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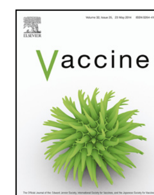

Antigenic and genetic comparison of foot-and-mouth disease virus serotype O Indian vaccine strain, O/IND/R2/75 against currently circulating viruses

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ABSTRACT

Foot-and-mouth disease (FMD) virus serotype O is the most common cause of FMD outbreaks in India and three of the six lineages that have been described are most frequently detected, namely Ind2001, PanAsia and PanAsia 2. We report the full capsid sequence of 21 serotype O viruses isolated from India between 2002 and 2012. All these viruses belong to the Middle East–South Asia (ME–SA) topotype. The serological cross-reactivity of a bovine post-vaccination serum pool raised against the current Indian vaccine strain, O/IND/R2/75,was tested by virus neutralisation test with the 23 Indian field isolates, revealing a good match between the vaccine and the field isolates. The cross reactivity of the O/IND/R2/75 vaccine with 19 field isolates from other countries (mainly from Asia and Africa) revealed a good match to 79% of the viruses indicating that the vaccine strain is broadly cross-reactive and could be used to control FMD in other countries. Comparison of the capsid sequences of the serologically non-matching isolates with the vaccine strain sequence identified substitutions in neutralising antigenic sites 1 and 2, which could explain the observed serological differences.

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1. Introduction

Foot-and-mouth disease (FMD) remains a globally important livestock disease affecting cloven-hoofed animals. It is enzootic in many regions, especially in developing countries where it imposes a trade barrier upon livestock and their products. The causative agent, FMD virus (FMDV) has a rapid mutation rate and exists in seven immunologically distinct serotypes, O, A, C, Asia 1, SAT (Southern African Territories) 1, 2 and 3, each with a spectrum of antigenically distinct strains.

FMDV is a single-stranded, positive-sense RNA virus (Genus *Aphthovirus*, family *Picornaviridae*). The viral genome is about 8.3 kb long, enclosed within a protein capsid. The capsid is composed of 60 copies each of four different structural proteins (VP1–4); VP1–3 are surface exposed while VP4 is internal. Crystallographic studies have identified the structure of the FMDV capsid [1–4] and immunological epitopes have been mostly found on surface-oriented interconnecting loops between structural elements. Studies employing monoclonal antibodies (mAb) have identified antigenic sites by sequencing mAb neutralisation resistant (mar) mutants [5–10]. Of the five antigenic sites reported so far for the most extensively studied serotype O, site-1 (G-H loop) is linear and trypsin-sensitive whereas the others are conformational and trypsin-resistant.

Serotype O virus is present in all continents where FMD is reported, and is antigenically less diverse [11] often exhibiting good cross-protection between strains. India has a large population of FMD-susceptible livestock (approximately 528 million) and FMD is endemic, although starting to be brought under control [12]. In the Indian sub-continent three FMDV serotypes (O, A and Asia1) are currently circulating [13], the majority of the outbreaks (∼80%) being caused by serotype O virus [13–15]. Two serotype O vaccine strains (v/s), O/TNN 24/84 and O/IND/R2/75 were incorporated

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in the vaccine used for regular vaccination purposes in India until October 2003 when the use of O/TNN/24/84 v/s was discontinued [16]. Currently only O/IND/R2/75 is being used for vaccination purposes throughout the country to control serotype O outbreaks. In this study we report the suitability of the O/IND/R2/75 v/s for use in the country and also provide evidence that this vaccine could provide protection against isolates from other countries. In addition, for the first time, we present detailed analysis of the full capsid sequence data of contemporary serotype O Indian isolates.

2. Materials and methods

2.1. Cells and viruses

Twenty-six serotype O viruses from the Indian sub-continent were used in this study (Supplementary Table 1A). One is the current v/s O/IND/R2/75 originally isolated from India in 1975; the other twenty five viruses were isolated over an eleven year period, 2002 to 2012. These samples were derived from bovine epithelial tissues except one each of caprine and porcine origin. In addition, 19 serotype O viruses submitted to the Food and Agriculture Organisation's World Reference Laboratory for FMD (WRLFMD) at the Pirbright Institute were also used in this study (Supplementary table 1B). Two are the v/s O1/BFS and O1/Manisa that were originally isolated in the UK and Turkey in1967and 1969 respectively; the 17 other viruses were isolated over an eleven year period (2001 to 2011). These viruses were from three continents; Asia ($n = 13$), Europe ($n = 2$) and East Africa ($n = 4$) (Supplementary Table 1B). These samples were mostly derived from bovine epithelial tissues except three of porcine origin and one of caprine origin. All the viruses were initially grown in primary bovine thyroid cells with subsequent passage in either BHK-21 or IB-RS2 cells. Stocks of virus were prepared by infecting IB-RS2 cell monolayers and were stored as clarified tissue culture harvest at $-70\,^{\circ}$C until required.

2.2. Polyclonal serum

Antisera were prepared against FMDV O Indian v/s O/IND/R2/75 by immunising five male calves of 12–15 months old with inactivated, purified 146S FMD virus particles in ISA-206 adjuvant (SEPIC, France). Bulk blood was collected on 21 day post-vaccination for preparation of sera. A pool of equal amounts of sera from these five animals was used in the serological tests. The pooled antiserum exhibited a homologous titre of $\log_{10}$ 2.48 by virus neutralisation test (VNT).

2.3. Two-dimensional micro neutralisation assay (2D-VNT)

The 2D-VNT was carried out using the 21-day post-vaccination serum pool following established methodology [17]. Antibody titres were calculated from regression data as the $\log_{10}$ reciprocal antibody dilution required for 50% neutralisation of 100 tissue culture infective units of virus ($\log_{10}SN_{50}/100\ TCID_{50}$). The antigenic relationship of viruses based on their neutralisation by antibodies is given by the ratio: 'r_1' = neutralising antibody titre against the heterologous virus/neutralising antibody titre against the homologous virus. Differences in the r_1-values obtained by the polyclonal antiserum were evaluated according to standard criteria [18]. All the tests were carried out in duplicates and repeated at least twice and the average of the two tests was used in further analysis.

2.4. Nucleotide (nt) sequencing and analysis of the sequence data

The sequences of the entire capsid coding region (P1) of selected Indian type O viruses ($n = 21$) were generated in this study. RNA extraction from the cell culture grown viruses and reverse transcription (RT) were performed as described [19]. PCR was carried out using the "KOD hot-start DNA polymerase" kit (Novagen) as recommended by the manufacturer, using the forward primer L463F (5′-ACCTCCRACGGGTGGTACGC-3′) and one of the reverse primers NK72 (5′-GAAGGGCCCAGGGTTGGACTC-3′) or EUR2B52R (5′-GACATGTCCTCCTGCATCTGGTTGAT-3′). PCR products were purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions and sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) using the PCR primers and additional internal sequencing primers (sequences available on request). Sequences (from the ABI 3730 machine) were assembled and analysed using SeqMan II (DNAStar Lasergene 8.0). Nucleotide sequences of the viruses were aligned using the CLUSTAL X multiple sequence alignment programme [20] and the predicted aa sequences were translated using BioEdit 7.0.1 [21]. In addition to the 21 sequences generated in this study, another five sequences (Supplementary Table 1A) available in Gene Bank were also used in the analysis. Out of these five capsid sequences O/TNN/24/84, which was one of the old Indian serotype O v/s contained the sequence of only VP1–3 (Acess. no. JQ818555). Therefore sequences of VP1–3 of all the isolates used in this study were aligned and used in all subsequent analysis in this study. Alignments were used to construct distance matrices using the Kimura 2-parameter nucleotide substitution model [22] as implemented in the programme MEGA 5.1 [23].

2.5. Bayesian phylogenetic analysis

The VP1–3 sequences of the viruses belonging to the Indian serotype O strain ($n = 26$) were aligned and subjected to jModelTest 0.1.1 [24]. The general time reversible (GTR) model for nucleotide substitution was used with a combination of gamma distribution and proportion of invariant sites (GTR + I + G) as this was found to be the best model for the Bayesian analysis of the sequence dataset. Analysis was performed using the BEAST software package v 1.5.4 [25] with the maximum clade credibility (MCC) phylogenetic tree inferred from the Bayesian Markov Chain Monte Carlo (MCMC) method. The age of the viruses were defined by the date of sample collection. In BEAUti v1.5.4, the analysis utilised the GTR + I + G model to describe rate heterogeneity among sites. In order to accommodate variation in substitution rate among branches, a random local clock model was chosen for this analysis [26]. BEAST output was viewed with TRACER 1.5 and evolutionary trees were generated in the FigTree program v1.3.1.

2.6. Data analysis

The proportion of synonymous substitutions per potential synonymous site and the proportion of non-synonymous substitutions per potential non-synonymous site were calculated by the method of [27] using the SNAP programme (www.hiv.lanl.gov). The amino acid (aa) variability of the capsid region of the serotype O viruses was determined as described by [28]. Statistical analyses used Minitab release 12.21 software.

3. Results and discussion

The population of FMD-susceptible livestock in India is very large. The Government of India is undertaking an FMD control programme (FMDCP) and in their last (10th) five-year plan covered 54 districts in several states [12,29,30]. There were 16 rounds of vaccination in these 54 districts using an inactivated trivalent vaccine containing strains of serotype O, A and Asia 1. Even though the programme was successful in reducing the incidence of FMD, the disease still occurred due to movement of animals and the existence

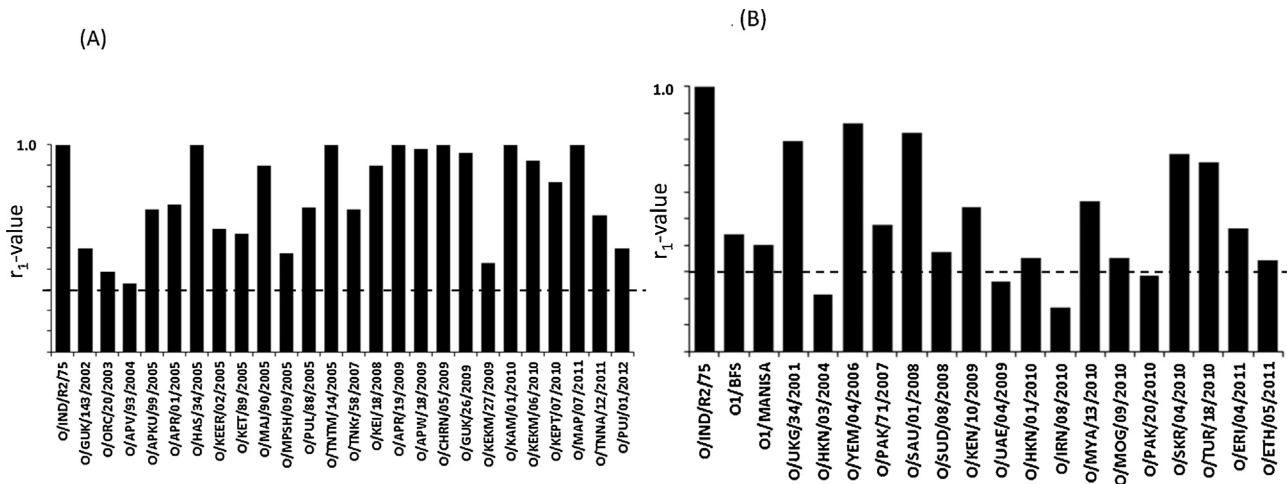

Fig. 1. Antigenic relationship (r_1) values of serotype O Indian isolates (A) and isolates from other countries (B) against O/IND R2/75 vaccine antisera. The horizontal dotted line indicates the cut-off value of 0.3, above which the vaccine is considered to be a good match.

of areas not covered under the control programme. In the 11th plan (started Oct 2010), an additional 167 districts were included and currently a total of 221 districts are targeted involving about 38% of the total cattle, buffalo and pig population in the country. In the next five-year plan (starting in 2015) the control programme is likely to include the entire country. The effectiveness of the control programme relies heavily on an efficacious and safe vaccine, which in the case of FMD requires that the vaccine strain can protect against the outbreak strains. Out of the three serotypes (O, A and Asia 1) prevalent in India, serotype O causes most of the outbreaks. Currently, three different lineages of serotype O viruses, namely Ind2001, PanAsia and Ind2011 are circulating in India [30].

3.1. Serological characterisation of the Indian serotype O viruses with O/IND/R2/75 sera

The cross-reactivity of the Indian serotype O viruses ($n = 23$) was measured by 2D-VNT using a pool of O/IND/R2/75 post-vaccination bovine sera. All the viruses exhibited strong cross-reactivity with the antisera indicating that the current v/s is a good match for the currently circulating field isolates (Fig. 1A). This indicates that the current Indian serotype O vaccine strain has a broad serological spectrum similar to an earlier report [16]. However two isolates, O/ORC/20/2003 and O/APV/93/2004 exhibited an r_1-value less than 0.4 (0.33 and 0.38, respectively). Therefore continuous monitoring of the outbreak strains and regular vaccine matching studies is essential to evaluate the suitability of the vaccine for use in the control programme in the country unless poor match can be compensated for by high antigen payload vaccines [31].

3.2. Serological cross-reaction between recent field isolates from other countries and the Indian type O vaccine strain

The cross-reactivity of some recent serotype O viruses from other countries was measured by 2D-VNT using the O/IND/R2/75 post-vaccination serum pool. About 79% of the viruses (15/19) reacted well with the antisera including viruses representing the MYA-98 strain (SEA topotype) and the EA-2 and EA-3 strains (AFRICA topotype). This indicates that the current Indian v/s is broadly cross reactive and might provide protection if used as a vaccine to control outbreaks in other countries (Fig. 1B). Four isolates, O/HKN/03/2004, O/UAE/04/2009, O/IRN/08/2010 and O/PAK/20/2010 exhibited an r_1-value lower than 0.3. O/HKN/03/2004 belongs to the Cathay topotype with very distinct host specificity, mainly affecting pigs [32]. O/PAK/20/2010 (PUN-10 sub-lineage) and O/IRN/08/2010 (FAR-09 sub-lineage) belong to the PanAsia-2 lineage within ME-SA topotype. It was surprising that O/UAE/04/2009 belonging to the Ind2001 lineage within ME-SA topotype was not a good match whilst the Indian serotype O isolates from the same lineage exhibited high r_1-values. This virus has a three nucleotide deletion in the G–H loop region at position VP1-139, similar to the report of Das and colleagues [13].

3.3. Genetic characterisation of the serotype O viruses

3.3.1. Full capsid sequence analysis

The full capsid sequences of the selected Indian viruses ($n = 21$) were generated in this study and the viral capsid sequences were assembled using DNASTAR Lasergene 10 EN. All the sequences were 2202 nt long except O/TNKr/58/2007 which had a 3-nt deletion at position 1978–1980 of P1 leading to the deletion of an aa at position 137 of the VP1 in the G–H loop which has been reported to be a dominant antigenic site (Site 1). Interestingly, an amino acid deletion at VP1-139 position has been reported previously in outbreak viruses belonging to the same lineage (Ind2001) from Gujarat state isolated in 2009 [13]. Compared to the oldest virus O5/IND/1962, the variation at nt level was 8.2% (O/GUK/143/2002) to 11.2% (O/TNN/24/84) and 2% (O/GUK/143/2002) to 5.2% (O/IND/75Madras) at aa level. Similarly, compared to the vaccine strain O/IND/R2/75, the variation was 3.7% (O/IND/75Madras) to 12.12% (O/KEKM/06/2009) at nt level and 3.7% (O/GUK/143/2002 and O/MPSH/09/2005) to 5.5% (O/IND/75Madras) at aa level. The sequence dataset was analysed by the Z-test for evidence of evolutionary selection using MEGA 5.1, but this was not found ($P > 0.1$) which probably explains why the vaccine still works on the recent isolates.

3.3.2. Phylogenetic analysis

Phylogenetic analysis of the capsid sequences revealed that all of the Indian serotype O viruses belong to the ME-SA topotype. The Indian viruses isolated from 2002 onwards clustered together, whereas the other virus strains did not (Fig. 2). A similar pattern was observed when phylogenetic trees were drawn using only VP1 sequences (data not shown).

3.3.3. Rate of nucleotide substitution per site

From the BEAST analysis, using a random local molecular clock, the rate of substitution of all the nt changes in the capsid coding region of the serotype O viruses from India was estimated to

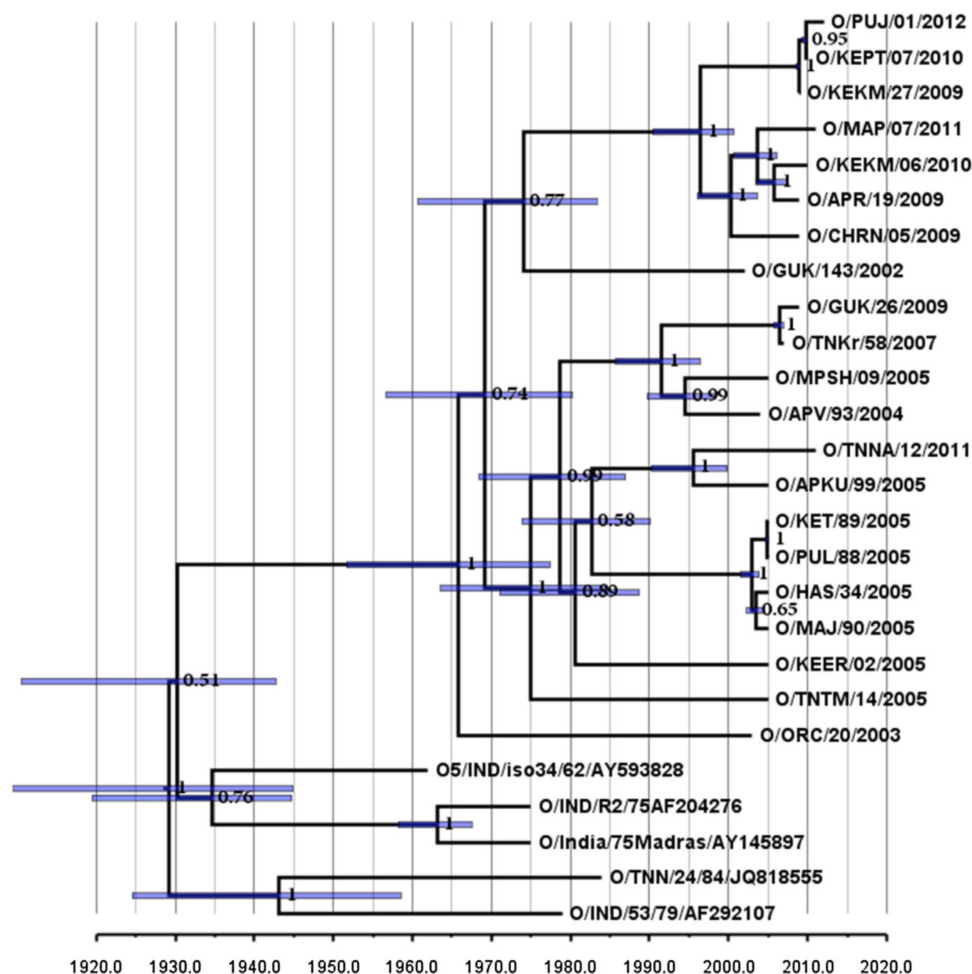

Fig. 2. Bayesian phylogenetic tree of the Indian serotype O viruses. The capsid sequences ($n = 5$) retrieved from Genbank are indicated by their respective accession numbers.

be 1.78×10^{-3}/site/year (95% HPD 1.23×10^{-3} to 2.41×10^{-3}). This is similar to the report of Hemadri and colleagues [14] for Indian serotype O FMD viruses and also by others in case of serotype O FMD viruses [33–36], however, it is lower than that of Indian serotype O viruses of the Ind2001 lineage reported by Subramaniam and colleagues [30]. The common ancestor of these viruses could have existed about 83.9 years ago, i.e. 1928 (95% HPD 67.1 to 102.48 years).

3.3.4. Serotype O Indian viruses

An unbiased analysis of the capsid sequences (VP1–3) of the 26 Indian serotype O viruses revealed 786 nt substitutions at 636 sites distributed across the region (Fig. 3A). Out of these, 80.05% of nt substitutions were found to be synonymous (silent) and 19.95% were non-synonymous (non-silent). Twenty three sites were identified to have been substituted three times and 104 sites were substituted twice. At eight sites, all the three bases of the codon were mutated encoding 3–6 different aa (Table 1A). Out of the eight sites with three nt substitutions (encoding 3–6 aa residues), two were present in VP2 and six in VP1 (Table 1A).

The analysis of the capsid aa residues of O Indian viruses revealed 157 substitutions at 104 sites across the capsid (Fig. 3A) with some sites having 2–7 alternative aa (Table 1A). Interestingly, sequences for VP2-133 and VP1-138 encoded seven different aa and exhibited nt changes at all the three positions within the codon as did VP1-140 and VP1-133,with changes at all the three positions of the codon giving rise to six and five alternative aa, respectively. The non-synonymous nt substitutions were not equally distributed across the capsid coding regions: there were several local areas where the d*N*/d*S* ratio was higher than in other parts of the sequence alignment (Fig. 3B). Two regions in VP3 (53 and 69–73), one region in VP2 (131–133) and four regions in VP1 (127–140, 171–174, 194–197 and 208–209) had d*N*/d*S* ratio of >0.7, indicative of sites under positive selection. Out of these regions the residues in

Table 1A
Capsid positions where multiple amino acid substitutions were observed. The changes are shown in parenthesis.

Viral protein	Positions with three amino acid substitutions	Positions with four amino acid substitutions	Positions with five amino acid substitutions	Positions with six amino acid substitutions
VP2	39 (A -> S/V/T)	–	–	133 (Q -> D/G/S/H/N/T)
VP1	96 (T -> N, A, K), 137 (A -> G/S/V), 144 (V -> I/K/L)	133 (N -> D/G/S/A)	140 (P -> D/G/S/A/T)	138 (D -> E/R/A/K/N/G)

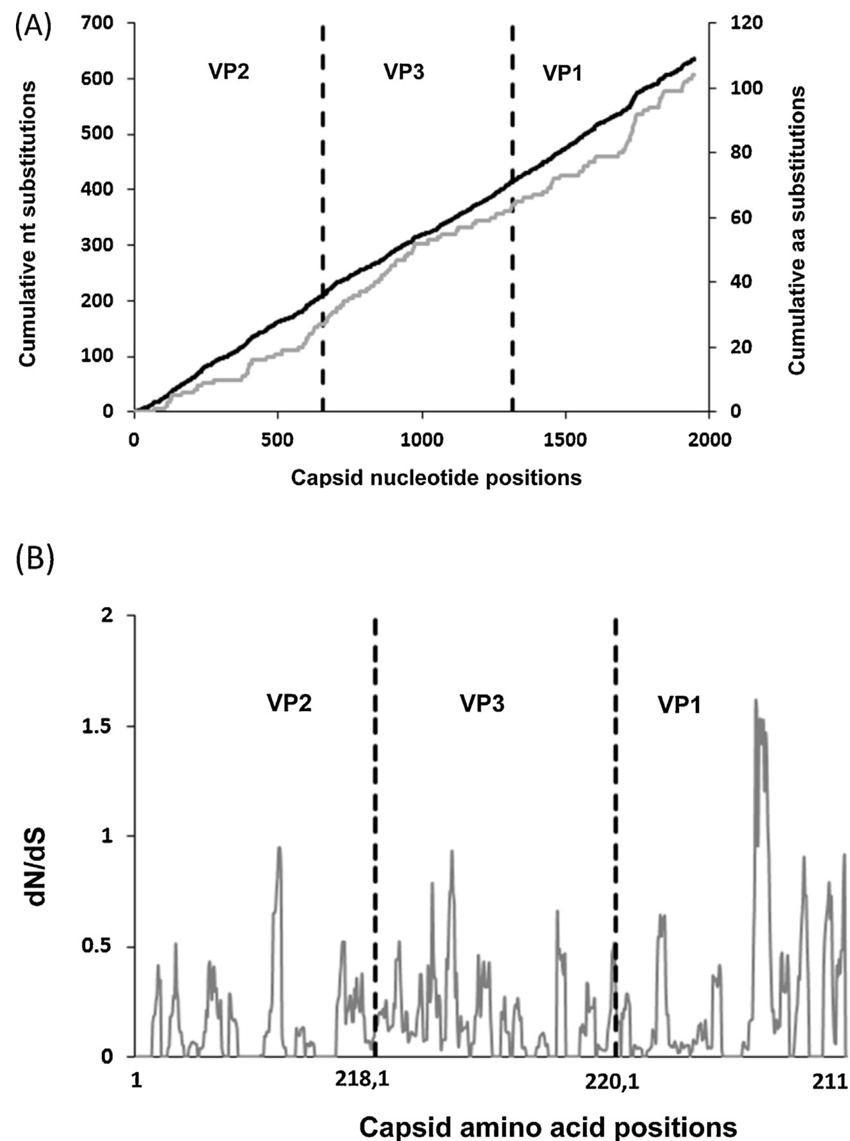

Fig. 3. (A) Cumulative changes in capsid coding regions of serotype O Indian isolates. The black line indicates total cumulative nucleotide changes, the grey line—cumulative amino acid changes. (B) d*N*/d*S* of capsid of serotype O Indian isolates. The vertical black dotted line indicates gene junctions.

VP1 127–140 had the highest d*N*/d*S* ratio (>1.5) indicating strongest selection pressure in case of serotype O viruses.

3.3.5. Amino acid variability of the capsid of the Indian serotype O viruses

Investigation of aa variability across the capsid of the Indian serotype O viruses revealed VP4 to be highly conserved (data not shown) and VP1 least conserved (Fig. 4A); similar to an earlier report of serotype A viruses [37]. The residues with a variability score greater than 0.3 (one each in VP2 and VP3, and 6 in VP1) are shown in Fig. 4B–C indicating that 75% of the residues with high variability scores were present in VP1 (Fig. 4A). All these residues were found to be surface-exposed (Fig. 4C).

3.4. Correlating genotype to antigenic phenotype

In an attempt to correlate genotype to antigenic phenotype, the capsid aa sequences of the serotype O viruses employed in this study were further analysed. A number of amino acid changes were observed between the v/s and the field isolates. However there was no linear correlation between the number of aa substitutions and the loss of serological cross-reactivity (data not shown) as the isolates displaying an equal no. of aa differences exhibited significantly different serological cross-reactivity indicating a large proportion of the substitutions are neutral and only a few located at particular position of the capsid have an impact on the antigenic phenotype of the virus [37–39].

3.5. Comparison between O/IND/R2/75 v/s and the serologically non-matching field isolates

In-vitro testing of four serotype O viruses (O/HKN/03/2004, O/UAE/04/2009, O/IRN/08/2010 and O/PAK/20/2010) with O/IND/R2/75 antisera generated r_1-values lower than 0.3 indicating lower expectation of protection efficiency. In addition two of the Indian serotype O viruses, namely O/ORC/20/2003 and O/APV/93/2004 also exhibited comparatively lower cross-reactivity with the O/IND/R2/75 antisera ($r1$-values <0.4). The capsid aa sequences of these six viruses were aligned with the v/s sequence and analysed further to investigate the molecular basis of the lower cross-reactivity of these viruses with O/IND/R2/75 vaccine strain. As most of these viruses (six isolates) exhibited

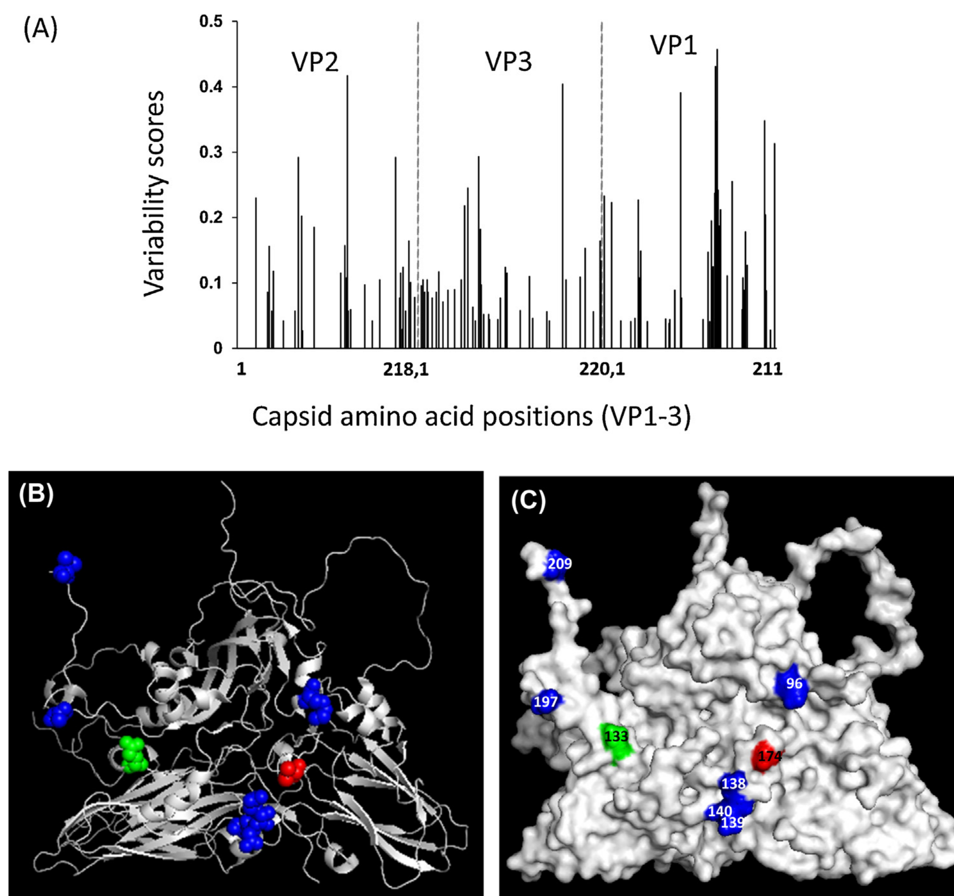

Fig. 4. (A) Capsid amino acid variability of serotype O Indian isolates. The vertical black dotted line indicates gene junctions. (B–C) 3-D structure of O BFS reduced protomer (1FOD, reduced) with highly variable capsid amino acid residues (with a score of more than 0.3) highlighted.VP1 residues—blue, VP3—red, VP2—green; (B) cartoon, (C) external surface. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

lower cross-reactivity with the antisera of the v/s, we specifically looked for amino acid residues in the field isolates which were different from the v/s. A total of 16 capsid amino acid residues; five in VP2, six in VP3 and five in VP1 (Table 1B, Fig. 5) were identified which may have an impact on the antigenic nature of the viruses. Three of these residues were eliminated as being completely buried (VP2-154, VP3-86 and VP1-123) in the structure. In addition, VP1-82 which is located close to the five-fold axis is less accessible to antibody binding and was therefore excluded. The remaining 12 residues were surface exposed (Fig. 5B) and are therefore good candidates to explain the inability of the antisera to cross-react with the field isolates. Three of the residues; VP2-70 and 134 involving antigenic site 2 and VP1-144 involving antigenic site 1 have been reported to be critical in serotype O mar-mutant studies [7,40]. Neutralising antigenic site 2 has been reported to be immunodominant in the polyclonal response of serotype O FMD-vaccinated animals followed by site 1 [41]. VP2-134 has also been reported to strongly influence the binding of neutralising antigenic site 2 mAbs in serotype O FMDV [19]. In addition VP2-74 and 191 have been recently shown to be linked to antigenic site 2 using a reverse genetics approach [39]. The S–P substitution at position 74 of VP2 could be significant as proline is known to be a helix-breaker. Though never reported to be a part of an epitope in any serotypes of FMDV, the residue VP3-219 and 220 are in close vicinity to residue VP3-218, which has been recently reported to be critical in serotype Asia 1 [10]. In addition, VP3-220 had been indicated to have an impact on the antigenic nature of serotype A FMD viruses [37]. Three residues in VP3 (96, 219 and 220) and one residue in VP1 (96) are located next to each other (Fig. 5B) and also in close proximity to the C-terminus of VP1 of an adjacent protomer; therefore potentially could form or be part of an epitope. A change in these residues may affect the overall conformation of the viral capsid and thereby alter the antigenicity of the virus.

Table 1B
Amino acid changes between O/IND/R2/75 v/s and serologically non-matching field isolates. Numbers where shown in parenthesis indicates no. of isolates with the substitution over total no. of isolates. At all othere positions the changes were observed in all the six serologically non-matching isolates.

VP2		VP3		VP1	
Position	Change	Position	Change	Position	Change
70	A–V/G	56	R–H	82	Y–H
74	S–P (4/6)	60	G–D	96	N–T
134	N–E/K	86	I–M	123	Q–H
154	V–M	96	H–Q	139	G–S/T
191	T–N/S (5/6)	219	R–T	144	I–V
		220	D–Q		

In summary, the current Indian serotype O vaccine strain O/IND/R2/75 is a good match with the circulating field isolates in India and with the majority of the field isolates studied from other, mainly Asian countries (except Cathay topotype viruses) and East Africa. This indicates that O/IND/R2/75 is broadly cross-reactive and, therefore could be used as a vaccine to control the disease in other endemic countries. However, comparatively lower cross-reactivity of the O/IND/R2/75 BVS with two of the Indian serotype O isolates and an isolate from United Arab Emirates that belong to Ind2001 lineage indicates that the antigenic phenotype

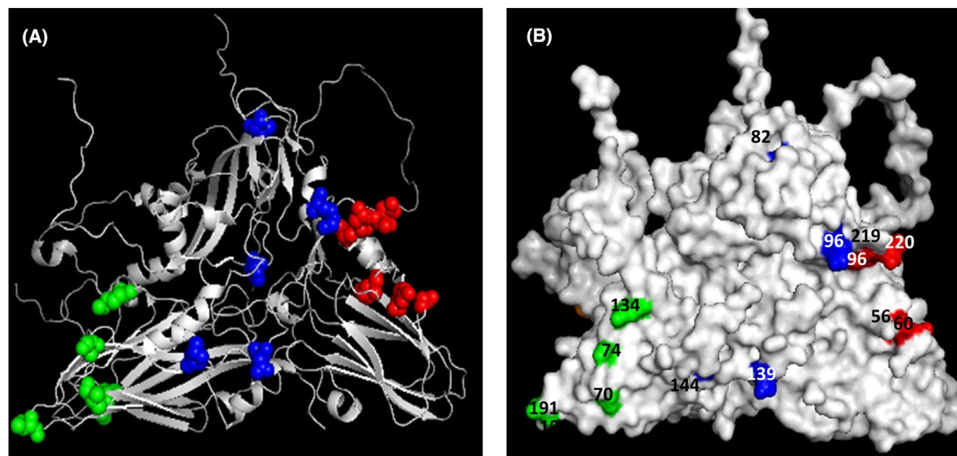

Fig. 5. 3-D structure of O1 BFS co-ordinates (1FOD, reduced) showing amino acid substitutions in serologically non-matching isolates.VP1 residues—blue, VP3—red, VP2—green; (A) cartoon, (B) external surface. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of the circulating viruses may change in the future. In the Middle East, O Manisa has been used as a v/s for over four decades; however recently-circulating viruses were not covered by the vaccine, requiring the development of O Pan Asia-2 vaccines by vaccine manufacturers. Therefore, close monitoring of the outbreak strains in the Indian sub-continent along with regular vaccine matching studies is crucial to evaluate the suitability of vaccine strains for use in FMD control programmes. The need to develop a new v/s should also be identified in a timely fashion to prevent future outbreaks. Comparisons of capsid sequences of the non-matching isolates with the vaccine strain sequence identified substitutions involving neutralising antigenic sites 1 and 2 which either individually or together underpin the observed antigenic phenotypes. Targeted mutagenesis studies involving a cDNA clone could confirm these findings.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.11.058.

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