately 300 µl of feces in 700 µl of 0.01M phosphate buffered saline containing 0.01M calcium chloride (PBS-CaCl₂), pH 7.4 (Appendix 1), mixed by using vortex (Remi Equipments, India), and then centrifuged at 10,000 rpm (8944 g) for 10 minutes at +4°C. The supernatant was stored in aliquots in sterile vials at -20°C for long storage or at +4°C for immediate use.

**4.1.4 Detection of RVA by ELISA**

Presence of RVA in fecal specimens was determined by antigen capture ELISA indigenously developed at National Institute of Virology (NIV), Pune (Kelkar et al., 2004).
**Chapter 4: Group B rotavirus infections in India**

4.1.4.1 **Principle**

The antigen capture ELISA test utilizes rabbit anti-SA-11 (Simian rotavirus strain) antiserum as coating antibody on the wells that allow binding of RVA if present in the test specimen. The binding of RVA is probed by the horse radish peroxidase (HRP) conjugated rabbit / guinea pig anti SA-11 polyclonal antibody added to the wells. Appearance of color after addition of substrate indicates the positivity of RVA in the specimen. If the specimen is negative for RVA, the HRP conjugate added to the wells remains unbound and is washed away. Hence no color develops on addition of substrate.

4.1.4.2 **Procedure**

i. Ninety-six well plates (Maxisorp, Nunc, Denmark) were coated with 100 µl hyper immune rabbit anti SA-11 serum diluted (1:5000) in 0.01M PBS pH 7.4 (Appendix 2.1). Plates were incubated overnight at +4°C. The unbound antibody was removed by washing the plates 5 times with 0.01M PBS pH 7.4 containing 0.05% Tween-20 (PBS-T) (Appendix 2.1).

ii. Post coating was carried out using 200 µl of 1.2% bovine serum albumin (BSA) (wt/vol) in PBS-T (Appendix 2.2). Plates were incubated at room temperature (RT) for 1 hr. The plates were again washed with 0.01M PBS-T, pH 7.4.

iii. 100 µl of 10% fecal suspensions (diluted 3:1 with 0.01M EDTA) (Appendix 2.3) were added to the identified wells in duplicate. SA-11 cultivated in MA-104 cell culture and uninfected MA-104 cells / PBS were included as positive and negative controls respectively and added to the wells in duplicate. The plates were incubated at 37°C for 2 hrs. Plates were washed 5 times with PBS-T.

iv. 100 µl of rabbit anti SA-11 IgG HRP conjugate was added to each well after diluting it at 1: 1000 in PBS-T containing 2.5% skimmed milk powder (SMP) (Appendix 2.4).

v. Plates were incubated at 37°C for 1 hr and then washed 5 times with PBS-T.

vi. 100 µl of substrate TMB (3, 3’, 5, 5’ tetramethyl benzidine dihydrochloride hydrate; Bangalore Genei, India) diluted at 1:20 with distilled water was
added to each well. After incubation for 5-7 minutes at RT in dark, the reaction was stopped using 1M phosphoric acid (Appendix 2.5).

vii. The reaction was evaluated by measuring the optical density (OD) at 450 nm with an ELISA reader (BioTek Instruments, Inc., USA).

viii. The Cut Off (CO) value for the test was calculated as mean of the negative control replica + 2SD (standard deviation). The samples with OD$_{450}$ > the CO value were considered positive while the samples with OD$_{450}$ ≤ the CO value were considered negative.

4.1.5 RNA extraction

4.1.5.1 Principle

RNA was extracted from fecal specimens using TRIZOL®-LS reagent (Invitrogen, Carlsbad, CA, USA) as per manufacturer's instructions. TRIZOL is a mono-phasic solution of phenol and guanidine isothiocyanate that maintains the integrity of RNA; disrupts cells and dissolves cell components during sample homogenization or lysis. Addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase, which is recovered by precipitation with isopropyl alcohol.

4.1.5.2 Procedure

i. 750 µl of TRizol® LS was added to the 250 µl of 30% fecal suspension in a tube of 1.5 ml capacity, mixed on a vortex and incubated at RT for 15-20 minutes. A negative control, consisting of same volume of nuclease-free water (Bangalore Genei, India) was included in each set of RNA extractions.

ii. 200 µl of chloroform was added to the mixture and the tube was inverted for 15 seconds. The mixture was allowed to stand for 15 minutes at RT.

iii. Tube was centrifuged at 13,000 rpm (15115 g) for 10 minutes at +4°C.

iv. The upper aqueous phase was transferred to a fresh tube (1.5ml) containing 500 µl of isopropyl alcohol.

v. The tube contents were mixed by inverting the tube 10-20 times, covered with aluminum foil and kept at RT for 30 minutes.

vi. The tube was centrifuged at 13,000 rpm (15115 g) for 30 minutes at +4°C.
vii. The supernatant was discarded and the RNA pellet was washed with 500 μl of 70% ethanol (Appendix 3) by centrifugation at 10,000 rpm (8944 g) for 20 minutes.

viii. The supernatant was poured off and the tube was re-centrifuged at 10,000 rpm (8944 g) for 2 minutes at +4°C to remove the remaining ethanol.

ix. Ethanol was removed and the pellet was air dried at RT, reconstituted in 10 μl of nucleases free deionized water (Bangalore Genei, Bangalore, India) containing 0.5 μl RNAsin, an RNAse inhibitor (40U/μl) (Promega, Madison, WI) and stored at -20°C till further use.

4.1.6 Semi nested reverse transcription polymerase chain reaction (RT-PCR)

The polymerase chain reaction (PCR) preceded by reverse-transcription was employed to detect RVB RNA using the protocol described by Gouvea et al., (1991) and NSP2 gene-specific primers designed for the study (Table 4.1).

4.1.6.1 One step RT-PCR

The cDNA synthesis and the first-round PCR amplification were performed using QiAGEN® one step RT-PCR kit (Qiagen, Hilden, Germany) (Table 4.2).

4.1.6.1.1 Principle

Reverse transcription is the process in which the single stranded RNA is reverse-transcribed into single-stranded DNA molecule (cDNA) complementary to the RNA template with the help of enzyme, reverse transcriptase. This is termed first strand synthesis. In the PCR step a thermostable DNA polymerase synthesizes single-stranded DNA molecules complementary to the cDNA molecules, thus generating double-stranded DNA template. This is termed second strand synthesis.

The QiAGEN® One Step RT-PCR Kit provides a convenient format that allows both cDNA synthesis and PCR amplification to take place in a single tube. The RT-PCR Enzyme Mix is an enzyme blend that contains Omniscript and Sensiscript Reverse Transcriptases with HotStarTaq DNA polymerase. The reverse transcriptase exhibits a high affinity for RNA and facilitates transcription through secondary structures. After reverse transcription, reactions are heated to 95°C for 15 min to activate HotStarTaq DNA polymerase and simultaneously
inactivate the reverse transcriptase. HotStarTaq DNA polymerase is a chemically
modified form of a recombinant 94-kDa DNA polymerase originally isolated from
*Thermus aquaticus* expressed in *E. coli*. The hot start procedure using
HotStarTaq DNA polymerase eliminates extension from non-specifically annealed
primers and primer dimers in the first cycle ensuring highly specific and
reproducible PCR.

### 4.1.6.1.2 Procedure

i. 4 µl of RNA was denatured at 95°C for 5 min and rapidly chilled on ice for
2 min. The nuclease free water served as negative control.

ii. 46 µl of master mix A (Table 4.3) containing the primers NSP2-AF and
NSP2-CR was added to this tube and cDNA was synthesized at 50°C for
30 minutes.

iii. Prior to amplification, HotStarTaq DNA Polymerase was activated by
heating at 95°C for 15 min. On the other hand, this heating step
inactivated the Omniscript and Sensiscript Reverse Transchptases and
the cDNA template was denatured.

iv. 50 µl of the final reaction mix was subjected to 35 thermal cycles for
amplification (Table 4.4) followed by a final chain extension step at 72°C
for 10 minutes.

v. After amplification, the first PCR product (321 bp) was further utilized as a
template for semi nested PCR.

### Table 4.1: List of primers designed for this study for amplification of RVB
NSP2 gene

<table>
<thead>
<tr>
<th>Forward primer (Primer ID / Position)</th>
<th>Reverse primer (Primer ID / Position)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCCATCAGACAGAGAATGTGGTGC A (NSP2-AF / 112-136)</td>
<td>TTGTCTGCGAAGCTAAAACATCC (NSP2-CR / 432-409)</td>
<td>321</td>
</tr>
<tr>
<td></td>
<td>CCAATCAGTCACAAGAGTCCATAG T (NSP2-8R / 340-316)</td>
<td>229</td>
</tr>
</tbody>
</table>
Table 4.2: List of reagents used in RT-PCR

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Reagent</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>QIAGEN OneStep RT-PCR Enzyme mix</td>
<td>Omniscript &amp; Sensiscript reverse transcriptases, HotStarTaq DNA polymerase</td>
</tr>
<tr>
<td>2</td>
<td>5X QIAGEN OneStep RT-PCR Buffer</td>
<td>Tris Cl, KCl, (NH4)2SO4, 12.5 mM MgCl2, DTT, pH 8.7</td>
</tr>
<tr>
<td>3</td>
<td>Deoxy Nucleotides (dNTP) mix</td>
<td>dATP+dTTP+dGTP+dCTP (contain 10 mM of each dNTP)</td>
</tr>
<tr>
<td>4</td>
<td>RNase free water</td>
<td>Ultrapure quality, PCR grade</td>
</tr>
</tbody>
</table>

Table 4.3: Master mix A for RT-PCR of NSP2 gene

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X QIAGEN OneStep RT-PCR Buffer</td>
<td>10 μl</td>
<td>1X</td>
</tr>
<tr>
<td>dNTP mix (containing 10 mM of each dNTP)</td>
<td>2 μl</td>
<td>400 μM of each dNTP</td>
</tr>
<tr>
<td>Primer NSP2-AF</td>
<td>2 μl</td>
<td>0.4 μM</td>
</tr>
<tr>
<td>Primer NSP2-CR</td>
<td>2 μl</td>
<td>0.4 μM</td>
</tr>
<tr>
<td>Enzyme mix</td>
<td>2 μl</td>
<td>-</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>28 μl</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>48 μl</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.4: Thermal cycles for RT-PCR of NSP2 gene

<table>
<thead>
<tr>
<th>No. of Cycles</th>
<th>Temperature (°C)</th>
<th>Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>15</td>
</tr>
<tr>
<td>35</td>
<td>94</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>72</td>
<td>10</td>
</tr>
<tr>
<td>Hold at +4°C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.1.6.2 Semi nested PCR

For the second round of PCR, Taq DNA polymerase (Roche, Germany) was used with primers NSP2-AF and NSP2-BR to amplify a region of 229 bp (Table 4.5).

4.1.6.2.1 Principle

The sensitivity and specificity of PCR can be increased by using nested / semi nested PCR. In both of the PCR, two separate amplifications are used. The
first uses a set of primers that yields a large product, which is then used as a template for the second amplification. The second set of primers anneal to sequences within the initial product producing a product shorter than the first one. The second round primers include one primer located within the amplified region and is used together with one of the first round primers. Semi nested PCR is similar to a nested PCR except that in the second PCR, one of the primers is a primer that was used in the first PCR. This technique uses three specific primers, rather than two, so has greater specificity than regular PCR. The second set of primers also serves to verify the specificity of the first product.

The specificity is particularly enhanced because this technique almost always eliminates any false/spurious non-specific amplification products. This is because, after the first round of PCR the non-specific products are unlikely to be sufficiently complementary to the semi nested primers to be able to serve as template for further amplification. Thus the desired target sequence is preferentially amplified. However, the increased risk of contamination is a drawback of this extreme sensitivity.

4.1.6.2.2 Procedure

i. 48 μl of master mix B (Table 4.6) containing the primers NSP2-AF and NSP2-BR (Table 4.1) was distributed to each tube.

ii. 2 μl of first round product was added to it and subjected to amplification cycles in GeneAmp® PCR System 9700 (Applied Biosystems, USA) as described in Table 4.7.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Reagent</th>
<th>Details</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Taq DNA polymerase</td>
<td><em>Thermus aquaticus</em> BM, (Roche, Germany)</td>
<td>5U/μl</td>
</tr>
<tr>
<td>2</td>
<td>10X PCR buffer</td>
<td>Tris Cl, KCl, (NH₄)₂SO₄, 12.5 mM MgCl₂, DTT, pH 8.7 (Roche, Germany)</td>
<td>50U/μl</td>
</tr>
<tr>
<td>3</td>
<td>Deoxy Nucleotides (dNTP)</td>
<td>dATP+dTTP+dGTP+dCTP (Promega, Madison, WI)</td>
<td>10mM</td>
</tr>
<tr>
<td>4</td>
<td>Nuclease free water</td>
<td>Ultrapure quality (Bangalore Genei, India)</td>
<td>PCR grade</td>
</tr>
</tbody>
</table>
Table 4.6: Master mix B for 2\textsuperscript{nd} PCR of NSP2 gene

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume/reaction</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR buffer</td>
<td>5 µl</td>
<td>1X</td>
</tr>
<tr>
<td>dNTP (containing 10 mM of each dNTP)</td>
<td>2 µl</td>
<td>400 µM of each dNTP</td>
</tr>
<tr>
<td>Primer NSP2-AF</td>
<td>2 µl</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>Primer NSP2-BR</td>
<td>2 µl</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.5 µl</td>
<td>2.5 U</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>36.5 µl</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>48 µl</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.7: Thermal cycles for 2\textsuperscript{nd} PCR

<table>
<thead>
<tr>
<th>No. of Cycles</th>
<th>Temperature (°C)</th>
<th>Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>35</td>
<td>94</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>72</td>
<td>10</td>
</tr>
</tbody>
</table>

Hold at +4°C

4.1.7 Agarose gel electrophoresis

Final PCR products were resolved and analyzed by electrophoresis using 2\% agarose gel (Invitrogen, USA) prepared in 1x TAE, pH 8.3 (Appendix 4.1) running buffer containing 0.5 µg / ml ethidium bromide (EtBr).

4.1.7.1 Principle

Electrophoresis is the separation of DNA fragments into different sizes employing a sieve-like matrix (agarose or polyacrylamide) and an electrical field. Blue ‘tracking dye’ in a loading buffer is mixed with DNA to make DNA loading easier and DNA migration visible. DNA possesses a net negative charge and under the pull of the electric current migrates towards the positively charged anode. The agarose gel acts as a sieve, allowing the smaller-sized fragments to migrate faster than the larger fragments, thus separating fragments by size. After electrophoresis, the DNA is viewed by staining with EtBr that intercalates between the bases of DNA.
4.1.7.2 Procedure
i. Agarose gel (2%) (Appendix 4.3) was casted on a horizontal gel electrophoresis unit (Biorad, USA) and 1x TAE (pH 8.3) running buffer was used as electrophoresis buffer.

ii. PCR products (10μl) were mixed with 2 μl tracking dye bromophenol blue (BPB) (Appendix 4.4) and loaded in identified wells.

iii. GeneRuler 100 bp Plus DNA ladder (1 μg/μl) (Fermentas, Lithuania) was used as DNA size marker for estimation of size of the PCR products.

iv. A current of 70 mA was applied till the dye front moved three-fourth distance of the gel.

v. The bands were visualized under UV transilluminator (Techno-Source, India) and the gel image was captured in gel documentation system (Syngene, USA).

4.1.8 Gel extraction and purification of PCR products
All PCR products were excised from the gel and purified using QIAquick gel extraction kit (Qiagen, Hilden, Germany).

4.1.8.1 Principle
The QIAquick system uses spin column technology with the selective binding properties of silica membrane. A solubilization buffer containing chaotropic salt dissolves the agarose gel slice containing the PCR product. In the presence of this chaotrope, the DNA fragment binds selectively to the silica membranes of spin columns. Consecutive purification procedure removes contaminants like unused primers, nucleotides, enzymes, mineral oil, salts, agarose, EtBr and other impurities from DNA samples. The adsorbed DNA is subsequently eluted in 20 μl of elution buffer.

4.1.8.2 Procedure
i. Three volumes (300μl) of QG (solubilization buffer) (pH ≤ 7.5) were added to 1 volume (100 mg of gel piece) of gel containing excised DNA fragment.

ii. The above mix was heated at 50°C in water bath for 10-15 minutes (or until the gel slice was completely dissolved).
iii. Isopropanol (100 μl) was added in equal proportion to gel volume (100 mg) and mixed.
iv. The mixture was added to QIAquick column to allow the DNA to bind to the silica membrane.
v. The column was centrifuged for 1 min at 10,000 rpm (8944 g) and the flow through was discarded.
vi. 500 μl of QG buffer was added and column was centrifuged for 1 min at 10,000 rpm (8944 g) and the flow through was discarded.
vii. 750 μl of PE (wash) buffer containing ethanol was added to the column. Incubation was carried out at RT for 5 minutes.
viii. The column was centrifuged for 1 min at 10,000 rpm (8944 g) and the flow through was discarded.
ix. The column was re-centrifuged for 1 min at 13000 rpm (15115 g) to remove traces of PE buffer.
x. The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube. DNA was eluted by adding 10 μl elution buffer (EB) (10mM Tris-HCl, pH 8.5).
xii. The columns were centrifuged for 1 min at 13000 rpm (15115 g).

4.1.9 Nucleotide sequencing

Both the strands of PCR products were sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster city, CA) employing an automated sequencer (ABI PRISM® 3100 Genetic Analyzer; Applied Biosystems). The kit consisted of Terminator ready reaction mix containing A-Dye Terminator labeled with dichloro [R6G], C-Dye Terminator labeled with dichloro [ROX], G-Dye Terminator labeled with dichloro [R110], T-Dye Terminator labeled with dichloro [TAMRA], deoxynucleotide triphosphates (dATP, dCTP, dTTP, dGTP), AmpliTaq DNA Polymerase, MgCl₂, Tris-HCl buffer, pH 9.0.

4.1.9.1 Principle

The nucleotide sequence is determined by the dideoxy nucleotide chain terminator method developed by Fred Sanger (Sanger et al., 1977). This method uses chemically altered radiolabeled "dideoxy" bases to terminate newly
synthesized fragments at specific bases. The 5’ carbon of an incoming dNTP is joined to the 3’ carbon at the end of the chain. Hydroxyl groups in each position form ester linkages with a central phosphate leading to chain elongation. Like a dNTP, a dideoxy dNTP is incorporated into the chain by forming a phosphodiester linkage at the 5’ end. However, the dideoxy dNTP lacks a 3’ hydroxyl group necessary for chain elongation. Hence, the addition of dideoxy dNTPs halts the chain elongation and incomplete products of various lengths that differ by a single nucleotide are separated on the basis of their sizes and DNA sequence can be read.

4.1.9.2 Preparation of cycle sequencing mix

The purified PCR product was subjected to cycle sequencing. Sequencing reaction for each primer used was prepared in separate 0.5 ml sterile thin walled microcentrifuge tubes (Tarsons, India) as follows,

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer (10 µM)</td>
<td>4.0 µl</td>
</tr>
<tr>
<td>Purified PCR product</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>5X Sequencing buffer</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>Nucleases free deionized water</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>Total</td>
<td>10.0 µl</td>
</tr>
</tbody>
</table>

The cycle sequencing reaction was carried out on Eppendorf Mastercycler gradient (Eppendorf, Germany). The program included initial denaturation at 94°C for 5 minutes followed by 25 thermal cycles of 96°C for 10 sec, 50°C for 5 sec, 60°C for 4 min and final hold at +4°C.

4.1.9.3 Post cycle sequencing purification of PCR products

Ethanol precipitation method that uses ethanol/EDTA-sodium acetate was employed for cleaning up reactions using Big Dye Terminator v3.1.

4.1.9.3.1 Principle

Ethanol precipitation is a widely used technique to purify or concentrate nucleic acids. In the presence of salt (in particular, monovalent cations such as sodium ions, Na⁺, ethanol efficiently precipitates nucleic acids and EDTA helps to stabilize the extension products during precipitation. The purified precipitate can be collected by centrifugation, and then suspended in a volume of choice. This
method removes the unincorporated dye terminators which if in excess in the sequencing reactions obscure data in the early part of the sequence and can interfere with basecalling. Ethanol/EDTA-sodium acetate precipitation is recommended to obtain good signal from base 1.

4.1.9.3.2 Procedure

**Master mix 1:** 10μl Nuclease free water +2μl 125mM EDTA (Appendix 5.1)

**Master mix 2:** 2μl 3M Sodium Acetate, pH 5.3 (Appendix 5.2) + 50μl absolute Ethanol

i. Cycle sequenced DNA (10μl) was mixed with 12 μl of Mix 1. This was followed by addition and proper mixing of 52 μl of Mix 2.

ii. The mixture was incubated at RT for 30 min.

iii. The mix was then centrifuged at 13,000 rpm (15115 g) for 30 min at RT and the supernatant was decanted.

iv. The DNA was washed with 200 μl of 70% ethanol by centrifuging at 13,000 rpm (15115 g) for 5 min at RT. The supernatant was discarded. This step was repeated twice.

v. Ethanol was removed completely by air-drying the pellet for 30 min at RT.

vi. The pellet was reconstituted in 20μl of Hi-Di™ formamide (Applied Biosystem, USA).

vii. The denaturation of DNA was carried out at 95°C for 5 min in boiling water bath, followed by snap chilling on ice for 5 min.

viii. The contents were mixed by vortex mixer (Remi Equipment, India), spun and loaded in ABI3100 genetic analyzer.

4.1.9.4 Detection of nucleotides in genetic analyzer

The nucleotide sequences were detected using ABI 3100 automated sequencer (Applied Biosystems, USA). The cycle sequenced products were subjected to capillary electrophoresis. During this process the extension products of the cycle sequencing reaction enter the capillary as a result of electro kinetic injection. A high voltage charge applied to the buffered sequencing reaction forces the negatively charged fragments into the capillaries. The extension products are separated by size. Shortly before reaching the positive electrode, the fluorescent-labeled DNA fragments, separated by size, move across the path of a laser beam. The laser beam causes the dyes on the fragments to fluoresce. An optical detection device detects the fluorescence. The Data Collection
Software converts the fluorescence signal to digital data and generates a four-color chromatogram. As each dye emits light at a different wavelength when excited by laser, all four colors, and therefore all four bases, can be detected and distinguished in one capillary injection. From the chromatogram, nucleotide sequence was read and compared with available sequences employing NCBI Blast tool and MEGA 5 program.

4.1.10 Phylogenetic analysis

Phylogenies are important for addressing various biological questions such as relationships among species or genes, the origin and spread of viral infection and the demographic changes and migration patterns of species. The advancement of sequencing technologies has taken phylogenetic analysis to a new height.

4.1.10.1 Identification of sequence by BLAST

Basic Local Alignment Search Tool, or BLAST (www.ncbi.nlm.nih.gov/blast), is an algorithm in bioinformatics for comparing primary biological sequence information, such as the amino acid sequences of different proteins or the nucleotides of DNA sequences. A BLAST search enables a researcher to compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold.

4.1.10.2 Procedure

The sequences obtained from the auto-sequencer with both the forward and reverse primers were checked manually and compared with that of the prototype strain. The nucleotide sequences were then subjected to BLAST analysis in NCBI database to determine the genotype or to identify a set of sequences that are homologous to a sequence of interest.

4.1.10.3 Alignments

The checked and corrected nucleotide sequences of NIV strains were adjusted in FASTA format and alignment of multiple sequences was carried out using Clustal W (Thompson et al., 1994). Clustal W is a multiple sequence
alignment program for DNA or proteins. It provides an integrated environment for performing multiple sequence and profile alignments and analyzes of the results.

4.1.10.4 Phylogenetic tree

4.1.10.4.1 Principle

A phylogenetic tree is a graphical representation of the evolutionary relationship between taxonomic groups. The term phylogeny refers to the evolution or historical development of a plant or animal species, or even a human tribe or similar group. A phylogenetic tree is a specific type of cladogram where the branch lengths are proportional to the predicted or hypothetical evolutionary time between organisms or sequences. Cladograms are branched diagrams, similar in appearance to family trees that illustrate patterns of relatedness where the branch lengths are not necessarily proportional to the evolutionary time between related organisms or sequences. Bioinformaticians produce cladograms representing relationships between sequences, either DNA sequences or amino acid sequences. However, cladograms can rely on many types of data to show the relatedness of species. Hence, it is important to understand that the cladograms generated by bioinformatics tools are primarily based on sequence data alone.

4.1.10.4.2 Procedure

The alignment file was converted to MEGA format using molecular evolutionary genomic analyzer software MEGA 5 (Tamura et al., 2011). The MEGA version file was used to analyze the data. Gene domain was selected in such a manner that all sequences carried start and end sequence number. Here the option to translate the nucleotide sequence in amino acids can also be selected. Phylogenetic tree was constructed by using Neighbour-Joining method with 1000 bootstrap support and Kimura 2-parameter, with pair-wise deletion method.

4.1.10.5 Genetic distance and percent identity calculation

Distance was calculated by choosing menu option 'compute distance' using P distance and pair wise deletion method in MEGA software. The distances and standard errors obtained in printable format were copied to clipboard. This distance chart was pasted in MEGA p-distance converter to obtain the percent identity between and within sequences.
4.1.11 Statistical analysis

The proportions across two different periods as well as two different age groups were compared using the Chi-square test with Yates’s correction and p values <0.05 were considered statistically significant.

4.1.12 Accession numbers

Sixty-five of 75 NSP2 gene sequences of RVB strains detected in sporadic cases have been deposited in GenBank under the accession numbers JQ686121-JQ686185. Sequences of the remaining 10 strains containing <200 bp could not be submitted to GenBank.

The nucleotide sequences of NSP2 genes of the strains detected in outbreak cases have been deposited in GenBank under the accession numbers, KF689785, JQ904239, JQ904240 (Daman), HQ425489-HQ425491 (Surat), HQ268794-HQ268804 (Mumbai), HQ425484-HQ425488 (Sangli) and KF689787, KF689788 (Bhor). The accession numbers of the sequences of NSP2 genes of 4 of the 15 RVB strains detected in the outbreak cases from Sholapur are KF689786, JQ904247-JQ904249, while those for 11 RVB strains could not be submitted due to un-availability of sequences ≥200 bp.

The reference strains included in the phylogenetic analyses are IDIR (U03558), ADRV (AJ867609), WH-1 (AY539861), Bang117 (GU391308), Bang373 (AY238393), CAL-1 (AY238383), IC-008 (GU377220), IDH-084 (GU377231), MMR-B1 (GU370058), GBRV (EU436849), CAL-10 (AY864914), CAL-30 (AY941790), DhakaB1 (DQ869562), DhakaB17 (DQ869565), DhakaB529 (GU478997), DhakaB77 (GU479007), DhakaB452 (GU479003), BRA12-UEL (EF577257), BRA14-UEL (EF577258), PB-71-H5 (AB673221), PB-S43-11 (AB673229), PB-91-Z4 (AB673230), PB-93-15 (AB673233), DB176 (GQ358725), RUBV226 (GQ358726), RUBV282 (GQ358727) and J19 (DQ113903).
Chapter 4: Group B rotavirus infections in India

4.2 Results
4.2.1 RVB positivity rates in acute gastroenteritis
4.2.1.1 Sporadic cases

From a total of 2101 fecal specimens collected from the sporadic cases of acute gastroenteritis, 75 (3.6%) were shown to contain RVB RNA by RT-PCR (Figure 4.1).

The rate of positivity in Pune (2004-2010), Alappuzha (2009) and Belgaum (2008-2009) cities was found to be 4.1% (36/870), 7.3% (8/110) and 4.1% (8/197) respectively. Although the positivity rate in Pune appeared lower (2.5%, 23/924) during 1994-1995 as compared to the rate in 2004-2010, the difference was not significant (p>0.05).

Yearly distribution of RVB RNA positivity showed relatively higher values in 1994 in comparison to 1995 (p=0.02) for adolescents and adults from Pune. However, no significant difference in the yearly positivity rates was noted in the years 2004-2010 (p>0.05) (Figure 4.2).

Figure 4.1: Electrophoretic migration pattern of RVB NSP2 PCR products (229 bp) visualized on agarose gel

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Negative control</td>
</tr>
<tr>
<td>2</td>
<td>RVB positive specimen NIV-0924341</td>
</tr>
<tr>
<td>3</td>
<td>100 bp DNA ladder</td>
</tr>
<tr>
<td>4</td>
<td>RVB positive specimen NIV-0948756</td>
</tr>
<tr>
<td>5</td>
<td>Reagent control</td>
</tr>
</tbody>
</table>

229 bp

100 bp DNA ladder

58
4.2.1.2 Outbreak cases
Among the 218 specimens collected during the outbreaks occurred in Mumbai, 11 (5.05%) (March (7/72; 9.72%) and October (4/146; 2.74%)) were tested positive for RVB RNA. From a total of 283 specimens collected during the first outbreak (February-April, 2010) occurred in Sholapur, 15 (5.3%) specimens were shown to contain RVB RNA, while none of the 57 specimens collected during the second outbreak (July-August, 2011) showed presence of RVB RNA.

RVB RNA was also detected in the specimens collected and referred to NIV, Pune during the outbreaks of gastroenteritis occurred in Daman (3 out of 5), Surat (3 out of 3), Sangli (5 out of 29) and Bhor (2 out of 2).

4.2.2 Age distribution of RVB infected patients

4.2.2.1 Sporadic cases
RVB positivity was significantly higher in adolescents/adults (20/538, 3.7% in the 1990s; 36/413; 8.7% in the 2000s) as compared to that of children (3/386; 0.8% in the 1990s; 0/457, 0.0% in the 2000s) (p < 0.005 for each comparison) in specimens analyzed from Pune city (Table 4.8). However, it was not significantly different in children and adolescents/adults from Alappuzha (3/332; 9.4% vs 5/78;
6.4%) and Belgaum (7/144; 4.9% vs 1/53; 1.9%). Overall, RVB infections were detected at a higher prevalence in adolescents and adults (62/1082; 5.7%) as compared to children (13/1019; 1.3%) (p < 0.001). In the group of children (between 0 and 10 years of age) the prevalence of RVB infection was highest in children ≤2 years of age (8/13; 61.5%). However, the rate of RVB positivity in adolescents and in different age groups (>18-29, 30-39, 40-49, 50-59 and ≥60 years) of adults was not different (>0.05).

Of the total 75 patients suffering from RVB infections, 51 (68%) were males and 24 (32%) were females.

Table 4.8: Rotavirus positivity among adolescent and adult cases of acute gastroenteritis in 1994-1995 and 2004-2010

<table>
<thead>
<tr>
<th>Year</th>
<th>No. Positive/No. Tested (%)</th>
<th>Children</th>
<th>Adolescents and adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>1994-1995</td>
<td>3/386 (0.8)%</td>
<td>20/538 (3.7)%</td>
<td></td>
</tr>
<tr>
<td>2004-2010</td>
<td>0/457 (0.0)%</td>
<td>36/413 (8.7)%</td>
<td></td>
</tr>
</tbody>
</table>

Note: a Vs b and c Vs d; p < 0.005

4.2.2.2 Outbreak cases

In the outbreaks occurred in Mumbai, RVB positivity was found to be distributed among children (n=6), adolescent (n=1) and adults (n=4). Among the RVB infected patients from Sholapur, positivity was found to be significantly higher in adults (86.7%, 13/15) as compared to that of children (13.3%, 2/15) (p < 0.005). All RVB infected patients identified during the outbreaks occurred in Daman, Surat, Sangli and Bhor belonged to adult age group.

4.2.3 Assessment of disease severity

By applying the criteria described earlier by Ruuska and Vesikari (1990), available information on clinical features presented by patients from sporadic cases of acute gastroenteritis showed mild to moderate severity of rotavirus disease, while all of the patients from outbreak cases presented with severe disease.
4.2.4 **Mixed infection**

Mixed infections of RVA and RVB were identified in 15/59 (25.4%), 1/8 (12.5%) and 2/8 (25%) specimens from Pune, Alappuzha and Belgaum, respectively. Adults from different age groups (n=4 for 18-29 years, and n=3 for each of the 30-39, 40-49 and ≥60 years age groups) showed the highest number (13/18, 72.2%) of mixed infections. Adolescents and children also showed presence of mixed infections, but at lower levels (2/18, 11.1%; 3/18, 16.7%).

One specimen collected in March, 2006 from Mumbai has shown mixed infection (9%, 1/11) of RVA and RVB. Mixed infection of RVA and RVB were also noted in 1 (7%, 1/15) of the specimens from Sholapur.

4.2.5 **Seasonality**

Monthly distribution of RVB infections identified in Pune in 1994-1995 and 2004-2010 is depicted in Figure 4.3. RVB positive cases were detected throughout the year. However, peak activity was found in the months of April and December during 1994-1995 and in the months of April, June and September during 2004-2010.

![Figure 4.3: Monthly distribution of group B rotavirus positivity in adolescent / adult patients with acute gastroenteritis from Pune, western India](image)

4.2.6 **Sequencing of NSP2 genes and phylogenetic analysis of RVB strains**

The presence of RVB RNA in 75 fecal specimens was confirmed by sequencing of the partial NSP2 gene (229 bp). Phylogenetically, all strains clustered with other RVB strains in an NSP2 lineage containing Indian-
Bangladeshi RVB strains belonging to VP7 genotype G2 (Figure 4.4). Nucleotide sequence identity of NSP2 genes among the 75 Indian RVB strains was 93.9-100%. These sequences showed 93.1-100 % and 92.6-95.1% nucleotide identity with their counterparts in other strains of Indian-Bangladeshi and the Chinese lineages respectively.

Phylogenetic analysis of NSP2 gene sequences of all of the 39 RVB strains detected in the outbreak cases also showed clustering with the strains from Indian-Bangladeshi lineage of VP7 genotype G2 (Figure 4.5) with 98.0-100% nucleotide identity. Identity with strains of Chinese lineage was noted to be 92.9-94.8%.
Figure 4.4: Phylogenetic dendrogram of partial group B rotavirus NSP2 gene (nt 137-340). The strains of the present study (n=65) are in blue colour and indicated by the symbols • (strains from Pune city), ○ (strains from Alappuzha city) and □ (strains from Belgaum city). The scale represents genetic distance.

Note: RVB strain names are according to the guidelines of the Rotavirus Classification Working Group (Matthijnssens et al., 2011).
Chapter 4: Group B rotavirus infections in India

Figure 4.5: Phylogenetic dendrogram of partial group B rotavirus NSP2 gene (nt 171-287 bp). The strains of the present study (n=39) are highlighted in blue colour. Strains from different regions were highlighted with different symbols: ■ (Daman), ◦ (Surat), ◼ (Mumbai), ◇ (Sangli), ▲ (Sholapur) and □ (Bhor). The scale represents genetic distance.

Note: RVB strain names are according to the guidelines of the Rotavirus Classification Working Group (Matthijnssens et al., 2011).
4.3 Discussion

Identification of non-RVA strains is known to be based on the characteristic electrophoretic migration patterns of their genomes or by electron microscopy of rotaviruses not reactive in common enzyme immunoassays for RVAs (Gouvea et al., 1991). Surveillance carried out to identify non-RVA strains in the 1980s made use of electropherotyping, EM, IEM and ELISA (Rodger et al., 1982; Hung et al., 1984, Dai et al., 1987; Fang et al., 1989). In the 1990s, highly sensitive RT-PCR assays were utilized for detection of RVB and RVC strains in the fecal specimens from human and animal species (Gouvea et al., 1991; Chang et al., 1997). Using electropherotyping aided with RT-PCR, limited studies conducted in Pune, western India and Kolkata, eastern India have reported variable (0.8-18.5%) frequencies of RVB infection in patients with acute gastroenteritis (Kelkar and Zade, 2004; Barman et al., 2006). Recently, different rates (0.5-26.2%) of RVB and RVC infections have been reported for humans or pigs from Ireland, South Korea, Myanmar, Bangladesh and Nepal (Rahman et al., 2005; Collins et al., 2008; Aung et al., 2009; Jeong et al., 2009; Saiada et al., 2011; Alam et al., 2013). The present study documents similar findings (2.5-7.3%) on RVB infections in three different cities located in three different states of India.

RVB infections were detected in both genders and in all age groups. Similar to the earlier report on RVA and RVB infections, the rate of infection was higher among children ≤2 years of age, compared to older children (between 2 and 10 years) (Bahl et al., 2005; Barman et al., 2006). It is interesting to note that RVB infection in Pune, western India was confined only to adolescent and adult cases of acute gastroenteritis during the seven consecutive years, 2004-2010 although a small proportion of pediatric population (0.9%) was found affected by this virus in the 1990s. This may indicate a low exposure of children to RVB in the recent past or low shedding of RVB in fecal specimens, below the detection limit of the assay employed in the study (Saif et al., 1994). It may be noted that our study conducted on the sporadic infections of acute gastroenteritis has limitations on account of the analysis restricted to small number of specimens and/or a short period of collection, especially from Alappuzha (2009) and Belgaum (2008-2009). However, it revealed similar rates of RVB infections (p> 0.05) in adolescents and different age groups of adults.
RVB was detected as sole viral pathogen in the specimens collected from Daman, Surat, Sangii and Bhor. Variable rates (0.0-17.2%) of RVB positivity were noted in the cases of outbreaks occurred in Mumbai, Sangii and Sholapur. This could be attributed to the different age groups (section 4.2.2) and sampling pattern associated with each of the outbreaks investigated in the study.

Mixed infections of RVA and RVB strains have been reported in humans and animals (Barman et al., 2006; Medici et al., 2011). In the present study, a significant proportion (24%) of RVB infected patients detected in sporadic cases was found to be co-infected with RVA as well. Patients infected with RVB or with both, RVB and RVA strains only experienced mild to moderate gastroenteritis. One each specimen having mixed infections of RVA and RVB collected during the outbreaks from Mumbai (March, 2006) and Sholapur (February-April, 2010) was from child patients below 1 year of age.

The year-wise trend of RVB RNA positivity showed relatively higher values for adolescents and adults in 1994 in comparison to 1995 (p=0.02/ <0.05). However, similar trend was not observed in the years 2004-2010 (p>0.05).

With respect to seasonality, the results of this study are in agreement with previous findings showing the absence of a seasonal pattern for RVB infections (Barman et al., 2006). In Bangladesh, incidence of RVB was found highest between August and October during 2003 (Rahman et al., 2007) and in March and May during 2008 (Saiada et al., 2011). In the present study, more RVB infections were noted in April and December in 1994-1995 and April, June and September during 2004-2010 in Pune as compared to other months (Figure 4.3).

The partial nucleotide sequences of the NSP2 gene of all RVB strains examined in the present study clustered together (Figure 4.4 and 4.5) and displayed a high nucleotide sequence similarity not only among themselves but also with other strains from eastern India, Bangladesh and Myanmar and thus revealed a single lineage of RVB circulating in the human population.

To summarize, this study has confirmed that human RVB strains are consistently causing infections sporadically and in outbreaks in western India at low to moderate levels, and also documented for the first time the circulation of RVB strains in southern India. To our knowledge, this is the first description of a long-term surveillance of RVB infections from India. Such studies would be useful for better understanding of the disease burden caused by RVB infections in sporadic and outbreak situations.