Infection, Genetics and Evolution 50 (2017) 1–6

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Circulation of multiple subtypes of bovine viral diarrhoea virus type 1 with no evidence for HoBi-like pestivirus in cattle herds of southern Italy

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ARTICLE INFO

Article history:
Received 12 November 2016
Received in revised form 2 February 2017
Accepted 8 February 2017
Available online 9 February 2017

Keywords:
Cattle
BVDV
Pestivirus
Subtypes

ABSTRACT

Pestiviruses of cattle include bovine viral diarrhoea 1 (BVDV-1) and 2 (BVDV-2) plus an emerging group, named HoBi-like pestivirus. In the present paper, the results of an epidemiological survey for pestiviruses circulating in cattle in southern Italy are presented. Molecular assays carried out on a total of 924 bovine samples detected 74 BVDV strains, including 73 BVDV-1 and 1 BVDV-2 viruses. Phylogenetic analysis carried out on partial 5′UTR and N^{pro} sequences revealed the presence of 6 different subtypes of BVDV-1 and a single BVDV-2c strain. BVDV-1 displayed a high level of genetic heterogeneity, which can have both prophylactic and diagnostic implications. In addition, the detection of BVDV-2c highlights the need for a continuous surveillance for the emergence of new pestivirus strains in cattle farms in southern Italy.

1. Introduction

Pestiviruses are enveloped, single-stranded, positive-sense RNA viruses that belong to the family *Flaviviridae,* genus *Pestivirus*. The pestiviral genome, approximately 12.3 kb in length, contains a unique open reading frame that is flanked by untranslated regions (UTRs) at the 5′ and 3′ ends and encodes for a long polyprotein (NH2-Npro-C-Erns-E1-E2-p7-NS2-3-NS4A-NS4B-NS5A-NS5B-COOH) that is processed by viral and cellular proteases, thus generating structural and non-structural proteins (Simmonds et al., 2011).

As a consequence of their RNA genome, pestiviruses display high mutation rates, which, in some cases, may lead to the emergence of new virus lineages. The genus Pestivirus contains four recognized species i.e., Bovine viral diarrhoea virus (BVDV) 1, BVDV-2, Classical swine fever virus (CSFV) and Border disease virus (BDV) (Tautz et al., 2015). Moreover, putative pestivirus species have been isolated from domestic and wild ungulates (Bauermann et al., 2013). Among these emerging pestiviruses are Bungowannah virus, detected in swine affected by stillbirth and neonatal death in Australia (Kirkland et al., 2007) and HoBi-like pestivirus, associated with BVDV-like clinical forms in several countries (Liu et al., 2009; Decaro et al., 2011, 2012a, 2012b; Stahl and Alenius, 2012; Weber et al., 2016). Atypical pestiviruses include also a divergent pestivirus, named Tunisian-like pestivirus, detected in small ruminants, Giraffe virus, associated with the outbreak of mucosal-like disease in Kenyan giraffes, and Pronghorn virus, isolated from a pronghorn antelope in the United States (Thabti et al., 2005; Vilcek et al., 2005; Becher et al., 2014).

Ruminant pestiviruses (BVDV-1, BVDV-2, BDV and HoBi-like viruses) in cattle are of great concern due to their impact on animal productivity (MacLachlan and Dubovi, 2011; Decaro et al., 2016). Pestiviruses are known to have a certain degree of host adaptation, with BVDV, BDV and CSFV being mainly recovered from cattle, small ruminants and swine, respectively. However, they are also characterized by an interspecies transmission (Moenning, 1990).

Several regions of the viral genome have been used for the genetic characterization of ruminant pestiviruses circulating in different areas of the world. Most frequently, this characterization is based on the comparison of nucleotide (nt) sequences from the 5′UTR, the viral autoprotease N^{pro} gene or the structural E2 gene (Tajima et al., 2001; Vilcek et al., 2001; Jackova et al., 2008). According to sequence comparison based on the 5′UTR region, 21 distinct BVDV-1 (1a–1u), 3 BVDV-2, 8 BDV and 3 CSFV subtypes are currently recognized (Paton et al., 2000; Jenckel et al., 2014; Deng et al., 2015; Giammarioli et al., 2015a; Peletto et al., 2016).

Several epidemiological surveys have proven that BVDV-1 is the predominant pestivirus circulating in European cattle population, although very recently BVDV-2 outbreaks have been reported (Polak et al., 2014; Schirrmeier, 2014; Aduriz et al., 2015; Decaro et al., 2016). BVDV-1b and BVDV-2a are the main subtypes detected in Europe (Kuta et al., 2013; Polak et al., 2014; Giammarioli et al., 2015a; Aduriz et al., 2015), while

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http://dx.doi.org/10.1016/j.meegid.2017.02.009
1567-1348/

BDV is displaying a more complicated evolutionary history with several subtypes being detected in domestic and wild ungulates (Giammarioli et al., 2015b; Peletto et al., 2016). The pestivirus epidemiology in Italy mirrors the European situation, with BVDV-1 being prevalent in cattle (Giammarioli et al., 2015a), while BVDV-2 has been found only sporadically (Luzzago et al., 2001; Decaro et al., 2004). Interestingly, BVDV-2c has been proven to circulate in southern Italy, mainly in sheep (Decaro et al., 2016), and Tunisian-like pestiviruses have been detected in small ruminants in Sicily (Ciulli et al., 2016).

In the present paper, the results of an epidemiological survey for emerging pestiviruses in cattle in southern Italy are reported, accounting for the circulation of different subtypes of BVDV-1 along with the sporadic detection of a BVDV-2c strain.

2. Material and methods

2.1. Sample collection

Samples were collected from 138 cattle herds during an epidemiological survey for bovine pestiviruses carried out in three different regions of southern Italy (Apulia, Basilicata and Sicily) in the period 2009–2016. A total of 920 bovine biological samples were analysed, consisting of 795 EDTA blood samples and 5 sera from herds with a history of reproductive failures, 2 faecal samples from cattle with enteritis, 2 placentas and 9 tissue samples from aborted foetuses, 25 respiratory specimens from calves with respiratory disease, 66 milk samples from cows with mastitis, 16 tissue samples (spleen and/or lungs) from dead animals.

2.2. RNA extraction

RNA was extracted from samples using the QIAamp® cador®Pathogen Mini Kit (Qiagen S.p.A.), according to the manufacturer's instructions.

2.3. Panpestivirus real-time RT-PCR

A real-time RT-PCR developed for detection of all pestiviruses circulating in ruminants (Losurdo et al., 2015) was used as a first screening. This assay has been proven to recognize all pestiviruses circulating in cattle, including the emerging HoBi-like strains, as well as BDV (unpublished data). Oligonucleotide sequences and positions are reported in Table 1.

Reverse transcription (RT) was carried out in a 20-μl reaction mix consisting of PCR buffer 1× (KCl 50 mM, Tris–HCl 10 mM, pH 8.3), $MgCl_2$ 5 mM, 1 mM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), RNase Inhibitor 1 U, MuLV reverse transcriptase 2.5 U, random hexamers 2.5 U (MuLV reverse transcriptase kit, Applied Biosystems, Life Technologies, Milan, Italy), and 1 μl of standard RNA or RNA extract. Reverse transcription was carried out at 42 °C for 30 min, followed by a denaturation step at 99 °C for 5 min.

Real-time PCR was performed in a 25-μl reaction mixture containing 12.5 μl of iTaq™ Universal Probes Supermix (Bio-Rad Laboratories Srl), 600 nM of primers, 200 nM of probe and 10 μl of c-DNA. The thermal protocol consisted of activation of iTaq DNA polymerase at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 48 °C for 30 s and extension at 60 °C for 1 min.

2.4. Triplex real-time RT-PCR for characterization of bovine pestiviruses

All pestivirus positive samples were subjected to a triplex real-time RT-PCR assay targeting the 5′UTR sequence for rapid characterization of BVDV-1, BVDV-2 and HoBi-like pestivirus (Mari et al., 2016). Oligonucleotide sequences and positions are reported in Table 1. RT and real-time PCR mixtures and thermal conditions were as for the panpestivirus assay, with the exception of the real-time PCR annealing and extension that were unified in a single step at 60 °C for 1 min.

Table 1
List of oligonucleotides used in this study.

Assay	References	Primer/probe	Sequence 5′-3′	Sense	Position	Specificity	Amplicon size (bp)
Panpestivirus real-time RT-PCR	Losurdo et al., 2015	Pesti-qF	GATGCCATGTGGACGAGGGC	+	229-248[a], 232-251[b], 116-135[c], 218-237[d]	BVDV-1, BVDV-2, HoBi-like, BDV	195[a], 192[b], 199[c], 193[d]
		BVDgen-R	TATGTTTTGTATAAAAGTTCA	−	403-423[a], 403-423[b], 294-314[c], 390-410[d]		
		BVDgen-Pb	FAM- CTCTGCTGTACATGGCACATG-TAMRA	−	368-388[a], 368-388[b], 259-279[c], 355-375[d]		
Multiplex real-time RT-PCR	Mari et al., 2016	Pesti-qF	GATGCCATGTGGACGAGGGC	+	229-248[a], 232-251[b], 116-135[c]	BVDV-1, BVDV-2, HoBi-like	160[a], 157[b], 164[c]
		Pesti-qR	CATGTGCCATGTACAGCAGAG	−	368-388[a], 368-388[b], 259-279[c]		
		BVD1-Pb	FAM- CAATACAGTGGGCCTCTGCAGCA-TAMRA	−	334-356[a]	BVDV-1	
		BVD2-Pb	VIC-GTGGCGTTATGGACACAGCCTG-BHQ2	+	307-328[b]	BVDV-2	
		BVD3-Pb	TexasRed-ATCAGGCTGTACTCCCAAAG-BHQ2	−	200-219[c]	HoBi-like	
RT-PCR amplification of 5′UTR and N^{pro}	Vilcek et al., 1994	324	ATGCCCWTAGTAGGACTAGCA	+	108-128[a], 108-128[b], −3-18[c], 98-118[d]	BVDV-1, BVDV-2, HoBi-like, BDV	288[a–c], 285[d]
		326	TCAACTCCATGTGCCATGTAC	−	375-395[a], 375-395[b], 266-286[c], 362–382[d]		
	Nagai et al., 2004	390	CTCTGCTGTACATGGCACATGGA	+	368-390[a], 368-390[b], 262-284[c], 355-377[d]	BVDV-1, BVDV-2, HoBi-like, BDV	1081[a,b], 1078[c],1075[d]
	Becher et al., 1997	1400	ACCAGTTGCACCAACCATG	−	1430-1448[a], 1430-1448[b], 1321-1339[c], 1411-1429[d]		
	This study	1516	GCCTGATAGGGYGYWGCAGAG	+	323-343[a], 324-344[b], 216-236[c]	BVDV1, BVDV-2, HoBi-like	
		1500	KTTTKGTYGTTTCACACATR	−	715-734[a]	BVDV1	412[a]
		1511	YATAGTCYTGATAGTAGACTGGC	−	643-665[b]	BVDV-2	342[b]

[a] Oligonucleotide positions and amplicon sizes are referred to the sequence of BVDV-1 strain NADL (GenBank accession no. M31182).
[b] Oligonucleotide positions and amplicon sizes are referred to the sequence of BVDV-2 strain New York (GenBank accession no. AF502399).
[c] Oligonucleotide positions and amplicon sizes are referred to the sequence of HoBi-like pestivirus strain Italy-1/10-1 (GenBank accession no. HQ231763).
[d] Oligonucleotide positions and amplicon sizes are referred to the sequence of BDV X818 (GenBank accession no. AF037405).