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Spectrum of VP1 region genetic variants in the foot-and-mouth disease virus serotype O populations derived from infected cattle tongue epithelium

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Summary. – RNA virus population exists as a complex distribution of non-identical but closely related sequences known as viral quasispecies. Variant strains are selected from this quasispecies population in response to changing environment. The quasispecies dynamics of a virus existing within an infected host differs from that in a cell culture-adapted population. This study was carried out to explore the genetic variations present in the VP1 coding region of the foot-and-mouth disease (FMD) virus serotype O derived directly from infected cattle tongue epithelium. Molecular clonal populations of two serotype O strains belonging to lineages Ind2001 (IND 30/2011) and PanAsia2 (IND 5/2011) were sequenced at VP1 coding region. For IND 30/2011, 19 clones were sequenced and analysis showed variations at 12 nucleotide positions (nt) resulting in 8 amino acid (aa) replacements. Similarly, for IND 5/2011 virus, 18 clones were sequenced, of which six showed nt variations leading to 3 aa replacements. Most of the variable positions mapped to the surface-exposed loops and some of them were found in the neutralizing antigenic sites (position 81, 149, 169, 186 and 202 of IND 30/2011 and 141 of IND 5/2011), which potentially could be beneficial in rapid adaptive evolution of the virus by giving rise to antigenic variants to overcome neutralizing antibodies. These findings encourage further research into the landscape of the viral quasispecies population *in vivo* and its implication for viral ecology.

Keywords: foot-and-mouth disease virus; VP1 region; variants in vivo

Introduction

Foot-and-mouth disease (FMD), primarily a disease of cattle, buffalo and pigs, is one of the most feared animal diseases owing to nearly 100% morbidity, rapid spread, severe decrease in livestock productivity, mortality in young animals and obstacles to the international trade in livestock and livestock products (Rowlands, 2003). The disease is caused by FMD virus, a prototype member of the genus *Aphthovirus* in the family *Picornaviridae* (Racaniello, 2001). The virus exists as seven antigenically and genetically distinct serotypes [(O, A, C, Asia1 and Southern African Territories (SAT)-1, 2, 3)]. Each serotype contains numerous lineages and strains showing variable degree of antigenic diversity (Domingo *et al.*, 2003). The virus genome is a single stranded positive-sense RNA molecule of ~ 8.5kbp size that encodes a single poplyprotein, which is protelytically cleaved into four structural and eight nonstructural proteins. Among the four structural proteins, the hypervariable VP1 protein is located around the icosahedral 5-fold axes and it contributes the most to the accessible surface of the virus. It houses integrin receptor-binding motifs and neutralizing antigenic sites, thus evolves under immune pressure (Kitson *et al.,* 1990; Mateu, 1995; Domingo *et al.,* 2003; Jackson *et al.,* 2003). Therefore, sequence analysis of VP1 region has been widely applied in epidemiological investigations to characterize field strains of FMD viruses. Due to the absence of proofreading activity of viral RNA-dependent RNA polymerase, the progeny virus population is expected to consist of numerous closely related

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Abbreviations: FMD = foot-and-mouth disease

mutant genomes existing in equilibrium around a theoretical consensus sequence. This complex and dynamic distribution of non-identical but related sequences is termed as viral quasispecies (Domingo *et al.,* 1978; Eigen and Schuster, 1979; Holland *et al.,* 1992). From this quasispecies population, antigenically divergent strains could be selected either in the presence or absence of immune pressure (Borrego *et al.,* 1993; Domingo *et al.,* 1993; Holguin *et al.,* 1997; Sarangi *et al.,* 2013), which might compromise vaccine efficacy (Feigelstock *et al.,* 1996).

The genetic variations in FMD virus have been extensively investigated in the cell culture-derived biological clonal populations giving emphasis on frequency of mutation, rate of evolution of capsid sequence and antigenic diversification with or without immune pressure (Borrego *et al.,* 1993; Domingo *et al.,* 1993; Holguin *et al.,* 1997; Arias *et al.,* 2001; Airaksinen *et al.,* 2003). The genetic composition of the quasispecies swarm generated in cell culture system is thought to be different from that of the virus replicating inside a host as the selection pressures and constraints shaping the course of evolution are different and many of the minor variants *in vivo* could be lost during propagation *in vitro* (Baranowski *et al.,* 2003; Domingo *et al.,* 2006; Carrillo *et al.,* 2007). Moreover, during propagation in cell culture additional mutations, which were nonexistent in the *in vivo* population, could arise. Therefore, the present study was undertaken to identify the variant genomes existing *in vivo* by molecular cloning and sequencing of the VP1 coding region amplified directly from infected cattle tongue epithelium.

Materials and Methods

Viruses. In this study, two serotype O viruses (IND 5/2011 and IND 30/2011) belonging to PanAsia-2 and Ind2001 lineage, respectively, were used in the form of clinical tongue epithelial samples derived from infected cattle in FMD outbreaks during the year 2011.

Determination of lineage and consensus sequence. For lineage determination, the RNA was extracted from tongue epithelium suspensions using RNeasy Mini Kit (QIAGEN). The reverse transcription of the viral RNA was carried out using Superscript™ III RNAse H reverse transcriptase (Invitrogen) employing NK61 reverse primer (complementary to position 3630–3649 of 2B region). The full-length VP1 coding region was amplified by PCR with NK61-ARS4 primers (1301 base pair) (ARS4 primer is complementary to nucleotides 2349–2369 of VP3 region) using Platinum Taq High Fidelity enzyme (Invitrogen) to minimize mis-incorporations (Knowles and Samuel, 1995). The consensus sequences for both viruses were determined from the purified PCR product directly on ABI 3130 genetic analyzer (Applied Biosystems) using NK61 and ARS4 primers (Knowles and Samuel, 1995).

Analysis of quasispecies composition. To study the quasispecies composition at VP1 coding region, the PCR amplicons were ligated to pGEM-T-Easy vector (Promega). The competent *Escherichia coli* JM109 cells (Promega) were transformed with the ligation mixture and plated on Luria Bertani-ampicillin agar plates. The recombinant colonies were screened by insert specific PCR and restriction enzyme digestion with *Eco*RI (Promega). 19 positive clones of IND 30/2011 and 18 clones of IND 5/2011 virus were selected and plasmids were extracted. The recombinant plasmids were sequenced on ABI 3130 genetic analyzer (Applied Biosystems). Nucleotide sequences were processed using ABI sequence analysis v5.3.1 software, and contigs were assembled using EditSeq programme of Lasergene core suite 10 (DNASTAR, Inc., USA). The VP1 region sequences (639 nucleotides) were aligned using clustal W algorithm (Thompson *et al.,* 1994) available in MEGA 5.05 program (Tamura *et al.,* 2011). In order to rule out the incorporation of error during sequencing step, the clones showing amino acid (aa) difference from the consensus sequence generated directly from PCR amplicons were re-sequenced.

Results

To study the within-host genetic variation at VP1 coding region of serotype O virus, molecular cloning technique was used. For IND 30/2011 (Ind2001 lineage) virus, 19 positive plasmid clones were sequenced and analysis of the results showed nucleotide variations at 12 different positions as compared to the consensus sequence derived directly from the PCR amplicon. When the deduced aa sequences were aligned, 8 variations were found. A total of 11 clones showed sequence exactly identical to the consensus sequence (derived from PCR products before cloning), whereas 5 clones showed one nucleotide substitution and 3 clones showed two nucleotide substitutions (Table 1). However, none of the nucleotide variations occurred in more than one clone. Nucleotide variations were observed at positions 33, 76, 166, 241, 369, 394, 446, 507, 543, 556, 606 and 636, resulting in aa variation at positions 26, 56, 81, 132, 149, 169, 186 and 202 (Table 1). Similarly, for IND 5/ 2011 (PanAsia lineage) virus, 18 clones were sequenced. 13 clones were found to be identical with the consensus sequence, while 5 clones showed one nucleotide difference and one clone showed two nucleotide differences. Out of the 6 nucleotide changes, 3 were non-synonymous mutations resulting in aa substitutions. Nucleotide substitution was observed at positions 157, 328, 342, 345, 421 and 450, and aa variations were seen at positions 53, 110 and 141. Most of the aa variations mapped to the surface-exposed loops and some of them were found in the neutralizing antigenic sites (Table 1).

Table 1. Nucleotide and amino acid variations observed in the VP1 coding region of different molecular clones of serotype O FMD virus compared to the majority sequence derived directly from PCR amplicon generated from tongue epithelium

Virus	Clone No.	Nucleotide position	Nucleotide change	Amino acid position	Amino acid change	Structural element
IND 30/2011	1	33	G→A	–	–	–
	9	76	A→G	26	R→G	N-terminus
	17	166	A→G	56	T→A	βC-βD loop
	19	241	A→G	**81**	**K→E**	βD strand
	5	369	C→T	–	–	–
	7	394	G→A	132	G→R	βG-βH loop
	4	446	A→T	**149**	**Q→L**	βG-βH loop
	4	507	G→C	**169**	**K→N**	βH-βI loop
	5	543	A→G	–	–	–
	19	556	T→C	**186**	**Y→H**	C-terminus
	13	606	A→C	**202**	**K→N**	C-terminus
	13	636	C→T	–	–	–
IND 05/2011	1	157	C→A	53	L→M	βC-βD loop
	2	328	G→A	110	A→T	βF-βG loop
	1	342	A→G	–	–	–
	5	345	T→A	–	–	–
	16	421	G→T	**141**	**V→L**	βG-βH loop
	10	450	G→T	–	–	–

Antigenically critical residues are shown in bold face.

Discussion

Natural populations of FMD virus from a single outbreak have been shown to be heterogeneous both genetically and antigenically and, moreover, an isolate obtained from an individual animal comprises more than one nucleotide sequence (Wright *et al.,* 2011; Morelli *et al.,* 2013). High mutation rate of FMD virus led to the proposal that FMD virus natural populations do exist as quasispecies (Domingo *et al.,* 1992). FMD virus strains are normally genetically described by a single sequence that represents the consensus of what is thought to be an underlying swarm of closely related genetic variants. Nevertheless, the consensus could change with time and space depending on the frequency of distribution of the variants in the quasispecies cloud. Conventional sequencing has provided insights into the genetic variation within population (Carrillo *et al.,* 2007). This is achieved through sequence analysis of either the biological clones (virus from individual plaques formed on the cell monolayer) or molecular clones (PCR products cloned in *E. coli*). Recently, with the advance of next generation sequencing (deep sequencing), the polymorphic structures of viral populations have been dissected with greater resolution. The technique has been used to compare viral population within two bovine epithelial samples from a single animal, to study intra-sample sequence diversity during serial transmission in bovine hosts and to detect site-specific polymorphism in the virus population generated after *in vitro* passages under antibody pressure (Wright *et al.,* 2011; Morelli *et al.,* 2013; Pandey *et al.,* 2014).

Characterization of the mutant spectra of virus quasispecies is finding increasing application in the understanding of virus pathogenesis and evolution of viruses (Forns *et al.,* 1999; Arias *et al.,* 2001; Domingo *et al.,* 2001). Arias *et al.* (2001) studied the mutant spectrum of FMD virus by nucleotide sequencing of three regions (internal ribosomal entry site, region between 2 AUG initiation codon in L and VP1 coding region) from 70 biological and 70 molecular clones. The results obtained by both methods were statistically indistinguishable with regard to the distribution of mutations among the three genomic regions analysed, type of mutation observed, mutational hot spot and mutation frequencies. Subsequently, molecular cloning technique has been used to study viral quasispecies of Asia 1 virus (Li *et al.,* 2011) and serotype O virus (Cottam *et al.,* 2009) and for many human viruses, e.g. hepatitis viruses, caliciviruses, enteric coronaviruses and papillomaviruses, which either do not grow in cell culture or grow poorly (Esteban *et al.,* 1999; Forns *et al.,* 1999; Flint *et al.,* 2000; Domingo *et al.,* 2001).

In this study, representative serotype O strains belonging to two predominant lineages circulating in India (Ind2001 and PanAsia) were analyzed in order to decipher the mutant distribution in the infected host-derived population of virus (Subramaniam *et al.,* 2013). Although it is possible that vari-

ations are present in the entire genome in the quasispecies population, only those variants that differ from the consensus in the capsid coding region could be considered important in the context of selection and emergence of antigenically divergent strains under immune pressure. Since VP1 protein is the most exposed part of the capsid surface, contains immunodominant antigenic sites and can tolerate extensive aa replacements, this preliminary investigation reports variations observed in the VP1 coding region (Domingo *et al.,* 1990; Mateu, 1995; Martin *et al.,* 1998).

Nucleotide sequencing of 19 positive clones of IND 30/2011 showed nucleotide variation at 12 different positions, which in turn resulted in 8 aa variations. Similarly for IND 05/2011 sequence, of 18 positive clones showed 6 nucleotide variations resulting in changes at 3 aa positions (Table 1). None of the nucleotide variations happened to be present in more than one clone, suggesting site-specific polymorphism to exist at a very low frequency in the population. Furthermore, none of the polymorphic loci was found to be shared between the two strains, thereby implying existence of a signature demographic structure for each strain of the virus. A total of only 3 transversions out of 12 substitutions were seen in case of IND 30/2011 clones, while a relatively higher proportion of transversions (4 out of 6 substitutions) were observed for IND 05/2011. Therefore, no specific bias in favor of either transitions or transversions in the population of variants could be proved. Most of the aa variations mapped to the exposed loops on the capsid surface, which are considered to be less constrained structural elements (Table 1). Notably, three of the 11 aa variations were found to be located on the hypervariable βG-βH loop of VP1.

Out of the positions showing aa variations, position 141 occurs in the βG-βH loop of VP1 upstream of the integrin-binding 'RGD' motif and position 202 at the carboxy terminal of VP1, which are known to be constituents of antigenic site 1 in serotype O FMD virus (Kitson *et al.,* 1990; Mateu, 1995). Likewise, position 149 occurs in the βG-βH loop of VP1 and has been reported to be the antigenic site 5 of serotype O FMD virus (Crowther *et al.,* 1993; Aktas and Samuel, 2000). Similarly, aa 81, 169 and 186 are known to be part of minor antigenic sites in serotype A virus (Thomas *et al.,* 1988; Baxt *et al.,* 1989) and may be of antigenic importance in serotype O as well. Codons 56 and 81 are predicted to be under positive selection pressure in serotype O virus and therefore could be experiencing adaptive evolution (Lewis-Rogers *et al.,* 2008). Alignment of VP1 sequence of Indian field isolates revealed variability at aa positions 26, 56, 81, 110 and 141, thereby supporting existence of genetic polymorphism at those positions in the naturally evolving virus populations as well. The presence of a higher number of clones with sequences identical to the consensus sequence provides support for the dominance of the consensus sequence in the population at a a given timepoint. More importantly, the presence of minor sequences/ antigenic variants in the quasispecies spectrum supports the possibility of their selection and likely dominance under hostile circumstances such as pre-existing host immunity. Further, such variant population demography suggests the intrinsic ability of the virus to mutate and rapidly adapt to changing environments. The findings of this study do encourage further research into genome-wide variant distribution, which may provide insights into virus evolution at population level *in vivo*.

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