**Recent molecular epidemiology of foot-and-mouth disease virus Asia 1**

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**Abstract:**

**Introduction:** Recently there has been an apparent increase of outbreaks due to foot-and-mouth disease (FMD) Asia 1. Spread has occurred into regions where this serotype has never or rarely been recorded - People’s Republic of China (including Hong Kong), Russian Federation and Mongolia. To determine the relationships between FMD virus isolates from the various outbreaks, a phylogenetic study using complete VP1 gene sequences was undertaken.

**Materials and Methods:** RNA extracted from either clinical samples or cell culture grown virus was subjected to RT-PCR using foot-and-mouth disease virus (FMDV)-specific primers targeting regions either side of the VP1 gene. The complete sequence of the VP1 gene was directly determined using standard automated sequencing techniques.

**Results:** The VP1 region of more than 140 FMD Asia 1 viruses were amplified by RT-PCR and directly sequenced. Phylogenetic trees were generated by the Neighbor-joining method using the MEGA 3.1 software package. Recent virus isolates fell into six genetic groups.

**Discussion:** FMDV serotype Asia 1 was recently responsible for multiple disease outbreaks throughout much of eastern Asia. Normally this serotype occurs in Southern and Southeast Asia and has been regularly reported in Afghanistan, India, Iran, Malaysia, Nepal, Pakistan and Thailand. However, at the end of 2004 through to 2006, in addition to some of these countries, outbreaks of Asia 1 were reported in several provinces or autonomous regions of the People’s Republic of China, Mongolia, Myanmar, several regions of eastern Russia, Tajikistan and Vietnam. Phylogenetic analysis of complete VP1 gene sequences from FMDV isolates responsible for these outbreaks showed that they were caused by viruses that belonged to six different lineages within the Asia 1 serotype. It appears from this study that some of these viruses have spread rapidly over large distances and are genetically very closely related to isolates collected 25 years ago in India.

**Introduction:**

Foot-and-mouth disease (FMD) virus (genus Aphthovirus, family Picornaviridae) principally infects cloven-hoofed animals. FMD is highly contagious and combined with high antigenic diversity of the virus makes this disease difficult to control. FMD virus (FMDV) is comprised of seven serotypes and infection or vaccination with one serotype does not confer protection against other serotypes (Brooksby, 1982; Cartwright et al., 1982; Mattion et al., 2004). This diversity is also observed at the genetic level and this characteristic is used to trace the origin of viruses (Knowles and Samuel, 2003).
FMD is a severe constraint to international livestock and livestock product trade. Although FMD has been eradicated in Europe, North America, parts of South America, Australasia and some island regions of Asia, it is still prevalent in many countries in Africa, Asia and South America. In all these areas serotypes O and A are present, whereas the Southern African Territories (SAT) serotypes, SAT 1, SAT 2 and SAT 3, are normally restricted to sub-Saharan Africa, although some rare escapes to the Middle East and Europe have been recorded, e.g. SAT 1 in the early 1960's (WRLFMD Records). However, apart from two incursions into Greece (in 1984 and 2000) (Anon., 1984; Anon., 2001), the Asia 1 serotype has remained restricted to Asia.

The intense trading of animals and animal products within Asia and their export to other regions, make this continent a major source and reservoir of FMDV sometimes providing new variants which impact a wider region, e.g. A22 in the 1960's (Arrowsmith, 1975), the O Cathay topotype in 1994-97 and the O PanAsia strain in 1998-2001 (Knowles and Samuel, 2003; Knowles et al., 2005). In this respect, the epidemiological surveillance of FMD in Asia is essential to assess the emergence of new strains that could threaten countries having a FMD-free status as well as to select the most appropriate vaccine strains to control this disease.

FMDV Asia 1 was first detected in samples collected in India in 1951-52 (Dhanda et al., 1957) and Pakistan in 1954 (Brooksby and Rogers, 1957). This serotype remained endemic in southern Asia and made frequent incursions into the Middle East and on two occasions into Europe (Ansell et al., 1994; WRLFMD records). Between 2000 and 2003, FMDV Asia 1 was reported in southern Asia by Afghanistan, Bhutan, Iran, India, Nepal and Pakistan; and for the last time in Southeast Asia in 2002 by Lao PDR. At the end of 2004 and in 2005-2006, in addition to countries in which this serotype is known to be endemic (e.g. India, Iran, Nepal and Pakistan), outbreaks of Asia 1 were reported for the first time (at least since 2002) by Afghanistan, several provinces or autonomous regions (AR) of the People’s Republic of China (Beijing, Gansu, Hebei, Hong Kong Special Administrative Region, Hubei, Jiangsu, Ningxia AR, Qinghai, Shandong, Tibet AR and Xinjiang AR), Mongolia, Myanmar, several regions of the Russian Federation (Amur, Chitinskaya, Khabarovsk and Primorsky), Tajikistan and Vietnam (Fig 1). FMDV Asia 1 is known or suspected to have been present in many countries in southern and Southeast Asia in the past; however, it has not been previously reported in Eastern Russia or Mongolia. Current reports suggest an increase in cases across this wide geographical area and in this study we report the phylogenetic characterisation of isolates responsible for many of these outbreaks.

**Materials and methods:**

Clinical samples containing FMDV Asia1 were received from Afghanistan, People’s Republic of China (including Hong Kong Special Administrative Region), Iran, Mongolia, Myanmar, Pakistan, Russia and Tajikistan by the Food and Agriculture Organisation World Reference Laboratory for FMD (UK), FGII All-Russian Research Institute for Animal Health (Russia), Lanzhou Veterinary Research Institute (People’s Republic of China), Indian Veterinary Research Institute (India), Plum Island Animal Disease Center (USA) and Pakchong Regional Reference Laboratory for FMD (Thailand).

Viral RNA extraction, reverse transcription-polymerase chain reaction (RT-PCR) and sequencing were performed according procedures used by each laboratory and further details can be obtained on request directly to the individual laboratories. The methods used in the WRLFMD are described below.

Various primer combinations (forward primers, As1-1C505F, As1-1C530F, As1-1C613F and As1-1C616F and reverse primers, EUR-2B52R and NK61) were used in a one-step RT-PCR (Table 1). Forward and reverse primer amounts were 20 and 40 pmol, respectively. We used two to four sequencing primers to ensure coverage of the VP1 region on both cDNA strands (Table 1).

**RT-PCR of vRNA:** Total RNA was extracted from 460 µL of a 10% epithelial suspension or cell culture by using RNaseasy kits (Qiagen Ltd., Crawley, West Sussex, UK), according to the manufacturer’s instructions, and resuspended in 50 µL nuclease-free water. This RNA (5 µL) was used as the template in a one-step RT-PCR (Ready-To-Go RT-PCR Beads; Amersham). The following thermal profile was used: 42°C for 30 min; 94°C for 5 min; 35 cycles of 94°C for 60 s; 55°C for 60 s; and 72°C for 90 s; followed by a final extension of 72°C for 5 min. PCR products were analysed by electrophoresis on a 1.5% agarose-Tris-borate-EDTA gel containing 0.5 µg/mL ethidium bromide. DNA weight markers (GeneRuler 100 bp DNA Ladder Plus, Ready-To-Use; Fermentas Inc, Hanover, MD, USA) were run alongside the samples to facilitate product identification and quantification. Post-PCR removal of deoxynucleoside triphosphates and primers
was achieved using the Qiaquick PCR purification kit (Qiagen) according to the manufacturer’s instructions, and resuspended in 30 µL nuclease-free water.

Sequence determination: PCR amplicons were sequenced by using the DTS Quick Start Kit (Beckman Coulter Inc., Fullerton, CA, USA) according to the manufacturer’s instructions and with the sequencing primers listed in the Table 1. The sequencing reactions were run on a CEQ8000 Automated Sequencer (Beckman Coulter) according to the manufacturer’s instructions.

Phylogenetic Analysis: An unrooted Neighbor-joining tree was constructed by using MEGA version 3.1 (Kumar et al., 2004). The robustness of the tree topology was assessed with 1,000 bootstrap replicates as implemented in the program.

Results and Discussion:

The phylogenetic analysis of the complete VP1 gene sequences from isolates of serotype Asia 1 included in this study showed that recent viruses belonged to six different groups (I to VI) (Fig. 2). These groups were all supported by bootstrap values of between 67 and 100%.

Viruses that were circulating in Iran in 2004 belonged to two different groups (I and II). In group I, one isolate collected in Iran in 2004 was closely related to some collected in Afghanistan in 2001. Other isolates collected in Iran during 2004 belonged to one of the sub-lineages of group II. These Iranian isolates revealed a close genetic relationship, with less than 7% nucleotide difference, to those collected in Afghanistan (2004), Tajikistan (2004), Hong Kong (2005) and Pakistan (2002-2004). This is the first report of FMDV Asia 1 in Hong Kong since 1980. It is also noticeable that the viruses collected in Tajikistan and Hong Kong in 2004-5 had less than 3% nucleotide difference, suggesting that this virus may have travelled a long distance in a short period of time. Interestingly, other Pakistani viruses collected in 2003 and 2005 from a related genetic sub-lineage were closely related to viruses responsible of outbreaks in Iran, Turkey, Armenia, Greece and Georgia between 1999 and 2001 (Fig. 2). Similar epizootics have occurred in the past: between 1972 and 1975 in Afghanistan, Iran, Turkey, Lebanon and Iraq; and between 1983 and 1985 in Armenia, Azerbaijan, Bahrain, Georgia, Greece, Israel and Lebanon. The ultimate sources of these epizootics have never been conclusively established, although the outbreaks are usually first seen in the east and appear to spread westwards (Ansell et al., 1994; Knowles and Samuel, 2003).

Group III contained only viruses that were collected in India between 2001 and 2004 and Bhutan in 2002. There is evidence for spread of viruses from the Indian sub-continent in the past (Ansell et al., 1994), however, none during the period of this report.

The virus responsible of the outbreak of FMD Asia 1 in Myanmar and Vietnam in 2005 was related to viruses originating from Southeast Asia that were collected in the same country in 2000 and in Thailand in 1998 (Group IV); this lineage was restricted to Southeast Asia. This finding suggests that this serotype might have persisted in some areas of Southeast Asia. However, the recent isolate collected in Myanmar was very different to the last collected in this country in 2001 (Fig. 2). Recent Vietnamese isolates were closely related to viruses collected in Thailand in 1998.

FMDV isolates collected in different places in the People’s Republic of China, the Russian Federation and Mongolia were very different from the Hong Kong isolates with 16.1 to 17.2% nucleotide difference and belonged to Group V. As far as we are aware this is the first report of Asia 1 in the Russian Federation and Mongolia. Surprisingly, viruses collected in the different provinces or regions of China, Russia and Mongolia were very closely related (0.8-1.7% nucleotide difference) to viruses from India (Tamil Nadu) collected in 1980-81 (Fig. 2). However, these viruses are very different to those that were recently collected in India during 2003-2004 (n=20) with 12.8 to 14.7% nucleotide difference. Further investigations need to be performed to determine the origin of this virus.

Surprisingly, FMDV isolates collected in Pakistan in 2003 and 2005 that belonged to group VI were very closely related to PAK/2/98 with 0.3% and 0.0% nucleotide differences, respectively.

Serotype Asia 1 is genetically and antigenically the least variable of the seven recognised serotypes of FMDV and is normally found only in Asia (Gurumurthy et al., 2002; Knowles and Samuel, 2003). However, the Asia 1 serotype has previously spread into Europe, most recently causing outbreaks in Turkey and Greece in 1999 and 2000, respectively. Fortunately, vaccine matching tests carried out at the FAO World Reference Laboratory for FMD using ELISA and/or VNT suggest that current vaccine strains will provide a good protection against this new strain of Asia 1, since recent isolates
of FMDV serotype Asia1 collected in Pakistan (n=4) during 2004 and in Hong Kong (n=2) at the beginning of the year, showed a good to very good antigenic match with bovine vaccinal serum (BVS) against the vaccine strain Asia1/Shamir, but less so with BVS against Asia1/IND/8/79 (Table 2).

These observations suggest that: i) Asia 1 is mainly responsible for clinical signs in cattle and Asiatic buffalo; ii) the viruses from groups II and V responsible for these outbreaks might have managed to spread large distances in a short time and the possibility of a future more worldwide spread cannot be discounted; iii) the close relationships of some old and recent isolates within groups V and VI raises the question of their origins, either as a slow evolutionary rate or as a reintroduction of laboratory/vaccine virus strains.

The spread of some of these FMDV of serotype Asia 1 throughout the Asian continent demonstrates the continuing need for active surveillance and for further development of regional control programmes. India and China with their very large livestock populations are expected to have a major role in FMD control in this part of the world.

Authors Conclusions:
- The recent apparent increase in incidence of FMDV Asia 1 has not been a single epidemic but due to the circulation and spread of multiple virus lineages.
- One of these strains, which appeared in China, Russia and Mongolia in 2005-06, is closely related to viruses from India in 1980-81.
- Vaccine matching by VNT has shown that the Shamir vaccine strain has wide coverage, although not all recent lineages have been tested.

Authors Recommendations:
- It is important that surveillance is continued throughout the region to monitor the spread of the different FMDV Asia 1 lineages.
- Additionally, the EUFMD should encourage submission of representative viruses to the WRLFMD for antigenic comparisons.

Acknowledgements:

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References:


Table 1. Oligonucleotide primers used for RT-PCR and cycle sequencing of FMDV Asia 1 isolates.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5’-3’)</th>
<th>Gene</th>
<th>Position</th>
<th>Use</th>
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<tbody>
<tr>
<td>As1-1C505F</td>
<td>TACACTGCTTCTTGACGTGCG</td>
<td>VP3</td>
<td>505-524</td>
<td>PCR</td>
</tr>
<tr>
<td>As1-1C530F</td>
<td>CCACRAGTGTGCGARRGGATGCG</td>
<td>VP3</td>
<td>530-551</td>
<td>PCR</td>
</tr>
<tr>
<td>As1-1C613F</td>
<td>GCCGGCAARGAYTGGAGTTYCG</td>
<td>VP3</td>
<td>613-635</td>
<td>PCR, sequencing</td>
</tr>
<tr>
<td>As1-1C616F</td>
<td>GCCAGGCCTTGGAGTTYCG</td>
<td>VP3</td>
<td>616-626</td>
<td>PCR, sequencing</td>
</tr>
<tr>
<td>As1-1D205F</td>
<td>GCRACGTACTACTYTCRAGACCT</td>
<td>VP1</td>
<td>205-228</td>
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<tr>
<td>As1-1D370R</td>
<td>GTTGTAYACTGTYGCCAGCACAG</td>
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<tr>
<td>NK72</td>
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<td>2A/2B</td>
<td>40-54 / 1-6</td>
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<tr>
<td>NK61</td>
<td>GACATGTCCTCCTGCATCTGC</td>
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<td>58-77</td>
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<td>EUR-2B52R</td>
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<td>2B</td>
<td>52-77</td>
<td>RT, PCR</td>
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Table 2. Vaccine matching by VNT.

<table>
<thead>
<tr>
<th>Genetic group</th>
<th>Field isolate</th>
<th>Asia 1 IND/8/79</th>
<th>Asia 1 Shamir</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>HKN/1/2005</td>
<td>0.35*</td>
<td>0.58</td>
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<tr>
<td>II</td>
<td>HKN/2/2005</td>
<td>0.39</td>
<td>0.87</td>
</tr>
<tr>
<td>II</td>
<td>IRN/10/2004</td>
<td>0.13</td>
<td>0.91</td>
</tr>
<tr>
<td>II</td>
<td>IRN/30/2004</td>
<td>0.58</td>
<td>&gt;1.00</td>
</tr>
<tr>
<td>II</td>
<td>IRN/31/2004</td>
<td>0.62</td>
<td>0.52</td>
</tr>
<tr>
<td>II</td>
<td>PAK/1/2004</td>
<td>0.13</td>
<td>0.74</td>
</tr>
<tr>
<td>II</td>
<td>PAK/2/2004</td>
<td>0.12</td>
<td>0.39</td>
</tr>
<tr>
<td>IV</td>
<td>MYA/5/2000</td>
<td>0.74</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>VI</td>
<td>PAK/20/2003</td>
<td>0.21</td>
<td>0.65</td>
</tr>
<tr>
<td>VI</td>
<td>IRN/58/99</td>
<td>0.17</td>
<td>0.41</td>
</tr>
<tr>
<td>VI</td>
<td>TUR/3/2000</td>
<td>0.36</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>(III)</td>
<td>BHU/36/2002</td>
<td>0.21</td>
<td>&gt;1.0</td>
</tr>
<tr>
<td>(I)</td>
<td>IRN/37/2001</td>
<td>0.23</td>
<td>0.79</td>
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<td>(II)</td>
<td>PAK/67/2003</td>
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<td>0.55</td>
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<tr>
<td>(II)</td>
<td>PAK/76/2003</td>
<td>0.11</td>
<td>0.48</td>
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*, r’ value
( ), no sequence data was obtained for these isolates, therefore probable genetic group is indicated.
Fig. 1. Recent outbreaks of FMD Asia 1 and the different genetic lineages involved.
Fig. 2. Neighbor-joining tree showing the relationships between the isolates of FMDV Asia 1 studied.