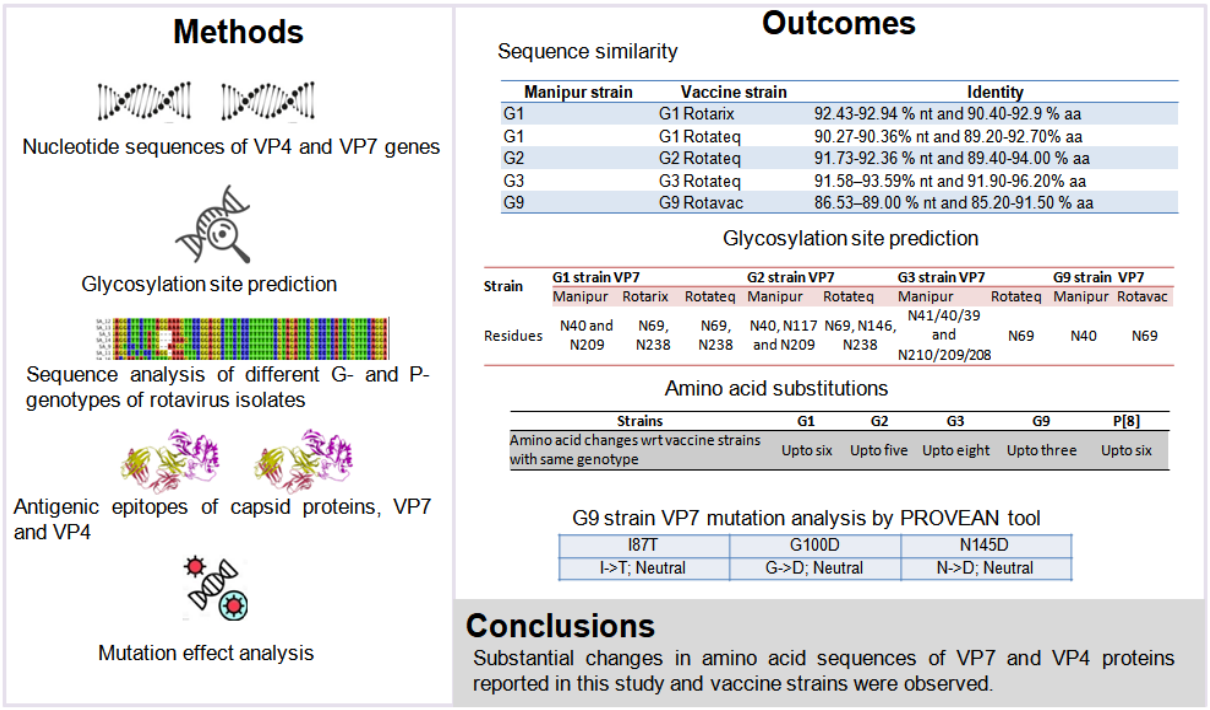
CHAPTER 4

GENETIC ANALYSES OF VP7 AND VP4 ANTIGENIC EPITOPES BETWEEN HUMAN ROTAVIRUSES CIRCULATING IN MANIPUR AND VACCINE STRAINS

Graphical Abstract



4.1. Introduction

Rotavirus (RV) triple layered particles consist of three concentric layers of protein. The outermost layer is composed of VP4 and VP7, VP6 make up the middle layer and the VP2 forms the inner layer. Group A rotavirus (RVA) VP7 and VP4 protein contains major neutralizing epitopes and the antibodies against VP7 and VP4 are known to neutralize the infectivity of RVAs. The group-specific VP6 antigen is highly conserved, abundant, immunogenic, and induces non-neutralizing antibodies. VP6 can generate self-assembled nano-sized structures forming virus-like particles (VLPs) [1]. Recently it has been reported

that VP6-specific IgG was much more efficient in neutralization than VP6-specific IgA in cell lines and *in vivo* model of murine rotavirus infection [2]. Indigenous oral and live attenuated RVA vaccines, Rotavac and RotaSIIL have been included in UIP of India. Rotavac™, developed by the Bharat Biotech International, Hyderabad is based on rotavirus strain 116E with G9P[8] genotype was licensed in 2015 and launched in 16 states including Manipur [3,4]. RotaSIIL® (Serum Institute of India) was licensed in 2017 and included in UIP in state of Jharkhand.

Studies have reported that rotavirus vaccination programs in children were associated with substantial reduction in rotavirus-associated hospitalizations in both developed and developing nations [5,6]. The global vaccines, Rotarix and RotaTeq have been shown to elicit heterotypic immunity and the efficacy are in the range of 80-97% in developed countries [7,8,9] but in developing countries, efficacy is less, and it is in the range of 48-60% [10,11,12]. Rotavac had shown efficacy against severe rotavirus gastroenteritis at 53.6% and 56.4% in the first year of life [3]. The efficacy of RotaSIIL against severe rotavirus gastroenteritis in an immunized Indian child was 33% [13] and 67% in Niger [14]. The lower efficacy of the vaccines can be analyzed in terms of antigenicity of the capsid proteins thereby investigating the antigenic epitopes of the circulating RV strains and the vaccine. Importantly, it has been noted that a significant difference in strain incidence between developed and developing countries, as well as reassortment with wild-type strains that results in the development of new strains or changes in virulence, pose challenges to the effectiveness of recently introduced RVA vaccines. Children who receive the rotavirus oral vaccine are said to develop cytotoxic T cells specific to the virus as well as serum and intestine serotype-specific neutralizing antibodies directed against VP7 and VP4. This is the likely mechanism of rotavirus vaccine mediated immune protection in children. There is a poor understanding about the relationship between the rotavirus vaccine strains and the circulating human RVAs. In this study we determined the sequences of VP4 and VP7 outer capsid proteins circulating in Imphal, Manipur during 2015 to 2019.

4.2. Materials and Methods

4.2.1. Study samples

Same as Chapter 3, section 3.2.2.

4.2.2. RNA extraction and RT-PCR

Protocol same as section 3.2.3, 3.2.4 and 3.2.6 of Chapter 3

4.2.3. Nucleic acid sequencing and phylogeny

Protocol same as section 3.2.8 of Chapter 3

4.2.4. Glycosylation site prediction

NetNGlyc - 1.0 server (https://services.healthtech.dtu.dk/service.php?NetNGlyc-1.0) with a threshold value of 0.5 was used to predict N-linked glycosylation sites in rotavirus VP7 and VP4 proteins. The server uses artificial neural networks that investigate protein sequences for the presence of consensus Asn-Xaa-Ser/Thr sequence which is the potential site for glycosylation.

4.2.5. Analysis of the antigenic epitopes of capsid VP7 and VP4 proteins

Sequence similarity of VP7 and VP4 genes of the strains were investigated using the sequence identity and similarity (SIAS) tool (http://imed.med.ucm.es/Tools/sias.html) for intragenotype similarities compared to rotavirus vaccine sequences; Rotarix (G1P[8]), Rotateq (G1-G4 with P7[5] and G6P1A[8]) and Rotavac (G9P[11]). Further, sequence alignment was done, and neutralizing epitope regions were analyzed between antigenic residues in the outer capsid proteins of VP4 and VP7 of vaccine strains and the strains circulating in Imphal, India.

4.2.6. Mutation effect analysis

Mutation analysis was carried out using the PROVEAN tool which is based on an alignment-based algorithm [15]. Sequence-based mutation effect prediction was further analyzed by modeling VP7 protein structure in I-TASSER [16] and comparing the modeled structures. To determine the interaction between VP7 capsid protein and IgG antibody, VP7 model structure was docked with Fab region of IgG (PDB ID: 3FMG) in Clustpro server and analyzed in PDBsum [17]. In PDBsum, hydrogen bonds and non-bonded contacts are calculated by HBPLUS, salt bridges, computed using the definition in Kumar & Nussinov (1999) [18] and interface areas are computed using a program called NACCESS.

4.2.7. Data availability

Reference vaccine strains were retrieved from GenBank: Rotarix® (JN849114 and JN849113), RotaTeq® (GU565057, GU565068, GU565079, GU565090, and GU565044) and 116E (FJ361209, FJ361204). VP7 and VP4 sequences identified in this study have been submitted to GenBank under accession numbers: VP7 sequences (OL584234 to OL584246) and VP4 (OL584247 and OL584248).

4.3. Results

4.3.1. Comparative sequence analysis of VP7 (G1-G4 and G9) and VP4 (P[6] and P[8]) genes of rotavirus strains from Manipur and vaccines strains

From the hospital-based rotavirus surveillance conducted among diarrhoeic children in Imphal during December 2015 to March 2019, VP7 and VP4 nucleotide sequences were deposited in NCBI database GenBank. The details of the sequences along with their accession numbers are given in Table 4.1. Intragenotype similarity analysis of VP7 and VP4 nucleotide and protein sequences based on amino acid identities between vaccine strains and circulating G9 strains is shown in Table 4.2.

Table 4.1. Summary of VP7 and VP4 lineage, date of isolation, and available patient information for rotavirus strains from Imphal analyzed in this study and rotavirus vaccine strains Rotavac, Rotarix and RotaTeq

Strain	VP7 lineage/ Genotype	Date of Isolation (month/year)	Age (months)	Gender	Genbank Accession no.
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P1A[8]	2/G1	-	-	-	JN849114
RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P7[5]	3/G1	-	-	-	GU565057
RVA/Hu/IND-Manipur/RM22112018/2018/G1P[4]	-/G1	10 / 2018	6	Female	OL584234
RVA/Hu/IND-Manipur/RM63012019/2019/G1P[8]	-/G1	07 / 2018	12	Male	OL584235
RVA/Hu/IND-Manipur/RM79032019/2019/G1P[6]	-/G1	03 / 2019	12	Female	OL584236
RVA/Hu/IND-Manipur/RM23112018/2018/G1P[6]	-/G1	-	-	-	OL584237
RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P7[5]	2/G2	-	-	-	GU565068.1
RVA/Hu/IND-Manipur/RM65022019/2019/G2P[4]	-/G2	02 / 2019	12	Male	OL584238
RVA/Hu/IND-Manipur/RM49012019/2019/G2	-/G2	01 / 2019	9	Male	OL584239
RVA/Hu/IND-Manipur/RM74032019/2019/G2P[10]	-/G2	02 / 2019	5	Male	OL584240
RVA/Vaccine/USA/RotaTeq-WI78-8/1992/G3P7[5]	2/G3	-	-	-	GU565079.1
RVA/Hu/IND-Manipur/RM252122016/2016/G3	-/G3	09 / 2016	12	Male	OL584241
RVA/Hu/IND-Manipur/RM53012019/2019/G3	-/G3	01 / 2019	11	Male	OL584242
RVA/Hu/IND-Manipur/RM32122018/2018/G3P[8]	-/G3	11 / 2018	8	Male	OL584243
RVA/Hu/IND-Manipur/RM50012019/2019/G3P[11]	-/G3	01 / 2019	11	Male	OL584244
RVA/Hu/IND-Manipur/RM78032019/2019/G3P[8]	-/G3	03 / 2019	16	Male	OL584245
A strain 116E/AG segment 9 viral protein 7	2/G9	-	-	-	FJ361209.1
RVA/Hu/IND-Manipur/RM61022019/2019/G9P[10]	-/G9	03 / 2019	9	Female	OL584246
RVA/Hu/IND-Manipur/RM83012016/2016/G9P[10]	-/G9	01 / 2016	16	Female	-
RVA/Hu/IND-Manipur/RM157032016/2016/G9P[4]	-/G9	03 / 2016	6	Female	-
Strain	**VP4 lineage**	**Date of Isolation (month/year)**	**Age (months)**	**Gender**	**Genbank Accession no.**
Rotavirus A strain 116E/AG segment 4 viral protein 4	NA	-	-	-	FJ361204
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P1A[8]	1/P[8]	-	-	-	JN849113
RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A[8]	2/P[8]	-	-	-	GU565044
RVA/Hu/IND-Manipur/RM64022019/2019/P[8]	-/P[8]	02 / 2019	13	Male	OL584248
RVA/Hu/IND-Manipur/RM66022019/2019/P[8]	-/P[8]	02 /2019	72	Male	-
RVA/Hu/IND-Manipur/RM419032018/2018/P[8]	-/P[8]	03 /2018	9	Male	-
RVA/Hu/IND-Manipur/RM32122018/2018/P[8]	-/P[8]	12 / 2018	8	Male	-
RVA/Hu/IND-Manipur/RM78032019/2019/P[8]	-/P[8]	03 / 2019	16	Male	-
RVA/Hu/IND-Manipur/RM38122018/2018/P[6]	-/P[6]	12 / 2018	9	Male	OL584247
RVA/Hu/IND-Manipur/RM63012019/2019/P[6]	-/P[6]	01 / 2019	12	Male	-
RVA/Hu/IND-Manipur/RM62022019/2019/P[6]	-/P[6]	02 / 2019	72	Female	-
RVA/Hu/IND-Manipur/RM23112018/2018/P[6]	-/P[6]	11 / 2018	8	Female	-

Table 4.2. The nucleotides and amino acids differences of VP7 and VP4 proteins of Manipur strains with rotavirus vaccine strains. Intragenotype similarities with Rotarix are colored in blue. Intragenotype similarities with RotaTeq are colored orange. Intragenotype similarities with Rotavac are colored green.

		Nucleotide /Amino acid identities (%) between circulating and vaccine strains: VP7 and VP4								
Strain name (Accession no.)	**G/P-genotype**	**G1 of Rotarix**	**G9 of Rotavac**	**G1 of RotaTeq**	**G2 of RotaTeq**	**G3 of RotaTeq**	**G4 of RotaTeq**	**P[8] of Rotarix**	**P[8] of RotaTeq**	**P[11] of Rotavac**
RM22112018 (OL584234)	G1	91.73/92.30	72.87/75.00	89.14/91.15	71.27/72.30	72.87/78.07	74.47/75.76	-	-	-
RM63012019 (OL584235)	G1	91.93/92.40	73.11/75.20	89.37/91.20	71.44/73.20	72.98/78.40	74.90/75.59	-	-	-
RM79032019 (OL584236)	G1	92.79/92.66	73.57/75.67	90.26/91.50	71.93/72.97	73.70/78.76	75.09/76.06	-	-	-
RM23112018 (OL584237)	G1	92.61/93.07	73.46/75.76	90.11/91.92	71.96/73.07	73.59/78.84	75.09/76.15	-	-	-
RM65022019 (OL584238)	G2	71.14/71.92	72.13/73.84	70.89/72.69	92.16/93.84	71.51/73.46	70.77/69.23	-	-	-
RM49012019 (OL584239)	G2	70.49/71.92	71.48/73.84	70.37/72.69	91.11/93.84	71.11/73.46	70.12/69.23	-	-	-
RM74032019 (OL584240)	G2	70.77/74.46	71.69/74.46	70.77/74.89	84.76/95.31	71.42/75.31	70.11/72.34	-	-	-
RM252122016 (OL584241)	G3	70.98/76.10	74.67/81.85	69.98/76.99	70.27/74.33	90.46/95.13	71.97/73.45	-	-	-
RM53012019 (OL584242)	G3	72.43/79.23	74.72/83.46	71.34/80.00	70.73/75.00	91.65/96.15	72.18/76.15	-	-	-
RM32122018 (OL584243)	G3	73.29/78.22	76.23/82.25	72.49/79.03	71.29/75.00	93.59/95.96	73.29/74.59	-	-	-
RM50012019 (OL584244)	G3	74.93/79.15	76.69/83.01	73.55/79.92	71.80/74.13	90.97/94.59	73.55/75.67	-	-	-
RM78032019 (OL584245)	G3	73.81/79.15	76.76/83.39	72.78/79.92	72.01/74.90	93.06/96.13	73.42/76.06	-	-	-
RM61022019 (OL584246)	G9	73.73/78.46	87.79/93.07	72.62/78.46	72.99/75.76	77.55/86.15	72.74/78.46	-	-	-
RM83012016	G9	72.36/70.20	86.94/86.44	70.83/69.69	72.22/69.69	75.97/75.25	71.38/69.19	-	-	-
RM157032016	G9	70.87/71.61	85.35/83.83	68.85/70.76	72.55/70.33	74.91/77.96	70.53/70.33	-	-	-
RM64022019 (OL584247)	P[8]	-	-	-	-	-	-	87.09/92.12	90.19/95.66	53.47/41.73
RM66022019	P[8]	-	-	-	-	-	-	87.85/87.68	90.83/91.04	54.64/41.41
RM419032018	P[8]	-	-	-	-	-	-	87.66/82.35	90.84/85.29	54.33/37.13
RM32122018	P[8]	-	-	-	-	-	-	89.20/88.23	92.06/92.15	55.00/40.78
RM78032019	P[8]	-	-	-	-	-	-	89.58/87.59	92.54/91.47	55.14/40.69
RM38122018 (OL584248)	P[6]	-	-	-	-	-	-	72.48/72.65	72.35/70.7	51.97/37.1
RM63012019	P[6]	-	-	-	-	-	-	70.25/67.07	71.35/65.84	52.93/34.56
RM62022019	P[6]	-	-	-	-	-	-	70.62/68.72	71.72/67.07	53.27/35.80
RM23112018	P[6]	-	-	-	-	-	-	69.98/67.21	71.07/65.57	52.93/35.24

The G1 strains showed 92.43-92.94 % nucleotide and 90.40-92.9 % amino acid identity with G1 Rotarix vaccine strain (lineage 2) and 90.27-90.36% nucleotide and 89.20-92.70% amino acid identity with the G1 strain in RotaTeq vaccine (lineage 3). The G2 strains showed 91.73-92.36 % nucleotide and 89.40-94.00 % amino acid identity with G2 RotaTeq (lineage 2). The G3 strains were close to the RotaTeq VP7 of the same genotype (91.58–93.59% nucleotide and 91.90-96.20% amino acid identity). G9 strains revealed 86.53–89.00 % nucleotide and 85.20-91.50 % amino acid identity with G9 Rotavac (lineage 2) (Table 4.2).

The VP8* segment of the P[8] strains were more similar to the RotaTeq P[8] (90.11–92.66% nucleotide and 86.67–95.67% amino acid identity) than to Rotarix P[8] (89.64–90.57% nucleotide and 87.69-90.31% amino acid identity). Whereas, the VP8* of the P[6] strains showed less similarity to the RotaTeq and Rotarix VP8*. The VP8* of the RM64022019 P[8] and RM38122018 P[6] strain showed close nucleotide similarity with P[11] of Rotavac; 92.03% and 91.50%, respectively but less amino acid similarity 48.21% and 49.12%, respectively (Table 4.2).

It was followed by phylogenetic analysis of the VP7 and VP4 genes of the strains in this study, different parts of India including north-east India, neighboring countries and a few more countries in the world and also VP7 and VP4 sequences of vaccine strains; Rotarix, Rotateq and Rotavc as shown in Table 4.3 and Table 4.4. The VP7 genes of four representative Manipur G1 strain in this study clustered within the same lineage and were found closely related to strains from India, the USA and Pakistan. The Manipur strains shared 94%-96.32% nt and 94.23%- 98.84% aa similarity. The three G2 Manipur strains were also found to cluster together were closely related to strains from India, USA and Russia and shared 93.22%- 98.39% nt and 90%- 99.23% aa similarity. The VP7 genes of five G3 Manipur strains form different clusters; strain RM252122016 was found clustering with strains from India and Pakistan; strains RM50012019, RM53012019 and RM78032019 were found clustering together and were closely related to strains from India, Malaysia, and Bangladesh. Strain RM32122018 was found clustering alone. The strains shared 82.75%-95.89% nt and 84.67%- 99.61% aa similarity. Out of the three G9 strains, RM830122016 and RM61022016 clustered together and were closely related to strains from India, Pakistan, Italy, and Ghana. The strains shared 65.71%-83.57% nt and 62.36%-78.13% aa similarity.

The VP4 genes of four representative Manipur P[8] strain clustered within the same lineage and were closely related to contemporary strains from the Dominican Republic. The Manipur P[8] strains shared 91.94%-95.11% nt and 91.48%- 94.81% aa similarity. The Manipur P[6] strain RM38122018 clustered with contemporary strains from China, Sri Lanka, and Thailand. The other three Manipur P[6] strains clustered together and were found closely similar to a strain from Bangladesh. The strains shared 82.71%-98.85% nt and 83.2%- 96.87% aa similarity.

Table 4.3. Alignment of antigenic residues in VP7 between the strains contained in Rotarix, RotaTeq and Rotavac and strains circulating in Imphal. Antigenic residues are divided into three epitopes (7-1a, 7-1b, and 7-2). Amino acids that differ between Rotarix, RotaTeq and Rotavac are indicated in boldface. Red colored residues are residues that are different from Rotarix, and Green colored residues are different from the most similar genotype in RotaTeq. Residues colored in Blue are different from both Rotarix and RotaTeq, Residues colored in Orange are different from Rotavac. Amino acid changes that have been shown to escape neutralization with monoclonal antibodies (Mcdonald SM 2009) are indicated with asteric

	VP7 antigenic epitope sites																														
	7-1a															7-1b							7-2								
Strain	**87**	**91**	**94**	**96**	**97**	**98**	**99**	**100**	**104**	**123**	**125**	**129**	**130**	**291**	**201**	**211**	**212**	**213**	**238**	**242**	**143**	**145**	**146**	**147**	**148**	**190**	**217**	**221**	**264**
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P1A[8]	**T**	**T**	**N**	**G**	**E**	**W**	**K**	**D**	**Q**	**S**	**V**	**V**	**D**	**K**	**Q**	**N**	**V**	**D**	**N**	**T**	**K**	**D**	**Q**	**N**	**L**	**S**	**M**	**N**	**G**
RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P7[5]	**T**	**T**	**N**	**G**	**D**	**W**	**K**	**D**	**Q**	**S**	**V**	**V**	**D**	**K**	**Q**	**N**	**V**	**D**	**N**	**T**	**K**	**D**	**Q**	**S**	**L**	**S**	**M**	**N**	**G**
RVA/Hu/IND-Manipur/RM22112018/2018/G1P[4]	T	T	**S**	G	**E**	W	K	D	Q	**N**	V	V	D	**N**	Q	N	V	D	N	T	K	D	Q	**N**	L	S	**T**	N	G
RVA/Hu/IND-Manipur/RM63012019/2019/G1P[8]	T	T	**S**	G	**E**	W	K	D	Q	**N**	V	V	D	K	Q	N	V	D	N	T	K	D	Q	**N**	L	S	**T**	N	G
RVA/Hu/IND-Manipur/RM79032019/2019/G1P[6]	T	T	**S**	G	**E**	W	K	D	Q	**N**	V	V	D	**N**	Q	N	V	D	N	T	K	D	Q	**N**	L	S	**T**	N	G
RVA/Hu/IND-Manipur/RM23112018/2018/G1P[6]	T	T	**S**	G	**E**	W	K	D	Q	**N**	V	V	D	**N**	Q	N	V	D	N	T	K	D	Q	**N**	L	S	**T**	N	G
RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P7[5]	**A**	**N**	**S**	**D**	**E**	**W**	**E**	**N**	**Q**	**D**	**T**	**M**	**N**	**K**	**Q**	**D**	**V**	**S**	**N**	**S**	**R**	**D**	**N**	**T**	**S**	**D**	**I**	**S**	**G**
RVA/Hu/IND-Manipur/RM65022019/2019/G2P[4]	**T**	N	S	**N**	E	W	E	N	Q	D	T	M	N	K	Q	D	V	**D**	N	**N**	R	D	N	T	S	D	I	S	G
RVA/Hu/IND-Manipur/RM49012019/2019/G2	**T**	N	S	**N**	E	W	E	N	Q	D	T	M	N	**N**	Q	D	V	**D**	N	**N**	R	D	N	T	S	D	I	S	G
RVA/Hu/IND-Manipur/RM74032019/2019/G2P[10]	**T**	N	S	**N**	E	W	E	N	Q	D	T	M	N	K	Q	D	V	**D**	N	**N**	R	D	N	T	S	D	I	S	G
RVA/Vaccine/USA/RotaTeq-WI78-8/1992/G3P7[5]	**T**	**T**	**N**	**N**	**S**	**W**	**K**	**D**	**Q**	**D**	**A**	**V**	**D**	**K**	**Q**	**D**	**A**	**N**	**K**	**D**	**K**	**D**	**A**	**T**	**L**	**S**	**E**	**A**	**G**
RVA/Hu/IND-Manipur/RM252122016/2016/G3	T	T	N	N	S	W	K	D	Q	D	A	V	D	K	Q	D	**T**	N	**N**	**N**	K	D	A	T	L	S	E	**D**	G
RVA/Hu/IND-Manipur/RM53012019/2019/G3	**I**	T	N	N	S	W	K	D	Q	D	A	V	D	**N**	Q	D	**T**	N	**N**	**N**	K	D	A	T	L	S	E	**D**	G
RVA/Hu/IND-Manipur/RM32122018/2018/G3P[8]	T	T	N	N	S	W	K	D	Q	D	A	V	D	K	Q	D	**T**	N	**N**	**N**	K	D	A	T	L	S	E	**D**	G
RVA/Hu/IND-Manipur/RM50012019/2019/G3P[11]	**I**	T	N	N	S	W	K	D	Q	D	A	V	D	K	Q	D	**T**	N	**N**	**N**	K	D	**P**	T	L	S	E	**N**	G
RVA/Hu/IND-Manipur/RM78032019/2019/G3P[8]	**I**	T	N	N	S	W	K	D	Q	D	A	V	D	K	Q	D	**T**	N	**N**	**N**	K	D	A	T	L	S	E	**D**	G
A strain 116E/AG segment 9 viral protein 7	**I**	**T**	**G**	**T**	**E**	**W**	**K**	**G**	**Q**	**D**	**A**	**I**	**D**	**K**	**Q**	**N**	**T**	**A**	**D**	**N**	**K**	**N**	**S**	**T**	**L**	**S**	**E**	**N**	**G**
RVA/Hu/IND-Manipur/RM61022019/2019/G9P[10]	**T**	T	G	T	E	W	K	**D**	Q	D	A	I	D	K	Q	N	T	A	D	N	K	**D**	S	T	L	S	E	N	G
RVA/Hu/IND-Manipur/RM83012016/2016/G9P[10]	**T**	T	G	T	E	W	K	**D**	Q	D	A	I	D	K	Q	N	T	A	D	N	K	**D**	S	T	L	S	E	N	G
RVA/Hu/IND-Manipur/RM157032016/2016/G9P[4]	**T**	T	G	T	E	W	K	**D**	Q	D	A	I	D	K	Q	N	T	A	D	N	K	**D**	S	T	L	S	E	N	G
	*	*	*	*	*	*	*	*	*					*	*	*		*	*	*		*		*	*	*	*	*	*

Table 4.4. Alignment of antigenic residues in VP4 (VP8*) between the strains contained in Rotarix, RotaTeq and Rotavac and strains circulating in Imphal. Antigenic residues are divided into four epitopes (8-1, 8-2, 8-3 and 8-4). Amino acid changes that have been shown to escape neutralization with monoclonal antibodies (Mcdonald SM 2009) are indicated with asteric.
Boldface: Amino acids that differ from Rotavac. Orange: Residues that are different from Rotarix, Green: Residues are different from the most similar genotype in RotaTeq. Blue: Residues different from both Rotarix and RotaTeq

	VP8* antigenic epitope sites																							
	8-1											8-2		8-3								8-4		
Strain	100	146	148	150	188	190	192	193	194	195	196	180	183	113	114	115	116	125	131	132	133	135	87	88	89
Rotavirus A strain 116E/AG segment 4 viral protein 4	T	S	A	A	V	T	F	N	PV	P	N	S	Y	A	Q	T	S	T	D	N	S	S	S	N	D
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P1A[8]	D	S	Q	E	S	S	A	N	L	N	N	E	R	N	P	V	D	S	S	N	D	N	N	T	N
RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A[8]	D	S	Q	E	S	N	A	N	L	N	D	E	R	N	P	V	D	N	R	N	D	D	N	T	N
RVA/Hu/IND-Manipur/RM64022019/2019/P[8]	**D**	S	**Q**	**D**	**S**	**N**	**A**	N	**L**	**N**	**G**	**E**	**R**	**N**	**P**	**V**	**D**	**N**	**R**	N	**D**	**D**	**N**	**T**	**N**
RVA/Hu/IND-Manipur/RM66022019/P[8]	**D**	S	**Q**	**D**	**S**	**N**	**A**	N	**L**	**N**	**G**	**E**	**R**	**N**	**P**	**V**	**D**	**N**	**R**	N	**D**	**D**	**N**	**T**	**N**
RVA/Hu/IND-Manipur/RM419032018/P[8]	**D**	S	**Q**	**D**	**S**	**N**	**A**	N	**L**	**N**	**G**	**E**	**R**	**N**	**P**	**V**	**D**	**N**	**R**	N	**D**	**D**	**N**	**T**	**N**
RVA/Hu/IND-Manipur/RM32122018/P[8]	**D**	S	**Q**	**D**	**S**	**N**	**A**	N	**L**	**N**	**G**	**E**	**R**	**N**	**P**	**V**	**D**	**N**	**R**	N	**D**	**D**	**N**	**T**	**N**
RVA/Hu/IND-Manipur/RM78032019/P[8]	**D**	S	**Q**	**D**	**S**	**N**	**A**	N	**L**	**N**	**D**	**E**	**R**	**N**	**P**	**V**	**D**	**N**	**R**	N	**D**	**D**	**N**	**T**	**N**
RVA/Hu/IND-Manipur/RM38122018/2018/P[6]	**D**	S	**S**	**E**	**Y**	**S**	**S**	N	**L**	**S**	**E**	**E**	**H**	**T**	**T**	**Q**	**S**	**T**	**E**	N	**N**	**N**	**I**	**N**	**Q**
RVA/Hu/IND-Manipur/RM63012019/P[6]	**D**	**N**	**N**	**E**	**Y**	**S**	**S**	N	**L**	**S**	**E**	**E**	**H**	**T**	**N**	**Q**	**S**	**T**	**E**	N	**N**	**N**	**T**	**N**	**Q**
RVA/Hu/IND-Manipur/RM62022019/P[6]	**D**	**N**	**N**	**E**	**Y**	**S**	**S**	N	**L**	**S**	**E**	**E**	**H**	**T**	**N**	**Q**	**S**	**T**	**E**	N	**N**	**N**	**T**	**N**	**Q**
RVA/Hu/IND-Manipur/RM23112018/P[6]	**D**	**N**	**N**	**E**	**Y**	**S**	**S**	N	**L**	**S**	**E**	**E**	**H**	**T**	**H**	**Q**	**S**	**T**	**E**	N	**N**	**N**	**T**	**N**	**Q**
	*	*	*	*	*	*			*			*	*		*		*			*	*	*	*	*	*

4.3.2. Glycosylation site prediction

All the G1 strain VP7 contained two potential N-linked glycosylation sites at N40 and N209. G2 VP7 also possessed three such sites at N40, N117 and N209. However, G2 strain RM74032019 possessed these sites at different positions; N92, N146 and N184. Further, G3 VP7 showed variation among the strains and the site was predicted at N41/40/39 and N210/209/208.

The glycosylation site of the VP7 G9 strain was predicted at N40 with a potential score of 0.7021. It was predicted that VP7 of G9 strain and G2 strain RM74032019 sequences may not contain a signal peptide in the sequence. Signal peptides in proteins are necessary to be recognized by the N-glycosylation machinery and increase the chance of glycosylation of potential motifs [19]. However, in all the vaccines strains glycosylation was observed at N69 and N238 in Rotarix and Rotateq G1& G2. In addition, RotaTeq G2 possessed a glycosylation site at N146 which was also predicted in G2 strain RM74032019.

4.3.3. Comparison of the VP7 (G1-G4 and G9) and VP4 (P[6] and P[8]) antigenic epitopes strains from Manipur and vaccines strains

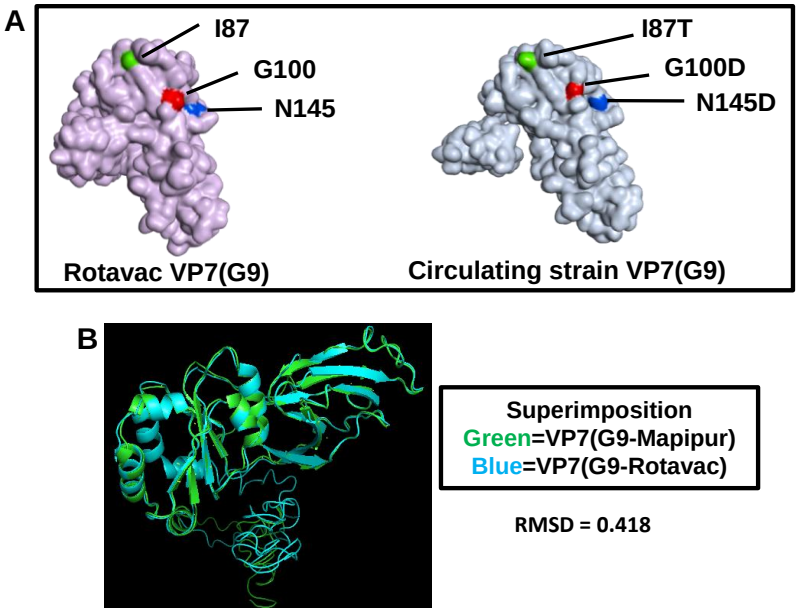
VP7 protein contains two structurally distinct epitope regions (71a and 71b and 7-2) and VP4 contains nine such epitope regions, four in VP8* (8-1 to 4) and five in VP5*. To determine the difference in these regions, sequence alignment was performed for deduced protein sequences of VP7 and VP4 of circulating strains in Manipur and the strains component of Rotarix, RotaTeq and Rotavac. Then, the epitope regions were examined as shown in Table 4.3 & Table 4.4 and those residues which have been shown previously to escape neutralization with monoclonal antibodies (mAb) [20] are indicated with an asterisk (Table 4.3 & Table 4.4). In VP7 of circulating G9, the amino acid difference was observed in residue numbers such as I87T, G100D, and N145D.

4.3.4 Mutation effect analysis

The mutation effect of amino acid substitution observed in VP4 and VP7 proteins was analyzed, and the results are shown in Figure 4.1 (inset table). Mutation effect analysis of the three amino acid substitutions was performed using the PROVEAN tool. As given in the inset table, the amino acid residue in position 87 is changed from Isoleucine (I) to Threonine (T),

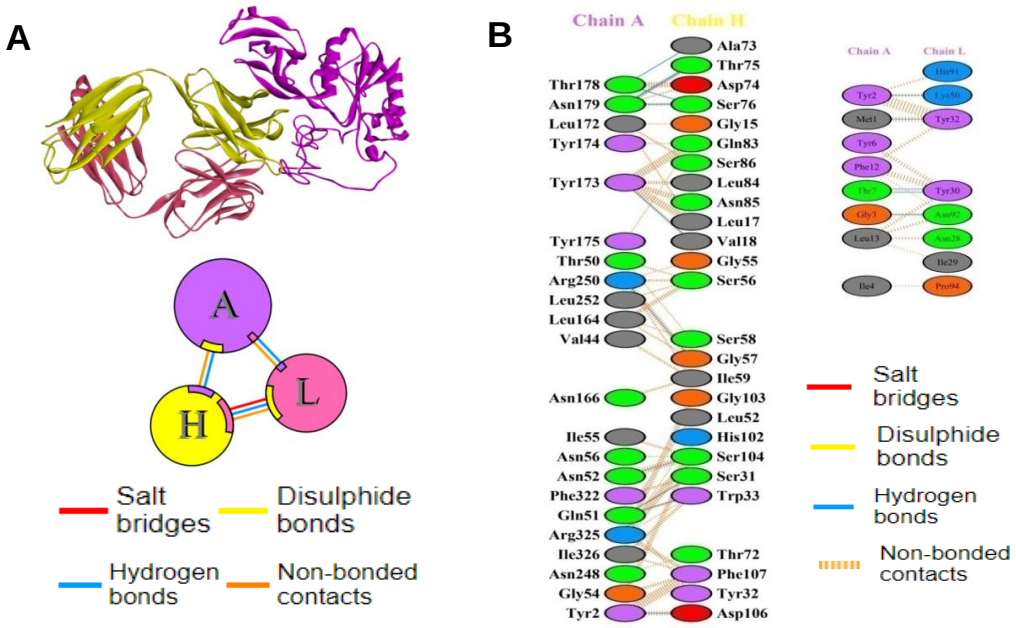
position 100, Glycine (G) substituted by Aspartate (D) and position 145, Asparagine (N) is changed to Asparatate (D). However, all three mutations were found neutral in nature. To visualize the sequence-based predictions, VP7 structures were modeled for both vaccine strain and circulating G9 strain in the I-TASSER web server. RMSD is the most widely used quantitative measure of similarity between two superimposed atomic coordinates of protein structures. The overall difference in the VP7 structures was performed by the superimposition of the VP7 protein structure of the Rotavac strain and circulating G9 strain in Pymol and root mean square deviation (RMSD) was found to be 0.418 Å.

Further, how VP7 interacts with Ab, docking was performed for VP7 structure and Fab (PDB ID: 3FMG) region of IgG. Then, Pubsum was used to visualize different amino acid residues in VP7 and IgG that are likely involved in interaction [15]. As shown in Figure 4.2 & 4.3, both the light and heavy chains of Fab are interacted with VP7 from the vaccine strain and the circulating G9 strain. The VP7 vaccine strain interacts with the heavy chain and light chain through 22 and 8 residues, respectively, resulting in 11 and 5 hydrogen bonds and 190 and 67 non-bonded interactions. Similar to this, the circulating G9 strain's VP7 interacts with the heavy chain and light chain via 14 and 7 residues, respectively, resulting in the formation of 7 and 2 hydrogen bonds as well as 95 and 30 non-bonded interactions.



I87T		G100D		N145D	
Non-polar, Uncharged and Hydrophobic (Isoleucine; I) to polar, uncharged, Hydrophilic (Threonine; T) mutation		Non-polar, neutral, (Glycine; G) to Polar, Negatively charged, Hydrophilic (Aspartate; D) mutation		Polar, Uncharged and Hydrophilic (Asparagine; N) to Polar, Negatively charged and Hydrophilic (Aspartate; D) mutation	
Secondary structure					
Rotavac Vaccine	G9 Manipur	Rotavac Vaccine	G9 Manipur	Rotavac Vaccine	G9 Manipur
Alpha helix	Alpha helix	Alpha helix	Alpha helix	Turns/Coil	Turns/Coil
Solvent accessibility					
Rotavac Vaccine	G9 Manipur	Rotavac Vaccine	G9 Manipur	Rotavac Vaccine	G9 Manipur
Partially exposed (40-49% RSA)	Partially exposed (30-39% RSA)	Exposed (60-69% RSA)	Exposed (60-69% RSA)	Partially exposed (20-29% RSA)	Partially exposed (40-49% RSA)
Mutation analysis by PROVEAN tool					
I->T; Neutral		G->D; Neutral		N->D; Neutral	

Figure 4.1. Mutation effect analysis of three amino acid substitutions by PROVEAN tool. A. Mutation sites, B. Superimpostion image of VP7 protein modelled structure of Rotavac strain and circulating G9 strain. The inset table is mutation effect analysis result; postion 87 where it changes from Isoleucine (I) to Threonine (T), position 100 Ghycine (G) substituted by Aspartate (D) and postion 145 where it changes from Asparagine (N) to Asparatate (D).

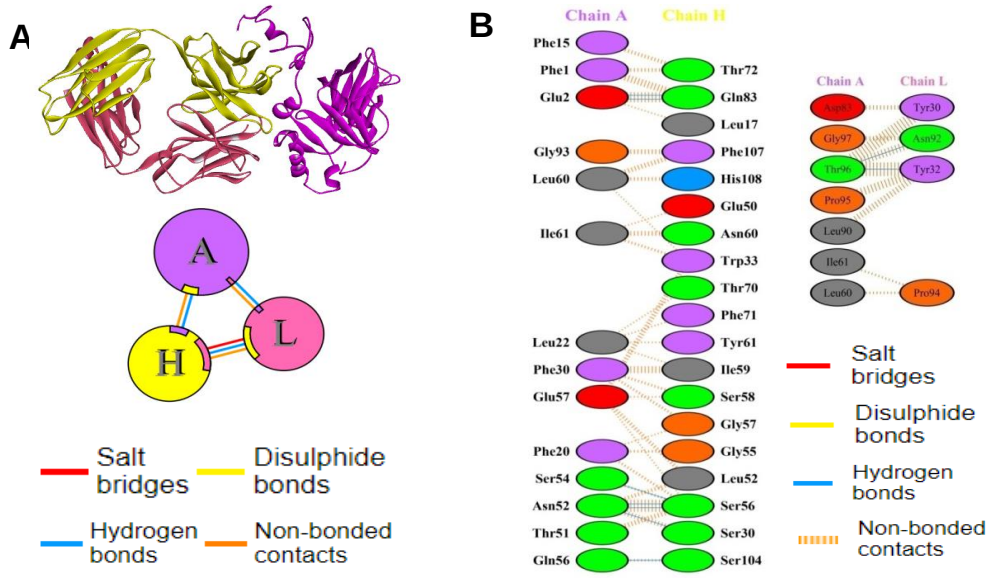


Residue colours

Positive (H,K,R); A,V,L,I,M = aliphatic; P,G = Pro&Gly;
negative (D,E); F,Y,W = aromatic; C = cysteine.

Chains	No. of interface residues	Interface area (Å^2)	No. of salt bridges	No. of disulphide bonds	No. of hydrogen bonds	No. of non-bonded contacts
A H H	22 : 26	1231:1240	-	-	11	190
A H L	8 : 8	388:385	-	-	5	67
H H L	28 : 33	1725:1695	3	-	11	170

Figure 4.2. Schematic diagram of interactions between VP7 protein of Rotavac strain and Fab region of IgG and interacting residues. Interacting chains (A; VP7, H; Heavy chain, L; Light chain) are joined by coloured lines, each representing a different type of interaction, as per the key above. The area of each circle is proportional to the surface area of the corresponding protein chain. The extent of the interface region on each chain is represented by a coloured wedge whose colour corresponds to the colour of the other chain and whose size signifies the interface surface area. Statistics for all the interfaces are given as an inset table. B. Residue interactions across interface coloured by residue type. The number of H-bond lines between any two residues indicates the number of potential hydrogen bonds between them. For non-bonded contacts, which can be plentiful, the width of the striped line is proportional to the number of atomic contacts.



Residue colours

Positive (H,K,R); negative (D,E); A,V,L,I,M = aliphatic; F,Y,W = aromatic; P,G = Pro&Gly; C = cysteine.

Chains	No. of interface residues	Interface area (Å^2)	No. of salt bridges	No. of disulphide bonds	No. of hydrogen bonds	No. of non-bonded contacts
A H	14 : 19	884:881	-	-	7	95
A L	7 : 4	244:272	-	-	2	30
H L	28 : 34	1737:1704	2	-	11	172

Figure 4.3. Schematic diagram of interactions between VP7 protein of circulating G9 strain and Fab region of IgG and interacting residues. Interacting chains (A; VP7, H; Heavy chain, L; Light chain) are joined by coloured lines, each representing a different type of interaction, as per the key above. The area of each circle is proportional to the surface area of the corresponding protein chain. The extent of the interface region on each chain is represented by a coloured wedge whose colour corresponds to the colour of the other chain and whose size signifies the interface surface area. Statistics for all the interfaces are given as an inset table. B. Residue interactions across interface coloured by residue type. The number of H-bond lines between any two residues indicates the number of potential hydrogen bonds between them. For non-bonded contacts, which can be plentiful, the width of the striped line is proportional to the number of atomic contacts.

4.4. Discussion

The effectiveness of the vaccine will depend on the ability to protect against the rotavirus strains circulating in the country. Further, long-term use of vaccine use could induce selective pressure on existing circulating strains, resulting in virus escaping antibody neutralization, increasing the prevalence of unusual genotypes and the emergence of novel strains. According to reports, the introduction of Rotarix in India was associated with a decrease in the homotypic strains of the vaccine [16]. Similar findings were also noted in other nations [17,18]; one of these findings was an increase in the incidence of rare and unusual strains in nations with high RotaTeq coverage [19,20].

In this study, we investigated the intragenotype similarity between the VP7 component of circulating G1, G2, G3 and G9 with VP7 components of Rotarix, Rotateq and Rotavac vaccines. Similarly, VP4 of P[6] and P[8] strains were compared with VP4 of the vaccine strains. Then, the genetic disparities in the antigenic epitope regions of the VP7 and VP4 proteins were compared and identified. Rotarix and RotaTeq were privately available in India since 2006 and the indigenous vaccine Rotavac™ was launched in 2015. In India, the efficacy of the global RVA vaccines was found to be low, and that of the indigenous vaccine was just 56%.

Significant intragenotype differences were found between VP7 and VP4 of circulating RVA strains and vaccine strains. Notably, the G9 strain displayed a large intragenotype difference at nucleotide and amino acid sequence level as compared to the G9 component of Rotavac. Similar to P[8] strain, P[8] component of Rotarix and Rotateq displayed a substantial intragenotype difference, both in nucleotide and amino acid sequence.. All the VP7 and VP4 sequences of circulating strains in this study form different clusters from Rotavac, Rotarix and RotaTeq VP7 and VP4 genes.

We observed up to six amino acid differences between VP7 of Manipur G1 strain, Rotarix, and RotaTeq. While six amino acid differences were found when the VP7 of Manipur G2 strain was compared with the same strain of RotaTeq. In the case of the VP7 of Manipur G3, up to seven amino acid differences were noticed in comparison with the RotaTeq G3 strain. However, relatively few differences were identified between VP7 of the Manipur G9 strain and the Rotavac G9 strain. For VP4, the most amino acid variation was observed in P[6]

strains than P[8]. Both P[8] and P[6] strains clustered, distantly from the P[8] of Rotarix and RotaTeq and P[11] of Rotavac. The Manipur P[6] strain RM38122018 was found distantly related to other P[6] strains in this study and clustered closely with porcine P[6] from China and P[6] strain from Sri Lanka.

G1 is one of the prevalent genotypes pre- [21] and post-vaccine introduction in the study area and northeast [22], eastern [23,24,25,26,27,28] and throughout India [29,30]. The aa substitutions in G1 VP7; N94S, S123N, K291N and M217T identified in this study have also been reported in Manipur strains previously (mani-375/07 and mani-140/06) [24], Kolkata (Kol-75-09, GRAVP731-32), Delhi (Dan279), Pune, etc. [31,26,32]. Substitutions of the circulating strains concerning the Rotateq G1 strain; D97E and S147N is likely indicating that Rotarix may be more effective than the G1 strain of Rotateq in children of Manipur.

Amino acid substitutions were observed in VP7 of the G2 strain of Manipur at A87T, D96N, S213D and S242N when compared to the Rotateq G2 strain. G2 is the most prevalent strains in India before and after vaccine introduction. All these four substitutions were observed in rotavirus G2 strains from the previous study in the same region (mani-384/07, mani-268/07, mani-257/07, mani-4/05) [21], other parts of the northeast, eastern (DIB/11-03-421) and different parts of India and world [33,34]. The G2 strain RM49012019 in this study had an extra mutation, K291N, while the strains from the previous study, mani-268/07 and mani-4/05, had N130D. This suggests that there are differences across G2 strains, which may affect how well the Rotateq G2 vaccine works for children in the study area.

In India, after the Rotavac vaccine introduction in UIP, the most prevalent strain shifted from G1 to G3. This study was conducted before the rotavirus vaccine introduction in UIP; however, the most prevalent strain was observed to be G3 but in the previous study by Mukherjee et al., (2010) G3 was not detected [21]. The substitutions observed were T87I, A212T, K238N and D242N. T87I mutation was possessed by a few strains and also observed in strains from Mangalore (NUROT-1) and Malaysia (L86). Mutations K291N and A146P were also observed in a few strains. Except for T87I, all the three mutations were observed in Lebanon strains [33].

Three amino acid changes were found at positions I87T, G100D, and N145D in the G9 strain used in this investigation, the same genotype as the native Rotavac vaccine. In addition to

these mutations, another N221S was observed in the G9 strains from a previous study; mani-475/08, mani-313/07, mani-230/07, mani-110/06 and mani-97/06 [21]. Further, in the strain mani-97/06, mutations T96I and T212P were also observed. Mutation T212P has been reported in Lebanon [34]. The three prominent G9 mutations are found in G9 strains that are found throughout India and in other countries like Ghana, Italy and Lebanon.

The substitutions observed in P[8] strains in this study are E150D, S190N, N/D196G, S125N, S131R and N135D which were also observed in P[8] strains throughout India and other countries like Rwanda, USA and the Dominican Republic. We observed additional substitutions such as A192S, N193D, N195T, R183G, N113D, and P114Q in the P[8] strains of the earlier investigation by Mukherjee et al. (2010) [21]. In the P[8] strains of the previous study by Mukherjee et al, additional substitutions were observed; A192S, N193D, N195T, R183G, N113D and P114Q.

In previous studies by Ciarlet et al. 1994 and 1997 [35,36], it has been shown that in animal RVA, glycosylation of residue 238 reduced neutralization by hyperimmune sera and mAb. Further, immunogenicity was altered dramatically for several other viruses by glycosylation of viral proteins, including influenza A, human immunodeficiency virus (HIV), and human respiratory syncytial virus (RSV) [37,38,39]. RVA glycoproteins' antigenicity is altered by glycosylation, which increases their resistance to neutralizing mAbs [40]. Additionally, these variations showed a gain or loss of glycosylated residues [40,41]; these modifications were also expected to be connected to the pathogenicity of RVA strains [42].

The VP7 of G1 vaccine strains was predicted to possess two N-linked glycosylation sites at residues 69 and 238. However, it was found conserved in the Manipur G1 strains and additionally sites were observed at residues 40 and 209. Similarly, all Manipur strains maintained three possible N-glycosylation sites present in the RotaTeqR vaccine VP7 component except G2 strain RM74032019 where vaccine mutation N146 is possessed. In the G3 strain, which is the most prevalent genotype detected in the region, a shift in the glycosylation site was observed when compared with the Rotateq G3 strain. Similarly, in the G9 strain shift in the glycosylation site was observed when compared with the Rotavac G9 strain. Thus, variation in glycosylation site was observed between the circulating strains and

the vaccine strains implying the diversity of RVA strains and likely linked to low effectiveness of the global vaccines.

4.5. Conclusions

This study is the first report on the analysis of RVA genetic diversity in the region. The strains in Manipur were similar to rotaviruses circulating in other states. The circulating strains were diverse and possess multiple amino acid substitutions in the antigenic epitope regions that have shown to escape neutralizing mAbs. Continuous surveillance of circulating RVAs is required to monitor the effectiveness of the introduced RVA oral vaccines.

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4.7. Manuscript of the chapter:

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