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Short Communication

Identification and molecular characterization of novel and divergent HoBi-like pestiviruses from naturally infected cattle in India

N. Mishra [a,*], K. Rajukumar [a], A. Pateriya [a], M. Kumar [a], P. Dubey [a], S.P. Behera [a], A. Verma [a], P. Bhardwaj [a], D.D. Kulkarni [a], D. Vijaykrishna [b], N.D. Reddy [c]

[a] National Institute of High Security Animal Diseases (formerly HSADL, IVRI), Indian Council of Agricultural Research, Anand Nagar, Bhopal, Madhya Pradesh 462021, India
[b] Laboratory of Virus Evolution, Duke-NUS Graduate Medical School, 8 College Road, 169857, Singapore
[c] Zoetis Pharmaceutical Research Private Limited, Plot No. 16, MIDC, K.U. Bazar Post, Thane Belapur Road, Turbhe, Navi Mumbai 400705, India

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ABSTRACT

HoBi-like pestiviruses have been sporadically reported from naturally infected cattle in South America, Asia and Europe. While the closely related bovine viral diarrhoea virus 1 (BVDV-1) and BVDV-2 have been reported from cattle in India, the prevalence and diversity of HoBi-like viruses have not yet been studied. Here we report the genetic diversity and molecular characteristics of HoBi-like viruses, through systematic surveillance in cattle ($n = 1049$) from 21 dairy farms across India during 2012–2013. On the basis of real-time RT-PCR, virus isolation and nucleotide sequencing results, of the 20 pestivirus positive cattle, HoBi-like viruses were identified in 19 cattle from four farms in three states and BVDV-1b in one cattle. Phylogenetic analysis of 5′-UTR and N^{pro} region identified the circulation of two lineages of HoBi-like viruses in India, that were distinct to those circulating globally, highlighting the independent evolution of at least three lineages of HoBi-like viruses globally. Antigenic differences were also evident between the two Indian lineages. In addition to revealing that HoBi-like virus may be more widespread in Indian cattle than previously reported, this study shows greater genetic divergence of HoBi-like viruses indicating a need for continued pestivirus surveillance in cattle.

1. Introduction

Pestiviruses are economically important pathogens of livestock. The genus *Pestivirus* in the family *Flaviviridae* consists of four approved species: Bovine viral diarrhoea virus 1 (BVDV-1), Bovine viral diarrhoea virus 2 (BVDV-2), Classical swine fever virus (CSFV) and Border disease virus (BDV). But atypical bovine pestiviruses (termed HoBi-like pestiviruses), identified in cattle and buffaloes over the last decade have not yet been assigned to species (Pletnev et al., 2011). Based on genetic and antigenic analysis, HoBi-like pestiviruses are more closely related to BVDV-1 and BVDV-2 (Schirrmeier et al., 2004; Liu et al., 2009b, 2010; Decaro et al., 2011), and therefore they have been proposed to be classified as a new species, BVDV-3 (Liu et al., 2009b; Bauermann et al., 2013). BVDVs are major pestiviruses in bovine, particularly in cattle and are prevalent worldwide. Depending on virus

* Corresponding author. Tel.: +91 755 2759204; fax: +91 755 2758842.
E-mail address: mishranir@rediffmail.com (N. Mishra).

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strain and host factors, the clinical manifestation of BVDV infection ranges from apparently healthy to death. The pestivirus genome is single stranded, positive sense RNA of approximately 12.3 kb in length, flanked by untranslated regions (UTR) at both ends. The single open reading frame (ORF) expressed as a polyprotein, is cleaved into four structural proteins and seven to eight non-structural proteins (Meyers and Thiel, 1996).

Since the first detection of an atypical bovine pestivirus, strain D32/00-'HoBi', in foetal bovine serum (FBS) originating from Brazil (Schirrmeier et al., 2004), genetically similar HoBi-like viruses have frequently been identified in commercial FBS batches, mostly of South American origin but also originating from Mexico, Canada and Australia and in contaminated cells (Stalder et al., 2005; Stahl et al., 2010; Xia et al., 2011; Mao et al., 2012). Although less frequently reported than BVDV-1 and BVDV-2, natural infections with HoBi-like virus cause clinical disease similar to that induced by classical BVDV-1 and BVDV-2. However, the recent association of HoBi-like viruses with severe respiratory and reproductive disease in cattle (Decaro et al., 2011, 2012) has raised concerns.

Natural infection of cattle with HoBi-like viruses has been reported since mid 2000s in Brazil, Italy, Thailand and more recently in Bangladesh (Cortez et al., 2006; Stalder et al., 2007; Stahl et al., 2007; Kampa et al., 2009; Decaro et al., 2011; Haider et al., 2014). However, there is no information on prevalence of HoBi-like virus in India, a country with the highest cattle population in the world, whereas BVDV-1 has been reported to be widely prevalent and BVDV-2 sporadically (Mishra et al., 2004; Behera et al., 2011). Surveillance for the prevalence of BVDV in dairy cattle during 2012–2013 resulted in the detection of HoBi-like viruses in 19 cattle in four farms across three states in India. Nucleotide sequencing and phylogenetic analysis of 5′-UTR and N^{pro} gene region showed that two HoBi-like virus lineages that are distinct to previously reported HoBi-like viruses are co-circulating in cattle in India indicating circulation of at least three lineages of HoBi-like viruses globally.

2. Materials and methods

2.1. Origin of samples and clinical history

Whole blood (with and without K_2 EDTA) samples from each cattle ($n = 1049$) were collected from 21 dairy farms across eight States (Punjab, Haryana, Uttar Pradesh, Andhra Pradesh, Tamil Nadu, Chhattisgarh, Maharashtra and Gujarat) in India. Approximately, 20% of the animals from each farm were randomly sampled. The animals were of 4–36 months of age and were apparently healthy or have had a history of reproductive problems (abortion, stillbirth, early embryonic death, retention of placenta, pyometra, repeat breeding), respiratory problems (coughing, nasal discharge, pneumonia) or diarrhoea. BVDV vaccination has never been employed in any of these farms. Blood and serum samples from each animal were aseptically collected from the jugular vein in sterile vacutainers using separate needles and were shipped in ice to the laboratory within 48 h.

2.2. Real Time RT-PCR for detection and differentiation of pestiviruses

A TaqMan assay (Hoffmann et al., 2006) with minor modifications (in thermal profile) was used for pestivirus RNA detection. Viral RNA was extracted from leukocytes using RNeasy mini kit (Qiagen, Germany) following manufacturer's protocols and subjected to the panpesti TaqMan assay using Light Cycler 480 (Roche, USA). The assay targeting the 5′-UTR was conducted in 25 μl reaction volume using the primers BVD190-F (Hoffmann et al., 2006), V326 (Vilcek et al., 1994), probe TQ-Pesti (Gaede et al., 2005), 2 μl of RNA and Superscript III Platinum one-step real time RT-PCR reagent set (Invitrogen, USA). The details of primers and probes used in this study are listed in the supplementary Table 1.

Blood leukocytes found positive for pestivirus RNA by the panpesti TaqMan assay were then tested by TaqMan real time RT-PCR in uniplex format using primers and probes specific to BVDV-1, BVDV-2 and HoBi-like pestivirus, Light Cycler 480 and SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen, USA) as reported earlier (Baxi et al., 2006; Liu et al., 2008). For HoBi-like pestivirus detection, 800 nM of primers T134-F and T220-R and 400 nM of probe T155r-P (Liu et al., 2008) were used.

2.3. Virus isolation

Virus isolation was carried out on Madin Darby bovine kidney (MDBK) cells maintained in EMEM (Eagle's minimal essential medium, Sigma) containing 15% horse serum (Invitrogen). After 4 days incubation at 37 °C, the cultures were frozen thawed thrice and the clarified supernatant was passaged again on MDBK cells. The viral growth was then monitored by immunoperoxidase monolayer assay (IPMA) in 96-well TC plates, as described earlier (Mishra et al., 2008), using a pool of pan-pestivirus reacting monoclonal antibodies (mAbs) WB103/WB105 (Veterinary laboratory Agency, UK). Leukocytes obtained from all the sampled in-contact animals were also subjected to virus isolation.

2.4. Virus neutralization assay

Serum samples ($n = 367$) obtained from the four pestivirus-positive cattle herds, were heat inactivated at 56 °C for 30 min and 1:5 diluted serum was subjected to virus neutralization assay as described earlier (Mishra et al., 2008) using 96-well TC plates, MDBK cells and 200 $TCID_{50}$ of BVDV-1 cattle isolate Ind S-1449 (Mishra et al., 2004), BVDV-2 cattle isolate Ind 141353 (Behera et al., 2011) and HoBi-like virus isolate IndBHA5309/12 (this study). Ten randomly selected BVDV antibody positive serum samples from each farm were further tested at serial two-fold dilutions to determine the neutralizing antibody titre against BVDV-1, BVDV-2 and HoBi-like virus.

2.5. Antigenic characterization

For antigenic typing, a virus neutralization assay was performed using 200 $TCID_{50}$ of HoBi-like virus strain