Molecular characterization of serotype Asia-1 foot-and-mouth disease viruses in Pakistan and Afghanistan; emergence of a new genetic Group and evidence for a novel recombinant virus

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A B S T R A C T
Foot-and-mouth disease (FMD) is endemic in Pakistan and Afghanistan. The FMD virus serotypes O, A and Asia-1 are responsible for the outbreaks in these countries. Diverse strains of FMDV, even within the same serotype, co-circulate. Characterization of the viruses in circulation can facilitate appropriate vaccine selection and tracing of outbreaks.

The present study characterized foot-and-mouth disease serotype Asia-1 viruses circulating in Pakistan and Afghanistan during the period 1998–2009. Phylogenetic analysis of FMDV type Asia-1 revealed that three different genetic Groups of serotype Asia-1 have circulated in Pakistan during this time. These are Group-II, -VI and, recently, a novel Group (designated here as Group-VII). This new Group has not been detected in neighbouring Afghanistan but viruses from Groups I and -II are in circulation there. Using near complete genome sequences, from FMD viruses of serotypes Asia-1 and A that are currently circulating in Pakistan, we have identified an interserotypic recombinant virus, which has the VP2-VP3-VP1-2A coding sequences derived from a Group-VII Asia-1 virus and the remainder of the genome from a serotype A virus of the A-Iran(D2009)7 sub-lineage. The Asia-1 FMDVs currently circulating in Pakistan and Afghanistan are not efficiently neutralized by antisera raised against the Asia-1/Shamir vaccine strain. Thus, new Asia-1 vaccine strains may be required to block the spread of the current Asia-1 viruses.

Article info
Abbreviations: FMD, foot-and-mouth disease; FMDV, foot-and-mouth disease virus; RT-PCR, reverse transcriptase polymerase chain reaction.
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The virus exists in seven distinct serotypes, i.e. O, A, C, Asia-1, SAT 1, SAT 2 and SAT 3, that do not induce cross protection against each other after either infection or vaccination (Bachrach, 1968; Domingo et al., 2003). The serotypes O and A have the widest global distribution and have been responsible for outbreaks in Europe, America, Asia and Africa. The SAT 1–3 viruses are normally restricted to sub-Saharan Africa whereas serotype Asia-1 is generally confined to Asia. The last reported outbreak due to serotype C FMDV was in Ethiopia during 2005 (Rweyemamu et al., 2008; WRL-FMD, 2006) but it did have a widespread distribution formerly. Within the serotypes, many subtypes can be identified that sometimes fail to induce efficient cross protection against other viruses from within the same serotype. In addition, the control of this disease is constantly challenged by the emergence of new strains.

FMD is endemic and widespread in both Pakistan and Afghanistan; the serotypes O, A and Asia-1 are responsible for these outbreaks (Ferrari et al., 2010; Jamal et al., 2010; Schumann et al., 2008; Valarcher et al., 2005, 2009). In order to achieve better control of the disease in such countries, it is essential to monitor the current variants of the prevalent serotypes of FMDV in the field to ensure that the most appropriate vaccine strains are used to combat the circulating viruses and to identify routes of infection. FMDV's serotypes O and A from Pakistan and Afghanistan circulating during 1997–2009 have been characterized in detail (Jamal et al., 2011a,b; Klein et al., 2007, 2008; Schumann et al., 2008; Waheed et al., 2010). However, only rather limited genetic studies on serotype Asia-1 FMDV from Pakistan and Afghanistan have been reported (Schumann et al., 2008; Valarcher et al., 2005, 2009). The present study has characterized the genetic diversity and geographical distribution of serotype Asia-1 FMDV in Pakistan and Afghanistan within the period, 1998–2009. In this work, the complete nucleotide sequences (633 nt) of the VP1 coding region from 18 FMDV serotype Asia-1 samples have been generated and compared with 27 sequences from samples derived from these countries and some sequences from other countries which were available from public databases and published (Schumann et al., 2008; Valarcher et al., 2005, 2009). Furthermore, the L-P1 coding sequences (ca. 2800 nt) of three serotype Asia-1 viruses were determined, of which two were extended to generate near complete genome sequences (ca. 7600 nt). These data provided evidence for interserotypic recombination between viruses of serotype Asia-1 and serotype A. In addition, the antigenic relationship between three distinct Asia-1 FMDVs and the vaccine strain, Asia-1/Shamir, was determined in virus neutralization assays using viruses rescued from the full length viral RNA (see Belsham et al., 2011).

### 2. Materials and methods

#### 2.1. Samples

Oral swab and epithelial samples were collected in Pakistan and Afghanistan between July 2008 and August 2009. A total of 24 samples (see Table 1) and other countries were obtained from the GenBank database (www.ncbi.nlm.nih.gov) and laboratory record number, followed by year of sampling (e.g. As/SIN/PAK/L5/2008).

Nucleotide sequence data for the VP1 coding region from other serotype Asia-1 FMDVs which originated in Pakistan and Afghanistan (see Table 1) and other countries were obtained from the GenBank database (www.ncbi.nlm.nih.gov).

#### 2.2. Sample preparation, RNA extraction, RT-PCR and sequencing

Sample preparation, RNA extraction, RT-PCR and sequencing of the VP1 coding region have been described earlier (Jamal et al., 2011a). The L-P1 coding sequences were also generated for three selected serotype Asia-1 viruses of which two were selected for further sequencing. The near complete genome sequences were determined by generating 15 separate RT-PCR products from each sample to provide overlapping cDNA fragments. These amplicons were sequenced in both directions and the sequences were then assembled using SeqMan Pro (DNASTAR). Primers used to generate nearly complete genome sequences are shown in Table 2. The sequences determined in this study have been submitted to the GenBank database and the accession numbers are shown in Table 1.

#### 2.3. Genome sequence analysis

Initial sequence analyses were performed as described (Jamal et al., 2011a). The serotype of the virus was identified by comparison of the VP1 coding sequences using BLAST (www.ncbi.nlm.nih.gov/blast). Further phylogenetic analyses were performed individually for the coding sequences for VP1 alone, for P1 (VP4-VP2-VP3), and for the Leader protease (L) using MEGA version 5.05 (Tamura et al., 2011).

The sequence data were aligned and then a heuristic method was used to search for the best-fitted substitution model for the data as described by Tamura et al. (2011). Initially, a total of 24 models for nucleotide substitution were evaluated and the best-fitted model was selected on the basis of the lowest Bayesian Information Criterion (BIC) value. A discrete Gamma distribution was used to model evolutionary rate differences among sites. For the VP1 and the Leader protease sequences, the Hasegawa–Kishino–Yano model (Hasegawa et al., 1985) was used, whereas the Tamura–Nei model (Tamura and Nei, 1993) was used for the P1 coding region. The Maximum Likelihood (ML) phylogenetic trees were constructed. The robustness of the tree topology was assessed with 1000 bootstrap replicates (Felsenstein, 1985). A maximum clade credibility phylogenetic tree was also constructed using the MRBAYES software (Ronquist and Huelsenbeck, 2003). Genetic distances between the FMDV genome sequences were calculated using Kimura’s 2-parameter model.

To test for evidence of genetic recombination, the online SimPlot 3.5.1 software (Lole et al., 1999) was used. Briefly, the nearly complete genome nucleotide sequences generated in this study and other selected sequences were aligned using ClustalW implemented in MEGA 4. Pair-wise genetic similarities were plotted between virus sequences using a window size of 200 nucleotides moving in steps of 20 nucleotides along the genome.

alignment. The pair-wise similarity values were plotted at the midpoint of the 200 nucleotide window. The recombination break points along the near complete genome sequence were identified by examining the points at which the similarities between the query and reference sequences markedly changed.

2.4. Antigenic characterization

Three serotype Asia-1 FMDVs, which were rescued using RNA extracted from the epithelial samples preserved in RNAlater (as described by Belsham et al., 2011), were characterized in cross-neutralization tests. The reference serum (raised against the Asia-1/Shamir vaccine strain) was titrated using homologous (Asia-1/}

3. Results

3.1. FMDV serotype Asia-1 in Pakistan and Afghanistan

As described previously (Jamal et al., 2011a), testing oral swab and epithelial samples from within Pakistan and Afghanistan.
generated 135 specimens which contained FMDV RNA. From these, the complete FMDV VP1 coding region was amplified by RT-PCR from 119 samples which were then sequenced and it was found that 69, 33 and 18 corresponded to serotypes A, O and Asia-1, respectively (with one sample containing both serotypes A and Asia-1). Analyses of the serotype O and A FMDVs have been described previously (Jamal et al., 2011a,b). The samples positive for FMDV serotype Asia-1 comprised 7 collected from Pakistan (including the sample with both serotype A and Asia-1) and 11 originating from Afghanistan. Each sample of Asia-1 FMDV was from clinically diseased animals. The presence of the Asia-1 serotype was detected in 2008 after a break of 2 years in Pakistan and 4 years in Afghanistan.

### 3.2. Diversity within FMDV serotype Asia-1

Using two different approaches, phylogenetic trees were constructed using the whole VP1 coding sequences (nt = 633) generated in this study in conjunction with previously determined sequences of serotype Asia-1 viruses circulating in Pakistan and Afghanistan. The Maximum Likelihood (ML) tree is shown in Fig. 1. The maximum clade credibility tree generated using the

<table>
<thead>
<tr>
<th>Primer's name</th>
<th>Primer's sequence (5’–3’)</th>
<th>Direction</th>
<th>Genomic position&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Genomic position&lt;sup&gt;b&lt;/sup&gt;</th>
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<sup>a</sup> Genome position (first nt at 5’ end) according to the FMDV As/Pak/1/54 sequence (Accession No. AY593795). Position of the primers used to amplify and sequence of type Asia-1 FMDV are shown.

<sup>b</sup> Genome position (first nt at 5’ end) according to the FMDV A22/Iraq sequence (Accession No. AY593763). Position of the primers used to amplify and sequence of type A FMDV are shown.

<sup>c</sup> Primers used to amplify and sequence the VP1 coding region (Jamal et al., 2011a).

<sup>d</sup> Primers used to amplify and sequence the P1 coding region (Jamal et al., 2011b).
The deduced amino acid sequences derived from the VP1 coding sequences were compared to investigate the consequences of the observed genetic divergence between the FMDV Asia-1 viruses under study. Group-specific signature amino acid residues for serotype Asia-1 are shown in Fig. 4. Group-I contained characteristic residues 33S, 44R, 47D, 149I, 155S, 170S, 193E and 210A (the numbering is for the VP1 amino acid sequence alone and the single letter amino acid designation is used); The Group-II viruses contained the characteristic residues 80I, 86I and 112V while Group-specific residues 33G, 41R, 47A, 58P, 59Y, 140A, 149V/I, 155A and 170N were present in the new Group designated here as Group VII.

The RGDLXXL motif within VP1 is responsible for the attachment of FMDV to the host cell (Fox et al., 1989; Jackson et al., 1997). It was found to be conserved in the Group-IV and Group-V viruses and each contained RGDLLA. However, amino acid substitutions in this motif were observed in the remaining Groups. The Group-I viruses contained RGDLAA, the Group-VII strains have RGDLAA (viruses circulating in 2008) or RGDLAV (viruses detected in 2009) while members of Group-VI contained RGDMAA. In the Group-II viruses, a variety of different motifs were found, these were RGDMAA, RGDVAAL, RGD TAAL and RGDVAAL.

3.4. Evidence for interserotypic recombination

The sequence information for the L-P1 coding region from the As/SIN/PAK/L2810/2009 and As/SIN/PAK/L2812/2009 viruses described above (see Fig. 3), showed much greater divergence from the As/SIN/PAK/L5/2008 within the L coding sequence than within the P1 coding sequence. Thus it seemed possible that these viruses were generated by recombination between a serotype Asia-1 virus and another virus serotype. In order to determine this and to identify potential sites of such inter-serotypic recombination events, defined portions of the genome sequences of serotype O, A and Asia-1 FMDVs were compared. The level of identities of different regions of the FMDV genomes between the putative recombining virus (As/SIN/PAK/L2810/2009) and selected viruses were calculated. As is evident from Table 3 and from the similarity and bootstrap plots shown in Fig. 5 (panels A, C and D), the 5’UTR, L, VP4, P2 and P3 regions of the As/SIN/PAK/L2810/2009 virus have very close nucleotide sequence identities with a serotype A virus (A/SIN/PAK/L4/2008) whereas, in contrast, the contiguous VP2, VP3, VP1 and 2A coding region of the As/SIN/PAK/L2810/2009 RNA showed close identity with the As/SIN/PAK/L5/2008 sequence (consistent with the serotype designation which is determined by the capsid sequences). From the SimPlot analysis (Fig. 5), it appears that the junctions for the recombination events between these viruses, as judged by the sharp switch in level of sequence identity, are close to nt 1407 and nt 3510. At each junction a short window (either 9 or 17 nt) within which the crossover event has occurred has been identified (see Fig. 5, panel B). These recombination sites are located at the beginning of the VP2 and 2B coding regions, respectively. It should be noted that the L-P1 sequence derived for the As/SIN/PAK/L2810/2009 virus only differs at one nucleotide position from the As/SIN/PAK/L2810/2009 virus, indicating it is a very similar recombinant (the L sequence is again closest to the serotype A strain (A/SIN/PAK/L4/2008) while the VP2, VP3 and VP1 coding sequences are derived from an Asia-1 virus). It would seem...
Fig. 1. Maximum Likelihood phylogenetic tree generated using the nucleotide sequences (nt = 633) for the complete VP1 coding regions of serotype Asia-1 FMDVs (note: viruses marked with circles and triangles originated from Pakistan and Afghanistan, respectively).
Fig. 2. TCS tree showing the relationships between the VP1 sequences of Asia-1 FMDVs (Group-II) circulating within Pakistan, Afghanistan, Tajikistan, Kyrgyzstan and Hong Kong between 2002 and 2009. Each circle/oval denotes a single nucleotide change. An open circle represents a synonymous change, whereas, a filled circle shows a non-synonymous change. Each amino acid residue change is shown for the non-synonymous substitutions. Double circles and a circle with a triangle show mutation and corresponding back mutation, respectively. *First and last letters represent change from one amino acid to the other, respectively. The numeral in the middle shows the position of the amino acid substitution within the VP1 polypeptide.
likely that these two recombinant viruses are the progeny of the same recombination event.

### 3.5. Antigenic characterization

Three serotype Asia-1 FMDVs were analysed in virus neutralization assays by titration of reference serum (raised against the Asia-1/Shamir vaccine strain) with the homologous virus and field strains. The $r_1$ values, showing the antigenic relationship between the isolates and the vaccine strains, determined for these isolates in this study and those determined previously for other isolates from Pakistan are shown in Table 4. It is apparent that the Asia-1/Shamir vaccine strain may not protect efficiently against the Group VII serotype Asia-1 viruses since the $r_1$ values are <0.3 (Rweyemamu, 1984).

### 4. Discussion

Foot-and-mouth disease is endemic in Pakistan and Afghanistan and the serotypes O, A and Asia-1 viruses are responsible for the outbreaks in these countries (Jamal et al., 2010; Schumann et al., 2008). In the present study, the nucleotide coding sequences for VP1 from a total set of 45 serotype Asia-1 FMDVs from Pakistan and Afghanistan, which were responsible for outbreaks in the period 1998–2009 (plus two viruses isolated in 1954 and 1985 from Pakistan) were analysed. Some 18 of these sequences were generated in the current studies.

FMDV serotype Asia-1 was first identified in 1954 from a sample which originated from Pakistan (Brooksby and Rogers, 1957), however, subsequently this serotype was found to be circulating in the present day Pakistan in the 1940s (Yasin and Huq, 1960). This serotype is still endemic in the Indian subcontinent where
Fig. 4. Alignment of deduced amino sequences for the whole VPI proteins of FMDV serotype Asia-1 viruses from Pakistan and Afghanistan. The Group specific residues are highlighted.
outbreaks occur regularly and spread to neighbouring countries
(Valarcher et al., 2009). Serotype Asia-1 appeared to move in the
early 1970s from Pakistan through Afghanistan and Iran to Iraq
plus Turkey (Firoozi Bandpay et al., 1974). It has been speculated
that the distribution of serotype Asia-1 within Asia is related to
the presence of Asian water buffaloes (Valarcher et al., 2009), how-
ever, in contrast, Jamal et al. (2010) found no association between
serotype Asia-1 and this species of animal. Furthermore, this sero-
type is endemic in Afghanistan despite the absence of water buffa-
loes there.

FMDV serotype Asia-1 is considered to be genetically and anti-
genically the least diverse serotype (Knowles and Samuel, 2003).
Indeed, Ansell et al. (1994) reported that 44 serotype Asia-1 FMDVs
isolated between 1954 and 1990 throughout Asia were less vari-
able compared to other FMDV serotypes in their VP1 coding se-
quences. It is noteworthy, however, that the RGDXXKL receptor
binding motif of serotype Asia-1 viruses was highly variable as
compared to serotypes O and A of FMDV (Jamal et al., 2011a,b)
although only the 2 leucine (L) residues within this motif changed.

Previous reports have classified FMDV serotype Asia-1 in differ-
ent ways. For example, Ansell et al. (1994) grouped serotype Asia-1
viruses isolated throughout Asia between 1952 and 1992 into 18
groups, while Mohapatra et al. (2008) classified serotype Asia-1
viruses from India, sampled over the last two decades, into seven
lineages and Valarcher et al. (2009) divided these FMDVs from
2003–2007 into six Groups. Unfortunately, there are no uniform
criteria for these classifications. Furthermore, serotypes O and A
FMDVs have been classified into lineages but serotype Asia-1
FMDVs have been classified as Groups (see Valarcher et al.,
2009). The present study revealed that three different Groups of
Asia-1 virus (as defined by Valarcher et al. 2009) with 95–100% se-
quence identity for the VP1 coding region within each Group were
in circulation within the region during the period 1998–2009.
Among these Groups, three have been present in Pakistan and
two in Afghanistan. Group-I viruses have been in circulation in
Afghanistan in 2001 and also in Iran in 2001 and 2004 but have
not been detected in Pakistan. Viruses from Afghanistan and Iran,
belonging to this Group show more than 99% identity to each other
within their VP1 coding sequences, which suggests that they have
a common origin. Group-II viruses were in circulation in 2002 and
2004 in Pakistan and in 2003 within Afghanistan. They also spread
further to Tajikistan (2003–2004), Uzbekistan (2003), Kyrgyzstan
(2004) and Hong Kong (2005), showing northwestern spread. Viruses
belonging to this Group were again detected in Afghanistan in
2009. These viruses are closely related and show <3% nucleotide
difference, which again suggests a common origin.

Phylogenetic trees (e.g. as in Fig. 1) cluster together closely
related viruses but the putative origin of each strain cannot be
ascertained. The TCS tree (as in Fig. 2), however, indicates the rela-
tionships between the different viruses due to its ability to track
variation within single outbreaks or during long term evolution of
viruses belonging to a single cluster. This helps to identify the
putative origin and transmission pathway of the virus. TCS trees
based on complete genome sequences have been used to deter-
mine the transmission pathways of serotype O FMDVs responsible
for the 2001 and 2007 outbreaks in the U.K. (Cottam et al.,
2008a,b). The TCS tree (Fig. 2) generated using the VP1 coding se-
quences, analysed over a longer period of time, indicates that the
source of the FMD outbreaks in Tajikistan in 2003–2004 and Hong
Kong in 2005 is the same, which may have originated in Afghan-
istan or Pakistan. Furthermore, a single nucleotide change between
the viruses responsible for outbreaks in Tajikistan and Kyrgyzstan
in 2003–2004 indicates that the virus spread from Tajikistan to
Kyrgyzstan. Very high sequence identities between the viruses
circulating in Afghanistan in 2003 and 2009 (Fig. 2) shows that
Group-II persisted in this country for a long time, despite the fact
that they were not detected during 2005–2008 but this may be
due to insufficient sampling. Group-VI viruses have been in circu-
lation in Pakistan in 1998, 2003 and 2005. These viruses show only
0–0.3% nucleotide difference in their VP1 coding regions between
each other, which suggests potential laboratory contamination,
laboratory escape or use of improperly inactivated vaccine. Inter-
estingly, the VP1 coding sequence of an isolate (As/PAK/2/1998)
from 1998 showed 100% identity with that of viruses detected in
2005. Furthermore, this virus is closely related to viruses circulat-
ing in Iran in 1999, Turkey in 2000 and Greece in 2000 (showing

Table 3
Nucleotide identity (%) of different regions of the As/SIN/PAK(L2810)/2009 genome compared with the other viruses (values in bold show highest identities).

<table>
<thead>
<tr>
<th>Viruses</th>
<th>5’ UTR</th>
<th>L-region (603 nt)</th>
<th>VP4 (255 nt)</th>
<th>VP2 (654 nt)</th>
<th>VP3 (657 nt)</th>
<th>VP1 (639 nt)</th>
<th>2A (54 nt)</th>
<th>2B (462 nt)</th>
<th>2C (594 nt)</th>
<th>3A (459 nt)</th>
<th>3B (213 nt)</th>
<th>3C (639 nt)</th>
<th>3D (1413 nt)</th>
<th>3’ UTR (87 nt)</th>
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<td>As/SIN/PAK/L5/2008 (JN006719)</td>
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<td>86.9</td>
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<td>98.6</td>
<td>98.4</td>
<td>96.3</td>
<td>95.9</td>
<td>93</td>
<td>88.2</td>
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<td>93.6</td>
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<td>87.1</td>
<td>84.6</td>
<td>86.9</td>
<td>82.1</td>
<td>87</td>
<td>93.3</td>
<td>92.8</td>
<td>91.5</td>
<td>98.1</td>
<td>97.5</td>
<td>97.6</td>
<td>94.4</td>
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<td>87.4</td>
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<td>87.8</td>
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<td>92.3</td>
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<td>75.4</td>
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</table>

* This is for the region adjacent to the coding sequence.
Fig. 5. Recombination analyses of the serotype Asia-1 FMDV strain, As/SIN/Pak/L2810/2009. Panel (A) similarity plot of As/SIN/Pak/L2810/2009 in comparison with the putative parents, As/SIN/Pak/L5/2008 (green) and A/SIN/Pak/L4/2008 (red). The sequences of two other viruses each belonging to serotype Asia-1 (As/Bam/L590/2009; shown in grey) and serotype O (UKG/11/2001; shown in blue) were also compared. The x-axis indicates nucleotide positions along the alignment and the y-axis denotes the percent identity. Panel (B) nucleotide sequence alignment around the junction sites of recombination (VP2 and 2B coding regions). At each junction a short window within which the crossover event has occurred is identified and is included within a rectangle. The numbering represents positions of the nucleotides in the alignment of the near complete genome sequences, corresponding to the x-axis of panels A and C. Panel (C) bootscan plot supporting the clustering of As/SIN/Pak/L2810/2009 with the parental viruses as in panel (A). The y-axis indicates the percent bootstrap values. Panel (D) representation of the origin of different regions of the As/SIN/Pak/L2810/2009 genome; the portions in green (from Group VII serotype Asia-1 virus) and red (from the A-Iran05MC07 sub-lineage) were acquired from the two parental viruses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
about 97.8–98.1% identity), which suggests its possible westward spread from Pakistan through Iran to Turkey and Greece. To our knowledge, this is the second and most recent incursion of serotype Asia-1 into a European country (an outbreak due to serotype Asia-1 virus had previously occurred in Greece in 1984). Closely related viruses have also been detected in Armenia in 2000 and in Georgia in 2000–2001 (Scherbakov et al., 2008), which suggests further spread of this strain from Turkey towards the north. Surprisingly, viruses belonging to Group VI have not yet been detected in Afghanistan. However, strains belonging to the Group-VII, as first detected in Pakistan in 2008–2009 within the present study, have now been reported from Afghanistan in May 2011 (WRL-FMD, 2011a), Iran in January–February 2011 (WRL-FMD, 2011b) and Bahrain in February 2011 (WRL-FMD, 2011c), which shows a westward spread and hence a potential threat to West EuroAsia. As now observed with serotype Asia-1, the periodic spread of FMD type A viruses in a westward direction has been documented in the past, for example, the A22 strain (Arrowsmith, 1975) and A-Iran-05 lineage (Knowles et al., 2009) spread from Iran to Turkey.

Overall, there is a high level of agreement between the phylogenetic trees shown here (Fig. 1) and those reported previously by Valarcher et al. (2009). However it should be noted that the sub-groupings of some serotype Asia-1 viruses as Groups VIa, VIb and VIc (as reported by Valarcher et al. 2009) do not form a single cluster/group in the analyses presented therein and here (see Fig. 1). Viruses within Group VIa (e.g. PAK/20/2003 and PAK/19/2005) indeed cluster within Group VI but the Group-Vic viruses (e.g. PAK/30/2002 and PAK/31/2002) form part of Group-II in our analyses (see Fig. 1). Furthermore, the TCS tree for the Group II viruses presented here (Fig. 2) is also consistent with the inclusion of the Group Vic viruses as part of Group II. The level of nucleotide sequence identity within the VP1 coding region between the Group Vic viruses and the Group II viruses is >95%.

The present study has detected a new Group of Asia-1 viruses circulating in Pakistan (here termed as Group VII). This new Group shows ~2% difference in the VP1 coding region compared to Group I viruses. In the WRL reports, the viruses classified here as Group VII are listed as belonging to an unknown Group (www.wrlfmd.org). Antigenic analysis recently reported by the WRL, Pirbright, UK shows that a virus (As/PK/29/2009) of this Group was not efficiently neutralized by antisera generated against the Asia-1/Shamir vaccine (WRL-FMD, 2010). The $r_1$ value has been used previously to ascertain antigenic relationships between the FMDV field isolates and the vaccine strains (Nayak et al., 2001; Parlak et al., 2007; Rweyemamu, 1984; Samuel et al., 1990). Using VNT, $r_1$ values of >0.3 have been shown to reflect a close antigenic relationship between the field isolates and vaccine strains, indicative of good protection by the vaccine, whereas values <0.3 reflect a distant antigenic relationship, indicating that the vaccine is unlikely to protect against the field isolates. The $r_1$ values reported in this work (see Table 4) reveal that the Asia-1/Shamir vaccine strain could give sufficient protection against the field strains circulating in Pakistan during 2003–2004. However, this vaccine strain may not give sufficient protection against the Asia-1 FMDVs circulating in Pakistan (Group-VII viruses e.g. As/PK/29/2009) and Afghanistan (Group-II viruses e.g. As/BAM/AFG/L590/2009) in 2008–2009. This may explain, in part, the recent westward spread of the Asia-1 serotype viruses.

The genetic heterogeneity within FMDV results from a number of different factors including the error rate of the viral RNA polymerase (coupled to the absence of proof-reading activity) during viral replication and the selection of variants as a result of various selection pressures. More dramatic changes in virus sequence can occur as a result of recombination between different FMDV genomes. The generation of recombinant viruses in the field has been reported earlier and such events have occurred within the coding region for the non-structural proteins (NSP) in the 3’ portion of the genome (Balinda et al., 2010; Giraudo et al., 1987; Krebs and Marquardt, 1992; Lee et al., 2009; Li et al., 2007), or in the 5’ end (Mohapatra et al., 2008) and even in the capsid coding region (Haydon et al., 2004; Tosh et al., 2002) of the genome. The recombination analyses in the present work (Fig. 5 and Table 3) indicate that recombination has occurred between serotype A and Asia-1 viruses at sites flanking the coding region for the surface exposed capsid proteins (VP2, VP3, VP1 plus 2A), to produce novel variants of the Group-VII viruses (e.g. As/SIN/PK/2810/2009). Both serotype Asia-1 and serotype A viruses were circulating in the same area at that time. Samples positive for both the serotype A and Asia-1 viruses were initially collected in December 2008 from the Hyderabab area of southern Pakistan, indeed one sample was found positive for both serotypes A (A-Iran05(ABC-07) sub-lineage) and Asia-1 (belonging to the Group-VII). We detected more serotype Asia-1 viruses from the same area in samples collected in July, 2009 but these showed 2% difference in the VP1 coding region from the Asia-1 viruses detected in 2008. Simultaneous infection of serotype A and Asia-1 FMDVs in an animal must have occurred to allow the formation of recombinant viruses (see Fig. 5). The recombination process requires that the viral RNA polymerase switches between two different FMDV RNA templates during viral replication. This usually occurs within highly conserved sequences and the sequence data provided quite small windows for the actual sites of recombination within the VP2 and 28 coding regions (Fig. 5, panel B), that resulted in the recombinant virus progeny. Analyses of near complete genome sequence of two of the Asia-1 viruses circulating in 2009 in southern Pakistan revealed a mosaic pattern within their genomes.

5. Conclusions

It is apparent from this work that three different Groups of FMDV serotype Asia-1 have been circulating in Pakistan and Afghanistan; emergence of a new genetic Group and evidence for a novel recombinant virus. Infect. Genet. Evol. (2011). doi:10.1016/j.meegid.2011.09.015
Afghanistan since 1998. Serotype Asia-1 was detected again in 2008/2009 after a lapse of 4 years in Afghanistan and 2 years in Pakistan. Detection of a new Group of Asia-1 viruses, termed here Group-VII, in the present study shows that the viruses of this serotype continue to evolve, as do the O- and A-Iran5 lineages (Jamal et al., 2011a,b) which also circulate in this region. A variant of the Group-VII Asia-1 viruses has arisen as a result of recombination between viruses belonging to Group-VII of serotype Asia-1 and a serotype A virus of the A-Iran5OG-07 sub-lineage, presumably due to simultaneous infection of an animal with these two viruses. The r1 value determinations indicate that the Asia-1/Shamir strain, which is being used in the current FMD Asia-1 vaccines, may not efficiently protect against the Asia-1 viruses circulating in Pakistan and Afghanistan in 2008–2009.

Acknowledgements

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References


Open session of the Research Group of the Standing Technical Committee on FMD. European Commission for the control of FMD, Food and Agriculture Organization of the United Nations, Rome, Italy, pp. 69–72.


