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Short communication

Clinico-molecular diagnosis and phylogenetic investigation of foot-and-mouth disease in small ruminant population of India

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ABSTRACT

Small ruminants (sheep and goats) are susceptible to foot-and-mouth disease (FMD), while studies with due emphasis on their role in the disease epidemiology have been meagre. The present study summarizes the results of clinico-molecular investigation of FMD in sheep and goats across several states of India during 2008–2014, where a total of 51 clinical epithelial tissue samples (vesicle/tongue/gum/foot epithelium) from sheep and 78 from goats were found positive for FMD virus (FMDV) serotype O in serotyping ELISA and multiplex reverse transcription-PCR. The VP1 region-based phylogenetic analysis demonstrated the involvement of O/ME-SA/Ind2001 lineage of serotype O virus in the outbreaks. The field viruses recovered from both small and large ruminant population during the same time period showed a close genetic relationship suggesting frequent inter-species transmission of virus. Since the disease often remains clinically camouflaged in small ruminants, the animals silently infected with FMD may pose potential threats to the in-contact livestock. Regular vaccination combined with surveillance and monitoring of protective antibody status in these species is therefore crucial to the effective control of FMD in the country.

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1. Introduction

Livestock husbandry is vital to the economy of the landless and marginal farming community. Nearly five million households in India are involved in rearing of small ruminants (sheep and goats) and associated activities. Sheep and goat husbandry immensely contribute to the agrarian economy of the country, especially in the arid and semi-arid regions, where crop or dairy farming remains economically unviable. Foot-and-mouth disease (FMD) is a highly infectious disease of cloven-hoofed animals with catastrophic effects on animals and herdsmen (Doel, 1999). The overarching effects of this dreaded menace have not only crippled the animal health and rural economy, but also the livestock industry and international trade (Zaher and Ahmed, 2014). As per the All India Report of 19th Livestock Census conducted in 2012, the total livestock population in the country is 512.05 million including 65.06 and 135.17 million heads of sheep and goats, respectively. Despite representing such a considerable proportion of India's FMD-susceptible livestock, there is still dearth of comprehensive reports elucidating their role in the epidemiology of FMD in the country. Notably, sheep have been incriminated as the cause of most of the exemplary outbreaks of FMD within and around the European Union member countries (Donaldson and Doel, 1992; Kitching, 1996; Taylor and Tufan, 1996; Ferguson et al., 2001), and also in North Africa as reported before (Mackay, 1994). In Turkey, 18.5% of the total FMD cases reported in 1996 were linked with small ruminants (Taylor and Tufan, 1996). In the 2001 epidemic in Great Britain, sheep was the first species infected in the affected farms (Ferguson et al., 2001). Other such examples clearly depicting the role of small ruminants in the transboundary spread of FMD virus (FMDV) include the introduction of FMD into Canada by sheep imported from the United Kingdom (UK) in 1875 (Krystynak, 1987); the 1978 and 1983 epidemics in Morocco (Donaldson, 1999); and the 1994 epidemic in Greece (Tsaglas, 1995). The spread of FMDV in the North Africa during 1989–1992 in Tunisia and then Algeria and Morocco was ascribed to the uncontrolled movement of massive number of sheep intended to suffice the meat demand during the religious festivals (Samuel et al., 1999). These historical examples are sufficient enough to stipulate the important role played by small ruminants in FMD epidemiology. FMD is enzootic in India and three serotypes of the virus such as O, A, and Asia 1 are prevalent with nearly 80% of the outbreaks caused by serotype O (Subramaniam et al., 2013). Despite numerous reports of FMD in cattle, there is a paucity of data available on FMD in small ruminants. The present study hence sum-

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Table 1
Results of antigen detection ELISA and mRT-PCR on clinical samples collected from sheep.

Place of sample collection	Number of clinical samples tested	Positive in antigen detection ELISA	Positive in multiplex RT-PCR
Karnataka	21	17	4
Tamil Nadu	12	4	8
Andhra Pradesh	16	4	12
Uttarakhand	2	0	2
Total	51	25	26

Table 2
Results of antigen detection ELISA and mRT-PCR on clinical samples collected from goats.

Place of sample collection	Number of clinical samples tested	Positive in antigen detection ELISA	Positive in multiplex RT-PCR
Tamil Nadu	17	4	13
Karnataka	19	5	14
Gujarat	2	0	2
Odisha	5	2	3
Uttar Pradesh	6	2	4
Uttarakhand	29	6	23
Total	78	19	59

marizes the clinico-molecular diagnosis and phylogenetic analysis of FMD outbreaks in small ruminant population in the recent years in order to understand the involvement of these animals in the disease epidemiology in India.

2. Materials and methods

2.1. Study areas and clinical sample collection

The states where suspected FMD outbreaks were reported in sheep and/or goats between 2008 and 2014 were visited and sampling was done along with questionnaire survey of the localites intended to gather information on livestock health and management practices followed in the region. A total of 51 clinical epithelial tissue samples (vesicles and/or erosive lesions on the dental pad/dorsum of the tongue/foot lesions in the coronary band/interdigital space) from sheep (from the states of Karnataka, Tamil Nadu, Andhra Pradesh, Uttarakhand) and 78 from goats (from the states of Tamil Nadu, Karnataka, Gujarat, Odisha, Uttar Pradesh, Uttarakhand) were collected in 50% phosphate buffered saline/glycerol medium (pH 7.5) (Tables 1 and 2) under cold chain. The samples were properly labeled and information regarding species, age, sex, and breed, herd type along with vaccination status was also recorded.

2.2. Virus diagnosis and serotyping

Supernatants of the homogenized clinical epithelial specimens were tested in a serotype differentiating antigen detection ELISA as per the protocol described earlier by Bhattacharya et al. (1996) for identification of the serotype of the virus. Samples found negative herein were further subjected to serotype differentiating multiplex reverse transcription-polymerase chain reaction (mRT-PCR) essentially as described previously (Giridharan et al., 2005). For this, RNA was extracted from supernatants of the homogenized clinical materials by RNeasy Mini Kit (Qiagen, Germany) and used in reverse transcription for synthesis of complementary DNA using FMDV specific reverse primer NK61 (Knowles and Samuel, 1995) and M-MLVRT (Promega, USA). Then, serotype differentiating mPCR was performed with three serotype specific forward primers namely DHP13, DHP15 and DHP9 against O, A and Asia 1, respectively and FMDV specific reverse primer NK61 using Hotstar Taq DNA polymerase (Qiagen, Germany). The amplified products were resolved on 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

2.3. Nucleotide sequencing and phylogenetic analysis

The VP1 region was amplified through PCR using *Pfu* polymerase (Fermentas, Germany). Since the involvement of serotype O was confirmed, the primer combination of ARS4 and NK61 (Knowles and Samuel, 1995) was used. The details of sequencing primers and thermal conditions applied are as described earlier (Hemadri et al., 2002). Cycle sequencing reactions of gel purified PCR products were carried out using 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 carried out using MEGA 6.06 software (Tamura et al., 2013) and employing the best fit nucleotide substitution model, TN93+G+I. Phylogenetic tree was reconstructed using Maximum Likelihood (ML) method and the robustness of the tree topology was evaluated with 1000 bootstrap replicates (Fig. 1).

3. Results and discussion

Tracing the source and route of virus movement during outbreaks of infectious diseases plays a crucial role in precise comprehension of the epidemiology and in exercising control measures. In this connection, nucleotide sequencing has been proven very useful and practical during the epidemiological study of FMD while investigating into the origin of outbreaks caused by serotype O and A in Europe over a period of twenty years (Beck and Strohmaier, 1987). Genome sequencing, being an important tool of epidemiological investigations, helps tracing the source of outbreaks (Samuel and Knowles, 2001). In the present study, a total of 25 (49.01%) clinical samples from sheep and 19 (24.35%) from goats were found positive for serotype O in antigen detection ELISA. A total of 26 ELISA-negative samples from sheep and 59 from goats were subsequently found positive for serotype O in mRT-PCR. The ML tree depicting phylogenetic relationships among serotype O isolates recovered from sheep and goats is shown in Fig. 1. The VP1 region-based phylogenetic analysis indicated the involvement of O/ME-SA/Ind2001 lineage in the outbreaks. 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 was responsible for several outbreaks in various species of animals during 2013 in the southern peninsular India. The isolates recovered from small ruminants clustered closely with the contemporary virus strains circulating in

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