Molecular epidemiologic investigation of foot-and-mouth disease in pig population of India

M. Rout*, S. Subramaniam, J.K. Mohapatra and B. Pattnaik

ICAR-Project Directorate on Foot and Mouth Disease, IVRI Campus, Mukteswar, Nainital-263 138, Uttarakhand, India.

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ABSTRACT
Foot-and-mouth disease (FMD) is a highly contagious and globally significant viral disease principally of cloven-hoofed livestock species. The present study describes the results of molecular epidemiologic investigation of FMD in pigs across various states of India between 2008 and 2014. During this period, a total of 37 clinical epithelial samples (vesicle/foot/snout epithelium) of FMD-suspected pigs were tested in a serotype differentiating antigen detection ELISA and samples found negative were further subjected to multiplex reverse transcription-polymerase chain reaction (mRT-PCR). A total of 29 (78.37%) samples were found positive for serotype O in antigen detection ELISA and 8 ELISA-negative samples were subsequently found positive for serotype O in mRT-PCR. The VP1 region-based phylogenetic analysis demonstrated the involvement of O/ME-SA/Ind2001 lineage in the outbreaks. The pig isolates clustered with the contemporary virus isolates collected from bovine indicating a close genetic relationship and therefore signifying inter-species transmission during the outbreaks.

Key words: Foot-and-mouth disease, Pig, Surveillance.

INTRODUCTION
Globally, agriculture being in a fulcrum position provides livelihood for more people than any other sector thus playing a vital role in poverty alleviation. The livestock within agricultural sector plays a paramount role for the rural households contributing to their income and welfare. Pig farming is one of the fastest growing livestock enterprises in the rural India that has become a dependable means of providing food, income and employment. Pig production being integral to their way of life, could be regarded as ‘bank account’ for the poor that can be useful during the crucial period when crop production betrays. As per the 19th Livestock Census All India Report of 2012, India has a total of 10.29 million heads of pigs. Such a huge pig population remains under progressive threat and is besieged by a large number of infectious diseases causing substantial economic losses to the farming community.

Foot-and-mouth disease (FMD) appears to be the most researched airborne viral disease in veterinary medicine (Donaldson, 1979) that affects cloven-hoofed animals and has spread across the globe, victimizing even the developed nations such as Britain and the Western European countries. This is the biggest threat to the health and productivity of livestock that hinders the international trade of animals and animal products. It immensely jeopardizes the livestock economy causing deaths in young animals and low productivity in adults (James and Rushton, 2002). FMD though endemic in the country, the vaccination-based control strategies mainly focus on cattle, while pigs are largely ignored. Three serotypes of the virus namely O, A, and Asia 1 are prevalent in India with nearly 80% of the outbreaks being attributable to serotype O (Subramaniam et al., 2013). Despite the enormous work that has been carried out on FMD in India, there appears to be little documentation of the disease in pigs with regard to characterization and phylogenetic analysis of the viruses involved in the outbreaks. More importantly, pigs being the amplifier hosts, exhale huge quantum of infectious aerosol virus and play role in spread of FMD (Alexandersen and Donaldson, 2002). The prime intention of this study therefore, was to document and investigate the FMD incidences in pigs so that their significance in the disease epidemiology is emphasized. For this purpose, the virus isolates recovered from the outbreaks in pigs were characterized based on nucleotide sequencing of VP1 gene (the most immunogenic and surface exposed protein) to comprehend the molecular epidemiology of FMD in pigs in India.

MATERIALS AND METHODS
Sample collection: The study involved FMD outbreaks occurring between 2008 and 2014 wherein pigs were affected (Table 1) across various states such as Tamil Nadu, Karnataka, Kerala, Andhra Pradesh, Tripura, Nagaland, Haryana, West Bengal, Mizoram and Assam in India. For molecular epidemiological investigation, a total of 37 clinical
Table 1. Results of antigen detection ELISA and mRT-PCR on clinical samples collected from pigs.

<table>
<thead>
<tr>
<th>Place of sample collection</th>
<th>Number of clinical samples tested</th>
<th>Positive in antigen detection ELISA</th>
<th>Positive in multiplex RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamil Nadu</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Karnataka</td>
<td>9</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>Kerala</td>
<td>9</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Tripura</td>
<td>3</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>West Bengal</td>
<td>3</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Haryana</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Assam</td>
<td>4</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>Mizoram</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Nagaland</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Andhra Pradesh</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>37</strong></td>
<td><strong>29</strong></td>
<td><strong>8</strong></td>
</tr>
</tbody>
</table>

epithelial samples (vesicle/foot/snout epithelium) from FMD-suspected pigs were collected aseptically in separate sterile plastic tubes containing 50% phosphate buffered saline/glycerol medium (pH-7.5) with proper maintenance of cold chain.

**Serotype differentiating antigen detection ELISA:** Each of the collected clinical specimens was triturated with sterile phosphate buffered saline (1gm of tissue/ml) in sterile mortar and pestle to prepare a tissue homogenate. After addition of 300μl of chloroform, the suspension was clarified by centrifugation at 3000 rpm for 15 minutes and the supernatant was collected in fresh tubes in several aliquots and stored at -80°C until used. One aliquot of supernatant of each homogenized clinical material was tested in the in-house serotype differentiating antigen detection ELISA following the procedure as described by Bhattacharya et al. (1996) for confirmation of the serotype of the virus.

**Serotype differentiating multiplex reverse transcription-polymerase chain reaction (mRT-PCR):** The mRT-PCR as described previously by Giridharan et al. (2005) was employed on ELISA-negative samples for further confirmation. For this, another aliquot of the supernatant of each homogenized tissue sample was utilized for total RNA extraction using RNeasy Mini Kit (Qiagen, Germany). The synthesis of complementary DNA was performed using M-MLV reverse transcriptase (Promega, USA) and serotype independent reverse primer NK61 (Knowles and Samuel, 1995). Then, serotype differentiating mPCR was performed with three serotype specific forward 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 products were analyzed by electrophoresis on 2% agarose gel stained with ethidium bromide.

**Nucleotide sequence comparison and phylogenetic analysis:** PCR amplification of VP1 region was performed using Pfu polymerase (Fermentas, Germany). For serotype O, the primer combination of ARS4 and NK61 (Knowles and Samuel, 1995) was used. The details of sequencing primers and thermal conditions applied were as described earlier (Hemadri et al., 2002). Cycle sequencing reactions of gel purified PCR products were assembled using the BigdyeV3.1 terminator kit and sequences were resolved on ABI 3130 genetic analyzer (Applied Biosystems, USA). Sequences were aligned using clustal W algorithm (Thompson et al., 1994). Phylogenetic analysis was conducted using MEGA 6.06 software (Tamura et al., 2013) employing the best fit nucleotide substitution model, TN93+G+I. Phylogenetic tree was reconstructed using Maximum Likelihood (ML) method to infer evolutionary history and elucidate the relationships of the sequences. The robustness of the tree topology was evaluated with 10000 bootstrap reiterations.

**RESULTS AND DISCUSSION**

In the surveyed FMD outbreaks, cattle were also affected along with pigs with clear erosive lesions in foot and mouth. The clinical signs in pigs were lameness with a drop in feed intake and some pigs remained depressed with fever. They developed vesicles around the coronets, snout and lips, while some animals developed erosive lesions in the legs. Epithelium on snout was necrosed leaving raw erosive ulcer (Fig. 1a). Severe erosions and ulcerations around the coronary band with sloughing of the claw and

![Fig 1a: Epithelium necrosed off leaving raw erosive ulcer on snout of FMD affected pig](image-url)
loss of horn from digit were observed in many of the affected pigs (Fig 1b). On the coronets of the feet, secondary infection and trauma in many cases converted them into raw jagged-edged ulcers. A total of 29 out of 37 (78.37%) samples were found positive for serotype O in antigen detection ELISA, while 8 ELISA-negative samples were subsequently found positive for serotype O in mRT-PCR, thereby providing laboratory confirmation of FMDV infection.

Fig 1b: Severe erosions and ulcerations around the coronary band of affected pig with sloughing of the claw and loss of horn from digit.

Thorough understanding of molecular structure and phylogenetic relationships of the FMDV strains is crucial in identifying their origin and movement, relationships with hosts, viral ecology and landscape epidemiology. These aspects have very important implications in designing control strategies for the disease and even in tracing the transmission pathways (Cottam, 2007). Pigs are thought to be the amplifier hosts of FMDV and are reportedly considered as high risk threats for the introduction and spread of the disease (Schembri et al., 2015). Rearing pigs and cattle together as seen in many farming systems makes the epidemiology and pattern of virus movement very complex.

VP1 is the main immunogenic protein of FMDV and can be used for characterization of the antigenic variants among circulating FMDV strains (Bachrach, 1968; Knowles et al., 2007). As it plays an important role in virus attachment, protective immunity and serotype specificity, nucleotide sequencing and phylogenetic analysis of this particular region has extensively been used to investigate the molecular epidemiology of FMDV worldwide (Du et al., 2007; Malirat et al., 2007; Ayelet et al., 2009). VP1 based sequence comparison is mostly being used for establishing genetic lineages of FMDV circulating in a region and also for deducing epidemiological link among the virus strains. There are many instances where VP1 sequence analyses have successfully been used to resolve the source of outbreak and also to track the virus movement. It is noteworthy that the VP1 gene sequences generated in this study were directly sequenced from porcine clinical samples without any cell culture adaptation thereby eliminating the probability of any mutation that usually accrue during propagation in cell culture (Sobrino et al., 1983).

The ML tree depicting phylogenetic relationships of serotype O isolates collected from pigs is shown in Fig 2. The isolates grouped within the ‘Ind2001’ lineage of Middle-East South Asia (ME-SA) topotype, precisely in the sub-lineage ‘Ind2001d’, which re-emerged in the year 2008 and has been dominating serotype O outbreaks in the country since then (Subramaniam et al., 2015). This sub-lineage provoked many outbreaks during 2013 in the southern peninsular India. The VP1 nucleotide sequences of selected FMDV serotype O strains isolated previously from India, Pakistan, Bhutan along with the Indian serotype O vaccine strain, INDR2/1975 were used to reconstruct the phylogenetic tree. The isolates from pigs clustered with the contemporary virus isolates collected from bovine. The closeness of the sequences presumably signifies that the virus strains did infect both species and also could be transmitted between species unlike the porcinophilic Taiwanese strain (O/Taiwan/97), which did not reportedly infect other cloven-hoofed species through natural route (Huang et al., 2000). This has implications in control policies with special reference to vaccination campaigns, in which all the susceptible domestic animal species including pigs should ideally be covered.

The ongoing FMD control programme in India covers only bovines with regard to vaccination ignoring a major fraction of agriculturally important FMD-susceptible animals including small ruminants and pigs. Hence, pigs are expected to have an inadequate level of protective antibody against the disease. Within the mixed farming set up, pigs attract the attention only in a few outbreaks of the disease in the country despite the report that up to 8.6 log10 TCID50 virus particles are shed per infected pig per day (Sellers, 1971). Hence, in situation where pigs become infected with FMD, a huge load of infectious virus through aerosol is liberated thereby facilitating the infection of other susceptible livestock present nearby. In some instances, the virus has also been transmitted from infected cattle to pigs as has been reported by Arora and Das (1981), where an outbreak of FMD caused by serotype Asia 1 in 1977 was reported to have occurred first in the dairy cattle and subsequently spread to pigs on the same farm. FMD in pigs in India has also been reported by Muthukumar et al. (2008). Saravana et al. (2009) described an outbreak of FMD in pigs in Namakkal, Tamil Nadu in 2007. Outbreak of FMD in an organized pig farm in Tripura state by serotype O virus was reported by Bhattacharya et al. (2001). Here it must be mentioned that the apparent prevalence estimate of FMDV 3AB nonstructural protein-antibodies (NSP-Abs) in randomly sampled bovine population of the ten states, where
the sampling from pigs was done between 2008 and 2014 (period of 6 years), ranges from 10.61% to 39.49% (Annual Reports, PDFM, 2008-2014). Therefore, the probability of bi-directional transmission of virus between cattle and pigs cannot be undervalued in an outbreak situation. Also the uncontrolled movement of livestock and no strict adherence to required zoosanitary measures in the country has facilitated the spread and made it very difficult to effectively control the disease. As long as the situation of FMD in pigs is not thoroughly evaluated, controlling infection in cattle will remain an uphill task, especially in an endemic country India.

Regular surveillance of FMD is thus very important to keep track of the circulating serotypes and strains of the virus. In India, where FMD control mainly focuses on vaccination, scientific monitoring of each outbreak regardless of the species affected backed by antigenic characterization and phylogenetic analysis will provide critical inputs for effective disease control strategies. If other species like cattle, goat or sheep flock are reared simultaneously with pigs, then the farmers should adopt preventive measures for all of them and keep a prudent vigil over the appearance of any typical clinical signs suggestive of FMD. Unfortunately, hardly any measures can prevent the airborne spread of huge aerosol infectious virus produced by pigs. In fact, there are no strict measures controlling animal movements and lack of records of comprehensive animal movements in any states of the country. However, exercising strict biosecurity measures can be able to restrict the spread. Movement of pigs should be controlled and kept to an absolute minimum. Infectious farms nearby particularly pig infected farms have been reported to pose a significant transmission risk to the neighbouring farms. Implementation of strict biosecurity measures during carcass disposal operation has been found to be very essential to reduce the risk of disease transmission to nearby farms (Hayama et al., 2015). The characteristics of a livestock area, including farm density and animal species, greatly influence the spread of FMD. Hence, consideration of the characteristics of the livestock area is again important in planning FMD control strategies (Hayama et al., 2016). Facilities where animals from different origins are commingled, such as sale-yards, animal fairs, communal grazing areas also pose a high risk for disease spread. Sale-yards are important risk factors for the introduction and dissemination of endemic and emerging animal diseases (Schembri et al., 2015). Sound on-farm management practices and biosecurity protocols are always the first line of defense against disease outbreaks (Schembri et al., 2015). Vaccination and quarantine measures should not only cover cattle but also all other FMD- susceptible animal species including pigs.
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