Phylogenetic characterization of foot-and-mouth disease virus recovered from mithuns and yaks in India

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ABSTRACT
The yak and mithun husbandry in India is confronted with several challenges including the prevalence of foot-and-mouth disease (FMD). The present study was initiated to investigate FMD outbreaks in semi-domesticated mithun and yak population in various parts of India. A total of 64 clinical samples (vesicle/tongue/foot epithelium/fluid) from mithun and 6 from yak were collected from suspected FMD outbreaks during 2008-2013. Supernatants of the homogenized clinical samples were tested in a serotype discriminating antigen detection ELISA and ELISA-negative samples were further subjected to multiplex reverse transcription-polymerase chain reaction (mRT-PCR). A total of 45 mithun samples and only 1 yak sample were found positive for serotype O in antigen detection ELISA. A total of 12 ELISA-negative samples from mithun and 4 from yak were later on found positive for serotype O in mRT-PCR. The phylogenetic analysis based on VP1 genome indicated the involvement of both O/ME-SA/PanAsia and O/ME-SA/Ind2001 lineages of serotype O in those outbreaks. These viruses were genetically similar to those contemporary virus isolates responsible for FMD in domestic livestock indicating a situation of virus sharing among different species of domestic and semi-domestic animals. Thus, mithuns and yaks should be synchronously considered and targeted along with cattle for the effective control of the disease in the country.

Key words: Foot-and-mouth disease, Mithun, O/ME-SA/PanAsia, O/ME-SA/Ind2001 lineage, Serotype O, Yak.

INTRODUCTION
Animal husbandry serves as a major component of Indian agriculture since time immemorial. Mithun (*Bos frontalis*), a free range semi-domesticated bovine species, also known as ‘Cattle of Mountain’ and considered as ‘Pride of north-eastern (NE) region of India’ plays a hefty role in social, cultural and economic life of the local tribes (Mondal and Pal, 1999). According to the 19th Livestock census conducted in 2012, India has 0.29 and 0.07 million heads of mithun and yak, respectively. Presently, Arunachal Pradesh has the highest mithun population in India followed by Nagaland, Manipur, Mizoram and Himachal Pradesh. However, as per the World Conservation Union, this species is vulnerable to extinction. Yak (*Bos grunniens* or *Poephagus grunniens*) has been considered as one of the threatened species globally as well. Although mithuns and yaks are the backbone of the tribal economy of NE region, they have historically been overlooked with respect to disease control measures, for which they are predisposed to an array of infectious diseases (Verma and Sarma, 1997). In India, Jammu & Kashmir has the highest yak population followed by Arunachal Pradesh, Sikkim, Himachal Pradesh and West Bengal.

Foot-and-mouth disease (FMD) is considered the most important infectious disease of cloven-hoofed (even-toed) animals and over 70 wildlife species (Jamal and Belsham, 2013). It is one of the major diseases putting Indian agriculture under stress. Furthermore, the disease compromises the health and welfare of livestock thereby incongruously influencing the livelihood of farmers and the consistency of food supply in large regions of the world. Thus, prevention and control of FMD in domestic livestock is genuinely crucial for all countries exclusively depending upon agricultural production and trade (Stenfeldt et al., 2015). Despite being a major impediment to the transboundary trade of animal products, much of the natural ecology and epidemiology of FMD in endemic regions is still poorly understood (de Carvalho Ferreira et al., 2017). Three serotypes of FMD virus (FMDV) such as O, A, and Asia 1 are circulating in India, where serotype O is blamed for nearly 80% of the outbreaks (Subramaniam et al., 2012). There is insufficiency of information available on the incidence of FMD and FMDV strain(s) circulating in mithun and yak population in the country even if frequent disease outbreaks do occur in these species. For this reason, in the present study, FMD incidences in mithuns and yaks during 2008-2013 were conscientiously investigated and the FMDV strains isolated from those suspected outbreaks were sequenced at VP1 coding region and phylogenetic comparison with the contemporary isolates from domestic.
animals was made. This was purposively believed to indirectly help in formulating better disease control and prevention strategy in these species in the country. Moreover, this could act as a useful service for the poor community and a benchmark for future researchers.

MATERIALS AND METHODS

Sample collection from various states: A total of 64 clinical samples (vesicular/tongue/foot epithelium/fluid) from mithuns (from the states of Kerala, Odisha, Arunachal Pradesh, Sikkim, Mizoram, Assam and Nagaland) and 6 from yaks (from the states of Jammu & Kashmir, West Bengal and Himachal Pradesh) were collected aseptically in 50% phosphate buffered saline/glycerol medium (pH 7.5) (Table 1) during suspected FMD outbreaks that had occurred between 2008 and 2013.

Detection of FMDV serotype in clinical samples: Supernatants of the homogenized clinical tissue materials were tested in a serotype discriminating antigen detection ELISA as per the protocol followed by Bhattacharya et al. (1996) to reveal of the serotype of the virus present in the samples. Samples found negative in typing ELISA were subsequently subjected to serotype differentiating multiplex reverse transcription-polymerase chain reaction (mRT-PCR) necessarily as described before (Giridharan et al., 2005). For this, total RNA was extracted from the tissue samples using RNeasy Mini Kit (Qiagen, Germany). Reverse transcription was performed using M-MLV reverse transcriptase (Promega, USA) and reverse primer NK61 (Knowles and Samuel, 1995) followed by mPCR using three serotype specific positive sense primers namely DHP13, DHP15 and DHP9 designed for O, A and Asia 1, respectively and the reverse primer NK61 using Hotstar Taq DNA polymerase (Qiagen, Germany). The PCR amplicons were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide.

Phylogenetic comparisons: The sequences of the VP1 coding region were generated from 8 FMDV isolates (6 from mithun and 2 from yak). The primer combination of ARS4 and NK61 (Knowles and Samuel, 1995) was used for amplification and NK61 for sequencing as described earlier (Hemadri et al., 2002). PCR products were purified using the QIAquick PCR purification kit (Qiagen, Germany) according to the manufacturer’s instructions and got sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA). Nucleotide sequences of the viruses were aligned using the CLUSTAL X multiple sequence alignment program and the alignments were used to reconstruct Maximum Likelihood (ML) phylogenetic tree using the model TN93+G+I available in MEGA 6.06 (Tamura et al., 2013). The robustness of the tree topology was evaluated with 1000 bootstrap replicates.

RESULTS AND DISCUSSION

A clear understanding of disease epidemiology is the prerequisite for successful disease control programme (Perry et al., 2002). Tracing the origin of any disease outbreak and the route of pathogen movement undoubtedly plays a key role in understanding its epidemiology, which in turn help in formulation of efficient control strategies. For this a distinct gene segment of the pathogen ought to be characterized so as to compare with many other strains of the agent. Characterization of FMDV evolution is often accomplished by phylogenetic analysis of the VP1 capsid gene/protein, which allows for the distinction of lineages within serotypes (Knowles and Samuel, 2003). Sequencing of FMDV VP1 region and clustering into lineages can be associated with a specific temporal and geographical distribution, from which the potential sources of infection may be traced out and epidemiological trends can be characterized (Cottam et al., 2008).

During the months of August and September 2012-2013, two outbreaks due to serotype O were recorded in cattle and yak of Kaza and Mandi districts of Himachal Pradesh. Two outbreaks of FMD caused by serotype O were confirmed in Arunachal Pradesh involving 95 cattle and 28 mithun. The outbreaks were recorded in Papum Pare and Lower Subansiri districts in April and June 2011. In October...

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<table>
<thead>
<tr>
<th>Species</th>
<th>Place of sample collection</th>
<th>Number of clinical samples tested</th>
<th>Positive in antigen detection ELISA</th>
<th>Positive in mRT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mithun</td>
<td>Kerala</td>
<td>1</td>
<td>1</td>
<td>-</td>
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<tr>
<td></td>
<td>Odisha</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Arunachal Pradesh</td>
<td>53</td>
<td>38</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Sikkim</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mizoram</td>
<td>5</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Assam</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Nagaland</td>
<td>2</td>
<td>2</td>
<td>-</td>
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<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>64</strong></td>
<td><strong>45</strong></td>
<td><strong>12</strong></td>
</tr>
<tr>
<td>Yak</td>
<td>Jammu &amp; Kashmir</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>West Bengal</td>
<td>2</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Himachal Pradesh</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>6</strong></td>
<td><strong>1</strong></td>
<td><strong>4</strong></td>
</tr>
</tbody>
</table>
2010, FMD due to serotype O was recorded in yak of Kargil district in Jammu & Kashmir. In the same year, two outbreaks in mithun were reported in Arunachal Pradesh. During the month of June and July 2009, two outbreaks due to serotype O occurred in Mizoram leading to high mortality in mithun population. During the same year in Arunachal Pradesh, disease outbreaks due to serotype O were again recorded in mithun and yak. The clinical FMD in the form of frank vesicular/erosive lesions in tongue and feet of mithuns and yaks along with lameness was evident in all the outbreaks. Severe drooling of saliva and vesicular lesions in the mouth parts was observed in affected mithun (Fig 1) and erosive lesions with blanched appearance in the tongue and lower dental pad was found in affected yak (Fig 2). These typical clinical signs/lesions of acute FMD have earlier been reported by Arzt et al. (2011) with variable severity depending on the causative strain and host species involved (Grubman and Baxt, 2004). In the present study, serotype discriminating antigen detection ELISA could detect serotype O in 45 samples of mithun (1 from Kerala, 1 from Odisha, 38 from Arunachal Pradesh, 1 from Sikkim, 1 from Mizoram, 1 from Assam and 2 from Nagaland) and only 1 sample of yak from West Bengal. A total of 12 ELISA-negative samples from mithun (from Arunachal Pradesh) and 4 from yak (2 from Jammu & Kashmir and 2 from Himachal Pradesh) were subsequently found positive for serotype O in mRT-PCR (Fig 3).

In the year 2007, FMD was documented in a zoological park in Kerala. The disease was first noticed in mithun, and then subsequently spread to blackbucks and wild boars kept in the zoo. Culling of mithuns and wild boars in the zoo could prevent the spread of the infection to other susceptible animals. The onset of outbreak in the zoo could be ascribed to poor biosecurity measures, zero visitor management protocol in place, outsourcing of feed and fodder, lack of prophylactic vaccination, and exposure of animal keepers to domestic animals and visitors. The viruses sampled from mithun were found to be serotype O. In phylogenetic tree, the isolates clustered in O/ME-SA/PanAsia II lineage and shared close genetic relationships (<5% nucleotide divergence at VP1 coding region) with the viruses recovered from domestic animals. During 2007, severe FMD outbreaks due to O/ME-SA/PanAsia II lineage were observed in the southern region of India (Subramaniam et al., 2012).

In 2009, when severe FMD outbreak with mortality in mithun was observed in Arunachal Pradesh, at the same time, the Government Mithun Breeding Farm at Sagalee remained free from FMD. Those farm animals were vaccinated regularly at six months interval. Suspected cases of FMD in domestic animals in the surrounding areas were also noted by local veterinarians. Deaths in mithun were due to severe maggot infestation and secondary bacterial infection after FMD associated lesions in nostrils, muzzle and inter-digital spaces of feet followed by concurrent starvation and inanition. Animals were not even in a position to breathe properly due to blockade of the nostrils, and were lying unattended in the jungle. It has been observed by the local farmers that mithuns in forests live in groups and they identify their family by smelling the muzzle, which might have catalyzed the spread of FMD in the population through inhalation of infectious virus. Phylogenetic analysis revealed
Fig 4: Phylogenetic tree estimated using Maximum Likelihood method at VP1 coding region depicting relationships of Indian FMDV serotype O isolates. Bootstrap values (>70%, out of 1000 replicates) is shown near the nodes. The isolates sampled from mithuns are marked with solid squares and those from yaks are marked with solid triangles.

Fig 3: Figure of mRT-PCR assay with 2% agarose gel electrophoresis of amplified partial VP1 gene segments corresponding to serotype O of FMDV. Here, Lane 16, 17 and 18 show positive controls against serotype O, A and Asia 1 that corresponds to 249, 376 and 537 bp, respectively. Lane 15: Negative control; Lane 19: 50 bp molecular weight marker. The positive amplification of VP1 gene segments of FMDV serotype O (249 bp) recovered from clinical samples of mithuns and yaks are shown in Lane 1 to Lane 14.
the clustering of the isolates of Arunachal Pradesh within O/ME-SA/PanAsia lineage under Middle East South Asia (ME-SA) topotype (Fig 4). Results of the phylogenetic analysis of serotype O isolates from mithun and yak indicate a high genetic homogeneity across the isolates recovered from cattle and other susceptible species indicating that the isolates are favorably commutable between species. However, no distinct association could be observed between the host species and viral lineages. By the mean time, definitive determination of the directionality of FMDV transmission could not be ascertained. In another occasion, two isolates collected from FMDV-infected yak in Jammu during 2010 bunched up within O/ME-SA/Ind2001 lineage. The O/ME-SA/Ind2001 lineage re-emerged in the year 2008 in India and has been causing maximum proportions of outbreaks in the country since then (Subramaniam et al., 2013). The analyses altogether confirm frequent virus transmission from domestic animals to mithuns and yaks in field situation.

The vaccination-based FMD control programme progressing in India covers only domestic large ruminants under its mandate. Despite a mixed farming and communal grazing system is followed by the farming community in the country, the disease in semi-domesticated mithun and yak has been reported in few outbreaks. Hence, these species should also be included under such control and surveillance programmes so as to achieve an effective control of the disease. It has been observed that compared to other domesticated animals, mithuns are more prone to FMDV infection due to its semi-wild nature sharing the grazing area with other wild animals like deer and wild pigs. The rate of morbidity in mithun is very high and the mortality rate may reach up to 60% and above. It has been hypothesized that FMDV infection in mithuns of Arunachal Pradesh is mostly due to their exposure to the ploughing bullocks brought from Assam especially during cultivation season (Kharif) (Tayo et al., 2013). Many workers have reported frequent outbreaks of FMD in mithun (Dutta et al., 1979; Verma and Sarma, 1997; Barman et al., 1999). Literature review indicated FMD in mithun caused by strain of serotype O lineage PanAsia I (OIE/FAO, 2010), serotype O (Hegde et al., 2011) and serotype Asia 1 (Verma and Sarma, 1997) in India. Joshi (1982) and Joshi et al. (1997) reported a number of outbreaks in different parts of Nepal among yaks and hybrids of yak with local cattle. Pal (1993) referred to an FMD outbreak caused by serotype A virus in yak of Sikkim during 1973. Prasad et al. (1978) previously reported FMD due to serotype O among yaks in Himachal Pradesh, where they were domesticated in the districts bordering Tibet and were in contact with cattle, sheep and goats. Serotype O FMDV had earlier been reported from yak sample obtained from Nepal (Report 1973-1974). The contact of mithuns and yaks with migratory cattle has been thought to have played an exceptional role in spreading the disease to these semi-domesticated animals as reported by Barman et al. (1999) and they have also recorded the morbidity rate of 22.90% and 24.51% in mithun and yak, respectively in their study. Verma and Sarma (1997) have described FMD due to serotype Asia 1 in mithun with morbidity as high as 42.08% and mortality up to 16.5% in Arunachal Pradesh. The intermingling of plain animals with mithun was suspected to have been the possible source of infection causing the outbreak. The prevalence could also be thought due to procurement of diseased cattle from various neighbouring villages along with probable trafficking of small ruminants that subclinically carry the virus (Kitching, 1998). Epidemiological studies of FMD in Vietnam and other countries in the Mekong region have linked many outbreaks to social and economical factors influencing animal movements (Di Nardo et al., 2011) and animal husbandry practices (Perry et al., 2002; Nampanya et al., 2013). The FMDV infection in mithun and yak may also be due to a synergetic combination of several factors including less biosecurity in the farms, more animal movement and frequent mingling in pasture with other susceptible animals, maintenance of less frequency of vaccination, vaccine in improper cold chain and veterinary care, which need to be significantly improved. It is noteworthy that FMDV serotype O is predominant in India and north-eastern states of the country involving different species of animals (Annual Report, PDFMD, 2015-2016).

An important factor of serious concern in the control of FMD is the occurrence of persistently infected, asymptomatic carriers within ruminant species (Stenfeldt et al., 2016). Mithuns and yaks being ruminants may also become carriers of FMDV after recovery from infection. The generally accepted definition of an FMDV carrier is an animal from which infectious FMDV can be recovered from oropharyngeal fluid during periods more than 28 days post infection, otherwise referred to as the persistent phase of infection (Stenfeldt et al., 2016). FMDV persistence has been demonstrated to occur in cattle, sheep, goats, Asian buffalo, and various wildlife species (Weaver et al., 2013). The length of this carrier state varies between species, with periods of up to 3.5 years in cattle (Alexandersen et al., 2002; Salt, 2004). Although the actual role of persistently infected cattle and other livestock in FMD ecology and epidemiology appears to be controversial and imprecise, these animals are still considered to be the potential sources of infection (Salt, 2004). Hence, oropharyngeal fluid from infected mithuns and yaks and those recovered from FMD should be collected and further examined for the presence of viral genome by
polymerase chain reaction or for virus isolation so as to declare a complete freedom from infection. Every single outbreak should be immediately diagnosed and meticulous scientific investigation must be ventured to make out the dynamics of FMDV circulation in these species along with regular well-timed surveillance to keep track on the circulating virus serotype(s) and strain(s).

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