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Short communication

First detection of kobuvirus in farm animals in Brazil and the Netherlands

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ABSTRACT

Animal kobuviruses have been described in pigs, cattle, sheep and bats in countries in Asia and Europe. The virus can be detected in fecal and serum samples of infected animals with or without diarrhea, but most of the clinical as well as epidemiological features of kobuvirus infection are still unknown. This study reports the first detection of kobuvirus in farm animals from Brazil and the Netherlands and the molecular analysis of the detected strains. In Brazil, 53% (61/115) of the pigs (suckling, weaned and sows) were shedding porcine kobuvirus in feces, while in the Netherlands 16.7% (3/18) of the tested weaned pigs were infected. Kobuviruses detected in fecal samples of pigs in Brazil showed association ($p = 0.0002$) with diarrhea. In pig serum, kobuvirus was detected at different ages (3, 21, 36, 60, 75, and 180 days), with an overall rate of 76.7% (23/30). The sequencing of amplicons detected in serum of pigs of different ages suggested reinfection and no persistent infection. Kobuvirus was also detected in sheep and cattle feces from Brazil and the Netherlands, respectively. Phylogenetic analyses of Brazilian and Dutch kobuviruses from pig, cattle and sheep revealed genetic variability, particularly in one strain detected in sheep feces, which was more closely related to human Aichi virus. The molecular and phylogenetic analyses performed with other published kobuvirus strains and the strains presented in this study, showed that, in most of the cases, kobuvirus seems to group according to host species, but not to geographical region of origin. The data presented in this study contribute to the comprehension of kobuvirus epidemiology and also to the molecular identification of kobuvirus strains circulating worldwide.

1. Introduction

Kobuvirus was first detected in fecal samples from humans with gastroenteritis after the consumption of raw oysters (Yamashita et al., 1991). Later, similar viruses were described in cattle, pigs, sheep, and bats (Yamashita et al., 2003; Reuter et al., 2008, 2010a; Li et al., 2010).

Kobuvirus is a genus of the Picornaviridae family that also includes 11 other genera: *Aphtovirus*, *Avihepatovirus*, *Cardiovirus*, *Enterovirus*, *Erbovirus*, *Hepatovirus*, *Parechovirus*, *Sapelovirus*, *Senecavirus*, *Teschovirus* and *Tremovirus*. Kobuvirus has two species: *Aichi virus*, which can infect humans, and *Bovine kobuvirus* that infects cattle and sheep. The porcine kobuvirus is a candidate species (Reuter et al., 2011).

Kobuviruses are non-enveloped with a 27–30 nm diameter and an icosahedral symmetry. The genome is a linear positive-sense, single-stranded RNA molecule of 8.2–8.4 kb with a VPg linked to the 5′ end and a poly (A) tail at 3′. UTR at both extremities are also present in the genome of kobuviruses (Reuter et al., 2011). The virus has only one open reading frame (ORF) encoding for a single polyprotein that is cleaved in structural (VP0, VP3 and VP1), and non-structural (2A–2C, 3A–3D) proteins (Yamashita et al., 1998).

The detection of kobuvirus in pig feces has been reported from a few countries in Europe and Asia (Reuter et al., 2008; Yu et al., 2009; Khamrin et al., 2009, 2010; Park et al., 2010), and also in pig sera (Reuter et al., 2010b). The prevalence of infection in pigs ranges from 30% to 99%. This large variation may be due to different ages of evaluated populations, presence or absence of gastroenteritis, and perhaps other factors. Also, in cattle just a few studies have been performed and most of these were conducted in a limited number of samples, which just allows the conclusion that the virus can be detected in fecal and serum samples (Yamashita et al., 2003; Khamrin et al., 2008; Reuter and Egyed, 2009; Mauroy et al., 2009; Park et al., 2011). In a recent study in cattle, the association between infection and age was also raised, as described in pigs (Jeoung et al., 2011). In both sheep and bat, kobuvirus was described in only one study (Li et al., 2010; Reuter et al., 2010a). Interspecies transmission between pig and cattle and vice versa has been suggested in two independent studies, however, the possibility of a passive infection could not be excluded (Khamrin et al., 2010; Park et al., 2011).

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The present study describes the presence of kobuviruses in feces of pigs, cattle, and sheep from Brazilian and Dutch herds and detection of porcine kobuvirus in serum of pigs of different ages. Additionally, a phylogenetic analysis of the detected strains was performed.

2. Materials and methods

2.1. Specimen collection

In Brazil, 115 fecal samples were collected from June/2009 to February/2010 from one pig herd, including suckling, weaned, and adults animals, with ($n = 37$) or without ($n = 78$) diarrhea. From the same pig farm, successive serum samples were collected from five animals of 3, 21, 36, 60, 75, and 180-day-old.

In July 2010, 23 fecal samples were collected from diarrheic ($n = 9$) and non-diarrheic ($n = 14$) sheep in one Brazilian sheep herd. The animals were from 1 to 7 months old.

In the Netherlands, fecal samples from 18 weaned piglets with ($n = 5$) and without diarrhea ($n = 13$) were collected from 12 herds in 2008. In 2007, fecal samples ($n = 9$) of 12- to 14-day-old asymptomatic calves were collected from five herds.

2.2. Kobuvirus detection

The nucleic acid extraction of the Brazilian samples was performed according to Boom et al. (1990). For the Dutch samples, the QIAamp® MinElute® Virus Spin kit (QIAGEN, Venlo, The Netherlands) was used. The RT-PCR was performed using the primers UNIV-kobu-F/R, which had been designed based on human, bovine, and porcine kobuvirus strains, and target a region of the RdRp gene (Reuter et al., 2009). Electrophoresis was performed in ethidium bromide stained 2% agarose gel and RT-PCR products were visualized under UV light.

2.3. Statistical analysis

Data analyses were performed by chi-square (χ^2) with confidence limits of 95%, $p < 0.05$ in EpiInfo version 3.3.2.

2.4. Sequencing and phylogenetic analyses

Purification was performed with GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Pittsburgh, USA) and Zymoclean™ Gel DNA Recovery Kit (Zymo Research, CA, USA) for the Brazilian and Dutch amplicons, respectively. Sequencing was performed in both directions using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) and run in ABI 3730 DNA Analyser. BioEdit vs7.0.9 and MEGA 4.1 software were used for the molecular and phylogenetic analyses, respectively.

3. Results and discussion

From the Brazilian porcine fecal samples, 61 (53%) of 115 were positive for kobuvirus (Table 1). There was association ($p = 0.0002$) between infection and diarrhea, with 29 (78.4%) of 37 diarrheic and 32 (41%) of 78 non-diarrheic samples presenting the virus. However, a causal relationship could not be proven, since 27 (44.3%) of the 61 kobuvirus infected pigs presented mixed infections including other enteric viruses (rotavirus, picobirnavirus and/or sapovirus – data not show). The highest (56/64; 87.5%) kobuvirus detection was from nursing piglets (until 21-day-old) indicating a high susceptibility of young animals to infection, possibly due to an inefficient immune response or other intrinsic age-related factors. However, further studies are necessary to support any idea on risk factors for this infection. Higher frequency rates in young piglets were also described elsewhere (Reuter et al., 2009; Park et al., 2010; An et al., 2011). In contrast, only two (11.8%) of the 17 tested sows were shedding the virus in feces at the time of sampling, which could indicate that young animals more often demonstrate productive infection, or that the fecal–oral route may not be the only mode of viral transmission from the mother to piglets in early life.

Table 1
Frequency of kobuvirus RNA in biological samples evaluated by RT-PCR assay.

Country	Host	Sample	Age	Positive/tested	Total
Brazil	Pig	Feces	4–21 days	56/64 (87.5%)	61/115 (53%)
			28–60 days	3/34 (8.8%)	
			>1 year (sows)	2/17 (11.8%)	
		Serum*	3 days	5/5 (100%)	23/30 (76.7%)
			21 days	1/5 (20%)	
			36 days	5/5 (100%)	
			60 days	4/5 (80%)	
			75 days	4/5 (80%)	
			180 days	4/5 (80%)	
	Sheep	Feces	1–7 month		9/23 (39.1%)
The Netherlands	Cattle	Feces	12–14 days		7/9 (77.8%)
	Pig	Feces	Weaned		3/18 (16.7%)

* Serum samples collected from five pigs with different ages.

In pig serum samples, kobuvirus was detected in all age groups (Table 1). This finding is in agreement with other studies performed with pig serum samples (Reuter et al., 2010b). All piglets were infected at the first sampling (3 day-old), but since only 11.8% of the sows were shedding the virus in the feces, this may indicate an alternative route of infection, for example through milk, blood (handling procedures), urine, saliva, or even aerosols as for other picornaviruses (Reuter et al., 2010b). At 21 days of age, only one of the five piglets presented kobuvirus in serum. This viral clearance indicates that the viremia of the first infection did not last until 21 days of age. The justification for such needs further study of immune-development in kobuvirus infection. The observed viral clearance also demonstrates that the animals were not persistently infected, i.e. kobuvirus not circulating in the blood during a pigs entire life. The 100% kobuvirus frequency at 36 day-old, showed kobuvirus reinfection in four (80%) of the five piglets. Weaning in Brazil is normally performed at 21 days, when the piglets go to the nursery with animals from other litters. The stress caused by changes in feeding and environment could lead to immunosuppression and predisposition for infections. In contrary to the high frequency in serum, and such as verified in sows, only 8.8% (3/34) of the weaned piglets tested positive for kobuvirus in feces. A possible explanation for this finding may be that some protection obtained after the first infection was preventing the shedding of the virus in feces, such as described in other enteric virus studies (Hodgins et al., 1999). These results also suggest the existence of another source of virus elimination. In this case, milk and blood could be excluded since the animals were not weaning and did not undergo any procedure for blood transfer. The importance of kobuvirus detection in serum needs further investigation since it has not been defined yet if the intestines are the main organ for virus replication and if the virus escaped from the intestinal tract to blood or was just eliminated via feces with replication occurring in other organs.

To confirm the possibility of reinfection, amplicons obtained from serum samples from one pig, but at different ages, were sequenced. The animal was negative at 21 and 60 days. The molecular analysis of three amplicons revealed that the animal was infected with the same strain at days 3 and 180, but with a different strain at 36 days of age. These results indicate at least three

different infections. Since the animal was re-infected with the same strain later in life, it is possible that kobuvirus immunity is not long-lasting. Co-infection with two different strains could also have occurred at 36 days, but in this case only one strain was detected in the RT-PCR. Since the identification of the strain was based on a fragment of the RdRp gene, which was shown to be the more conserved gene in porcine kobuvirus, it is very likely that these two strains are different and do not just result from the constant changes in the RNA genome of kobuvirus. In a recent study, it was shown that the estimated mean substitution rate in this part of RdRp gene of porcine kobuvirus is 1.3×10^{-2} substitutions/site/year, which indicates that the two infections at 3 and 36 days (33 days apart) were not resulting from virus mutations (Park et al., 2011).

The sequencing of 15 and eight amplicons from porcine fecal and serum samples, respectively, confirmed the RT-PCR specificity. Despite the fact that all samples were obtained from the same pig herd, the porcine kobuvirus detected in Brazil showed genetic variability (90.9–99.3%). The sequences from fecal samples appeared to be more variable, compared to the serum samples: 91.5–100% versus 97.2–100%. This result reveals the circulation of different strains at the same time. On the other hand, the detection of 18 identical sequences (six of eight serum and 12 of 15 fecal samples), and just a few different ones, suggests the predominance of one strain. In the phylogenetic analysis, all sequences clustered with other porcine kobuvirus strains including the prototype S-1-HUN (Fig. 1).

In sheep, the 216 bp RT-PCR fragment was amplified in nine (39.1%) of the 23 stool samples evaluated. Molecular and phylogenetic analyses were performed using three sequences. Two sequences grouped with bovine kobuvirus strains and with the TB3 strain, the only other sheep kobuvirus strain described (Reuter et al., 2010a). However, these sequences showed a relatively high (71.6–95.8%) genetic variability to each other. BRA11-sheepKobu was distant from bovine/sheep strains and formed a cluster with Aichi virus, the prototype of human kobuvirus (Fig. 1). Unlike other studies which describe the detection of bovine kobuvirus in a pig fecal sample and vice versa, the grouping of BRA11-sheepKobu (from sheep) with Aichi virus, does not suggests interspecies transmission (Khamrin et al., 2010; Park et al., 2011). The pairwise distance between BRA11-sheepKobu and Aichi (0.334) and TB3 (0.309) strains, and the amino acid phylogenetic analysis that clustered these strains separately, indicate that BRA11-sheepKobu possibly evolved differently from other bovine/sheep kobuvirus strains. However, further studies including the capsid gene must be performed to identify the strain origin.

In the Netherlands seven (77.8%) of the nine calf stool samples presented the virus, and three (16.7%) of the 18 pig fecal samples tested positive (Table 1). The kobuvirus frequency in bovines was higher than described in other studies from Japan (16.7%), Thailand (8.3%), Hungary (6.25%), and Korea (34.6%) (Yamashita et al., 2003; Khamrin et al., 2008; Reuter and Egyed, 2009; Jeoung et al., 2011). However, since only a few samples were tested, this may not reflect the real prevalence of infection in Dutch cattle. In swine, the rate of kobuvirus infection was lower compared to the overall Brazilian frequency. However, taking into account only weaned pigs, the rate of kobuvirus infection in the Netherlands (16.7%) was higher than what was found (8.8%) in Brazilian weaned piglets. In order to perform the identification of the kobuvirus strains, two amplicons of each host species were sequenced. The bovine kobuvirus sequences showed genetic variability (92.3%), but both grouped with the bovine prototype U-1. The porcine strains were also different (90.2%) but in the phylogenetic analysis grouped with other porcine kobuvirus strains (Fig. 1).

In general, a phylogenetic analysis of just a small fragment of a conserved gene of a virus, such as RdRp, is not enough to characterize a strain. For such, the sequencing of larger gene parts encoding structural protein would be preferable. However, the grouping of

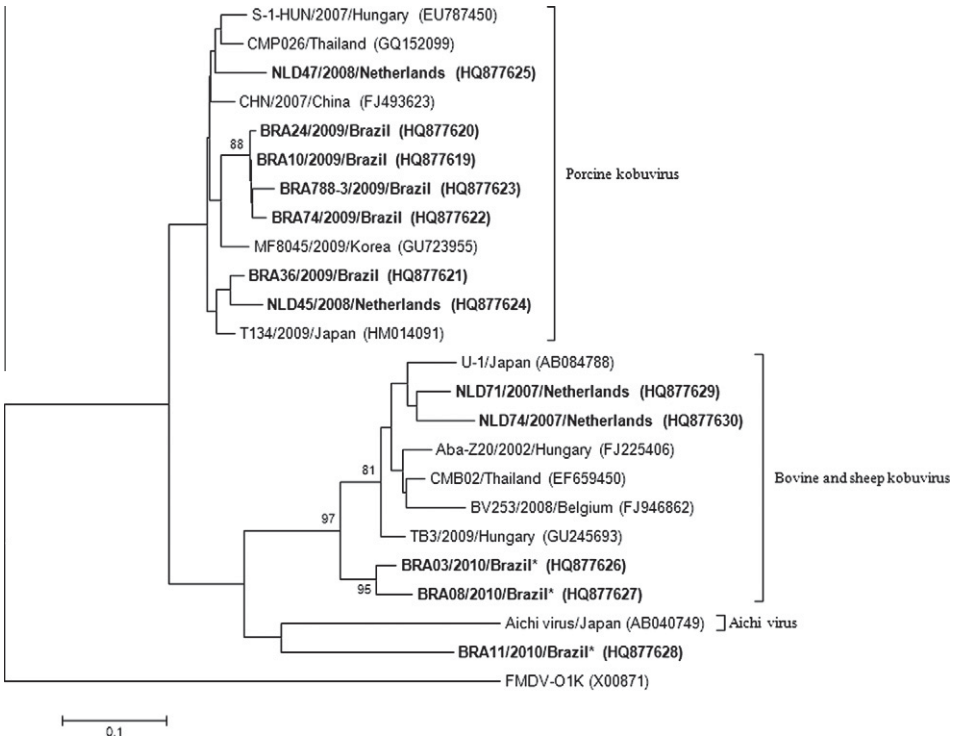

Fig. 1. Neighbor-joining phylogenetic tree reconstruction using the Tamura–Nei model based on partial (141 nt) polymerase 3D gene. Bootstraps values (1000 replicates) higher than 70% are shown. GenBank accession numbers of reference strains, Brazilian and Dutch kobuvirus are indicated between parentheses. New sequences from Brazil and the Netherlands are in boldface. *Kobuvirus detected in sheep.

kobuvirus strains according to host species and the relatively low number of interspecies transmissions described in other studies, suggests that kobuvirus infections are not species-specific but seem to be rather well-adapted to their hosts. Evolutionary analysis of different regions of the kobuvirus genome also indicates host adaptation (Reuter et al., 2010b). The porcine strains show less variability than bovine/sheep kobuvirus. For the latter group it has recently been proposed to classify it into at least four clusters (Jeoung et al., 2011). Kobuvirus strains presented in this study were from America and Europe and in the phylogenetic analysis grouped with strains from Asia and other European strains. From this observation it can be concluded that the strains do not seem to group according geographical regions.

This is the first description of kobuvirus in the American continent and in the Netherlands. Since the virus was detected in different animal species from two distinct and not related countries, it is possible that kobuviruses circulates worldwide. The successive detection of the virus in sera from pigs in different ages and the high rate of viral shedding only in suckling piglets can contribute to the understanding of kobuvirus epidemiology. The second description of kobuvirus in sheep in the world and its genetic variability reinforces the need of prevalence studies in other animal species than pigs and cattle. However, the real importance of kobuvirus for animal health is still largely unknown and need further clinical and epidemiological studies.

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