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Kobuvirus shedding dynamics in a swine production system and their association with diarrhea

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ABSTRACT

Porcine kobuviruses are widely distributed in swine, but the clinical significance of these viruses remains unclear, since they have been associated with both diarrheic and healthy pigs. In addition, there is a paucity of data on *Kobuvirus* prevalence in Canadian pig herds. In this study, a total of 181 diarrheic and healthy piglets were monitored and sampled on four occasions, intended to represent the different stages of production. The piglets were sampled at the nursing farms (birth to weaning stage), at the nursery farms (post-weaning stage), and at finishing farms (at the beginning and the end of the fattening stage). Fecal and environmental samples were collected during each life stage. Following viral extraction, *Kobuvirus* detection by RT-PCR was conducted, and positive samples were sequenced. During the late-nursing stage (6–21 days old), piglets with diarrhea shed more *Kobuvirus* than healthy individuals. Piglets shed more *Kobuvirus* during the post-weaning stage (nursery farms) than during any of the other life stages. This was evidenced in individual samples as well as in environmental samples. Over 97% of the sampled piglets shed *Kobuvirus* at least once in their lifetime. All piglets shedding a *Kobuvirus* strain or mix of strains at the nursing stage did not appear to shed another porcine kobuvirus strain at a later life stage. Overall, our findings throw light on *Kobuvirus* shedding dynamics and their potential role in neonatal diarrhea at the nursing stage, which appears to be the point of entry for kobuviruses into swine production systems.

1. Introduction

Kobuvirus is a genus in the *Picornaviridae* family composed of small, non-enveloped viruses with a single-stranded, positive-sense genomic RNA (Reuter et al., 2009; ICTV, 2019). The *Kobuvirus* genus is divided into six species, *Aichivirus A* to *F*, infecting a variety of hosts, including humans, cattle, pigs, sheep, goats, ferrets, bats, dogs, and cats (Reuter et al., 2011; Khamrin et al., 2014; Lu et al., 2018). *Aichivirus C* is the only *Kobuvirus* specie reported infecting pigs and is therefore also referred to as "porcine kobuvirus" (Khamrin et al., 2014). The 8.2kb porcine kobuvirus genome encodes a single polyprotein consisting of a leader protein, three structural capsid proteins (VP0, VP3, and VP1) and seven non-structural proteins (2A to 2C and 3A to 3D) (Yamashita et al., 1998; Reuter et al., 2009).

Since the discovery of *Kobuvirus* in swine from Hungary in 2007, porcine kobuvirus has been found to be widely distributed around the world, including in Asia, Europe, Africa, and the Americas (Khamrin et al., 2009; Barry et al., 2011; Verma et al., 2013; Amimo et al., 2014; Zhou et al., 2016). The detection rate of porcine kobuvirus varies greatly, from as low as 13.1% in Kenya (Amimo et al., 2014) to as high as 99% in Thailand (Khamrin et al., 2009). A few studies have sampled pigs from different life stages and reported the following rates of *Kobuvirus*-shedding pigs: 29.3% in Vietnam (Van Dung et al., 2016), 52.4% in Italy (Di Bartolo et al., 2015), 53% in Brazil (Barry et al., 2011), 56.7% in Europe (Zhou et al., 2016), and 87.3% in Czech Republic (Dufkova et al., 2013). Untargeted high-throughput sequencing studies have also revealed porcine kobuvirus in pig fecal samples (Chen et al., 2018; Theuns et al., 2018).

Kobuviruses are thought to be transmitted by the fecal–oral route, infecting the gastrointestinal tract. Transmission through breastfeeding, blood, or food has also been reported (Reuter et al., 2011; Khamrin et al., 2014). The clinical role of kobuviruses is still unknown, since they have been detected in both diarrheic pigs (Khamrin et al., 2009; Park et al., 2010; Cromeans et al., 2014; Van Dung et al., 2016; Almeida et al., 2018) and non-diarrheic pigs (Dufkova et al., 2013; Goecke et al., 2017; Jackova et al., 2017). A statistically significant association has

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been reported between the detection of *Kobuvirus* and clinical signs of diarrhea (Zhou et al., 2016). In some cases, kobuviruses have been reported as the sole enteric pathogen detected in diarrheic pigs (Park et al., 2010; Almeida et al., 2018), cattle (Ribeiro et al., 2017), and felines (Lu et al., 2018), emphasizing the need for further investigation.

Swine producers rely on healthy herds to meet the increasing demand for pork and pork products, and diarrhea in young piglets remains one of the most predominant pathological conditions (Zhou et al., 2016), causing important economic losses through retarded growth, high cost of treatment/management and sometimes even death. Therefore, the pathological role of *Kobuvirus* in piglets warrants further investigation. In addition, while porcine kobuviruses are thought to be shed at a higher frequency between 3 and 8 weeks of age (Dufkova et al., 2013; Di Bartolo et al., 2015), little is known about the shedding dynamics in other stages in a production system, the possible sources of entry onto farms, and how *Kobuvirus* shedding and the strains involved may be related to diarrhea. The aim of this study was to evaluate the presence of porcine kobuvirus in a Canadian swine production system along the major stages of a pig's life and to determine its role in porcine diarrhea. Additionally, phylogenetic analyses of *Kobuvirus*-positive samples were conducted to better understand the *Kobuvirus* strain diversity at each of the pig life stages.

2. Methods

2.1. Study design and sampling protocol

In this study, the presence of *Kobuvirus* was assessed from pig rectal swabs and fecal samples. In addition, composite environmental samples and surface swabs were taken throughout the farms. All farms included in the study were part of a farrow-to-finish swine production system, namely, nursing farms (piglets less than 3 weeks old and their sows), nursery farms (piglets 3 to 9 weeks old), and fattening farms (pigs 9 to 23 weeks old). Each nursing farm was selected by a veterinarian on the basis of diarrheic episodes occurring on the farm, and diarrheic and healthy piglets from each of the selected farms were sampled. Once collected, each sample was categorized in one of three groups, depending on the piglet's diarrhea status and their pen mates'. Piglets with symptoms of diarrhea were in group 1, clinically healthy piglets sampled in a pen where at least one other piglet from the same litter had diarrhea were in group 2, and healthy piglets in a healthy litter were in group 3. Fecal samples were also taken from sows that had a piglet sampled in their pen. The piglets were then monitored individually, once at the nursery stage and twice during the fattening period (at the beginning and at the end).

The study was conducted between November 2013 to January 2015, where rectal swabs or fecal samples of 8 g or less were collected from 181 piglets at the nursing stage (< 3 weeks of age). When the nursing piglets were sampled, ear tags were placed on both ears to facilitate tracking. The piglets were then monitored at the nursery stage (5 weeks of age), early and late fattening stages (12, and 20 weeks of age), when fecal or rectal swab samples were also collected. Only 126 piglets had individual samples taken at the 4 life stages; 55 of them either died or were lost while being transported from one farm to another.

Of the 126 followed piglets, 43 were in group 1, 15 in group 2 and 63 in group 3. However, the complete 181 piglets sampled in the nursing farms were analyzed before removing the 55 dead or lost piglets to assess the diarrhea effect on nursing piglets separately. Due to the high variability of the age of the pigs sampled at the beginning in the nursing farms, only this life stage was analyzed with all 181 piglets divided in two groups depending on their age (Table 1).

In addition to the individual samples from the piglets, 85 fecal samples were also collected from the piglets' respective sows. A total of 11 nursing farms, 14 nursery farms, and 14 fattening farms were sampled, with some farms visited more than once, for a total of 59 visits. Environmental surface swabs of 300 cm^2, which were made up of dust and debris from around the farms including shovels, wood panels, and the surface of feed delivery tubes, were collected from the nursing (n = 70) and nursery (n = 72) farms by means of individually sterilized metal clamps and sterile sponges (Nasco Whirl-Pak Speci-Sponge Bags; Fischer Scientific, Ottawa, ON, Canada) pre-moistened with 5 mL of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Mississauga, ON, Canada). Composite samples, which were made up of feces and debris collected from the pen floors, were collected with sterile wood sticks in weeks 5 (n = 76), 12 (n = 41), and 20 (n = 25). A total of 142 composite samples from the pen floors and 142 swabs from different surfaces found throughout the farms were sampled. Once collected, all samples were placed in individual sterile bags, transported on ice, and stored at −80 °C until treatment. Overall, a total of 928 samples were collected and processed.

Table 1
Piglets sampled in nursing farms (< 3 weeks), divided by age and diarrhea status.

	Age at sampling (days)		
Group	early (< 6)	late (6-21)	total (0-21)
1	39	22	61
2	12	11	23
3	40	57	97
total	91	90	181

Group 1: piglets with diarrhea at < 3 weeks.
Group 2: healthy piglets < 3 weeks sampled in a pen where there was diarrhea.
Group 3: healthy piglets < 3 weeks sampled in a healthy pen.

2.2. RNA extraction

The fecal and composite samples were prepared in a 20% (w/v) phosphate buffered saline (PBS) (WISENT Inc., St-Bruno, QC, Canada) suspension that contained a maximum of 8 g of fecal matter per sample. Rectal swabs with less than 1 g of fecal matter were suspended in 2 mL of PBS. The samples were vortexed for 30 s and centrifuged at 16 000 × *g* for 5 min. Viral RNA was extracted from the supernatants with a QIAamp Viral RNA Mini Kit (Qiagen, Mississauga, ON, Canada). The environmental swabs were mixed with 15 mL of DMEM containing 0.1 g of polyvinylpolypyrrolidone (PVPP) (Sigma-Aldrich, St. Louis, MO, USA), and 100 µL of a murine norovirus (MNV) was spiked at 10^4 PFU/mL as an internal process control. The environmental sponge swab samples were mixed by hand, and 15 mL was collected and then vortexed for 30 s, filtered with 0.45-µm and 0.22-µm filters (Sarstedt, Nümbrecht, Germany), and concentrated on an ultrafiltration device (Amicon Ultra-15 100 kDa; Fisher Scientific) by centrifugation at 5000 × *g* for 10 min. Viral RNA from the environmental samples was extracted with an RNeasy Mini Kit (Qiagen). All kits were used according to the manufacturer's recommendations.

2.3. RT-PCR detection

Viral RNAs were reverse-transcribed (RT) using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's recommendation with random hexamers and 5 µL of total viral RNA. *Kobuvirus* was detected from the resultant cDNA by PCR using a pair of "universal" *Kobuvirus* primers (UNIV-kobu-F and R) (Reuter et al., 2009) amplifying a 217-nt fragment located in the conserved 3D region encoding the RNA-dependent RNA polymerase. The PCR reactions were performed using a Taq PCR Core Kit (Qiagen) in a 20-µL final volume using 2 µL of cDNA, 2 µL of PCR buffer 10×, 0.5 µM of each of the primers, 0.2 mM of each dNTP, 0.5 mM of $MgCl_2$, and 4 U of the enzyme mix. The thermal cycling conditions were as follows: an initial denaturation at 95 °C for 3 min; followed by 40 cycles of 94 °C for 30 s, 53 °C for 90 s, and 72 °C for 60 s; and then a final elongation at 72 °C for

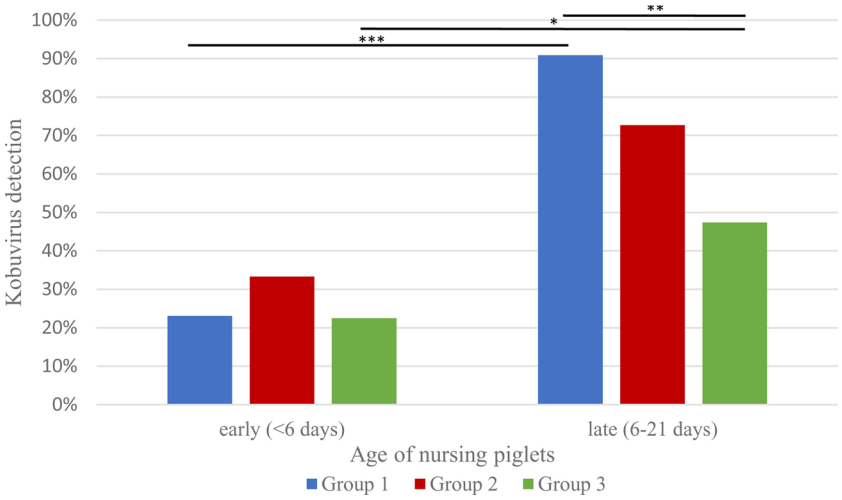

Fig. 1. *Kobuvirus* detection in pigs between early and late nursing stage, depending on their health status. Group 1: piglets with diarrhea; group 2: piglets without diarrhea from a litter that had at least one piglet with diarrhea; group 3: piglets without diarrhea from a healthy litter. Chi-square significant differences are indicated on the graph as follows: ***: $p < 0.0001$; **: $p = 0.003$; *: $p = 0.02$.

10 min. Detection of MNV was performed as described previously (Kingsley, 2007). All amplifications were performed on an Eppendorf 5331 MasterCycler Gradient Thermal Cycler (Brinkmann Instruments Canada, Mississauga, ON, Canada). Negative controls (extraction and amplification) were incorporated at each step. Amplicons were analyzed on a 2% agarose gel and submerged for 5 min in a 1.0 µg/mL ethidium bromide solution.

2.4. PCR primer design

Multiple existing primer sets from Europe and Asia (Yu et al., 2011; Di Profio et al., 2013) were tested in order to amplify a longer and more diverse porcine kobuvirus PCR fragment (VP1) with no success (data not shown), creating the need to develop a new PCR system. A total of 6 primer sets designed in different parts of the porcine kobuvirus genome were tested (data not shown) and all *Kobuvirus*-positive pig fecal samples were re-amplified using the selected primer set with the highest positive re-detection rate which was located in the same 3D region as the "universal" primers. This new primer set: (kobu-FW7001 [5′-GCC GTTCACTCTTTGTCCAAC-3′] and kobu-RW7976 [5′-CCAGTAGTCTTC ATTACCTGATCTC-3′]) amplifies a longer, 976-nt *Kobuvirus* fragment. The PCR reagents and the concentrations of the new primer pair were the same as the "universal" primers, and the thermal cycling conditions were as follows: an initial denaturation at 94 °C for 3 min; 40 cycles of 94 °C for 60 s, 52.3 °C for 60 s, and 72 °C for 90 s; and then a final elongation at 72 °C for 10 min.

2.5. Sequencing

All positive PCR amplicons obtained for the 976-nt *Kobuvirus* fragment (n = 162) were sequenced using BigDye v3.1 chemistry on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at CRCHUL (Centre de recherche du Centre hospitalier de Québec – Université Laval, Quebec City, QC, Canada). The 135 successfully sequenced samples were edited with BioEdit software, version 7.2.6, to remove poor-quality bases leading to a final consensus length was 845 nt. Nucleotide alignment, nucleotide similarity percentages, and phylogenetic trees were performed with MEGA 7.0.26 software (Kumar et al., 2016), using ClustalW for alignment with previously published complete kobuvirus genomes from human (*Aichivirus A*, AB010145.1), bovine (*Aichivirus B*, AB084788.1), and swine (*Aichivirus C*, EU787450.2, KC204684, KM977675) kobuviruses. All 97 porcine kobuvirus sequences presented in the two phylogenetic trees from this study were deposited in the GenBank database under accession numbers MK695526 to MK695622.

2.6. Statistical analysis

Statistical tests used on the *Kobuvirus* detection results were Pearson's chi-square test in GraphPad Prism software, using a significance level of $p = 0.05$. The statistical confidence of the phylogenetic relationships was determined by bootstrap analysis with 1000 replications and using the neighbor-joining method with the Kimura 2-parameter model for the phylogenetic tree in MEGA software. Nucleotide differences and similarity percentages were calculated for each type of farm (nursing, nursery, and fattening), once using the pairwise comparisons between all strains, and a second time using the pairwise comparison of strains originating from samples from the same farm. Statistical analyses of nucleotide difference data were carried out in GraphPad Prism software, version 6.07, using the unpaired *t*-test statistical test with Welch's correction.

3. Results

3.1. In nurseries, Kobuvirus is found at a higher frequency in older piglets with diarrhea

Of the 181 piglets sampled on the nursing farms, a total of 77 (42.5%) were found positive for *Kobuvirus* using the "universal" primers. The nursing farm samples were not significantly different in terms of *Kobuvirus* shedding in feces ($p > 0.05$) depending of their diarrhea status and their pen mates', with 29/61 (47.6%) positive for group 1, 12/23 (52.2%) positive for group 2, and 36/97 (37.1%) positive for group 3. These results were not significantly different from the *Kobuvirus* detection rate in sow samples, which was 33/85 (38.8%). A higher percentage of piglets were shedding *Kobuvirus* when their respective sows were also shedding the virus, at 41/64 (64.1%), compared to when their sows were not, at 36/108 (33.3%) ($p < 0.0001$); 9 piglets did not have a sample taken from their sow. There was a positive correlation between diarrhea and the presence of *Kobuvirus* in late nursing (between 6 and 21 days of age) ($p = 0.0003$); this correlation was not observed in very young piglets (< 6 days of age) (Fig. 1). Moreover, a higher percentage of *Kobuvirus*-shedding piglets was observed in late nursing in comparison with early nursing in both group 1 ($p < 0.0001$) and group 3 ($p = 0.02$) (Fig. 1).

3.2. Kobuvirus presence is at its peak in piglets from the nursery farms

Of the 181 sampled piglets in the nursery farms, 126 were successfully followed at the four life stages sampled and further analyzed. From the nursing farms (< 3 weeks of age) to the nursery farms (5 weeks of age), a higher percentage of *Kobuvirus*-shedding piglets were

Table 2
Kobuvirus detection in followed pigs depending on their life stage and diarrhea status.

Group	*Kobuvirus*-positive piglets (%)			
	< 3 weeks*	5 weeks**	12 weeks	20 weeks*,**
1	22/43 (51.1)	28/43 (65.1)[b]	15/43 (34.9)[b,c]	8/35(18.6)
2	9/15 (60.0)	10/15 (66.7)	6/15 (40.0)	2/15 (13.3)
3	26/68 (38.2)[a]	48/68 (70.6)[a]	39/68 (57.4)[c]	11/57 (16.2)
total	57/126 (45.2)	86/126 (68.3)	60/126 (47.6)	21/126 (16.7)

Group 1: Piglets with diarrhea at < 3 weeks; Group 2: Healthy piglets < 3 weeks sampled in a pen where there was diarrhea; Group 3: Healthy piglets < 3 weeks sampled in a healthy pen.

* All three groups are significantly different from < 3 weeks compared to 20 weeks ($p < 0.002$).

** All three groups are significantly different from 5 weeks compared to 20 weeks ($p < 0.002$).

[a] $p = 0.0002$.

[b] $p = 0.005$.

[c] $p = 0.02$.

observed only in group 3 (Table 2). From the nursery (5 weeks of age) to early fattening (12 weeks of age), fewer piglets shedding *Kobuvirus* were observed only in group 1 (Table 2). All groups shed less *Kobuvirus* in late fattening (20 weeks) than in both the nursing stage and the nursery stage. The only significant difference between groups 1, 2 and 3 at the same life stage was identified in early fattening, where there was a higher percentage of pigs shedding *Kobuvirus* in group 3 than group 1 (Table 2).

A total of 224/504 (44.4%) *Kobuvirus*-positive samples were detected from all four life stages in the followed animals. A total of 123/126 (97.6%) pigs shed *Kobuvirus* at least once, and 79/126 (62.7%) shed the virus more than once (Table 3). Only three pigs (2.4%) did not have a *Kobuvirus*-positive fecal sample at any of the four stages, and these animals were all from group 3.

3.3. Up to 100% of the farms are positive for Kobuvirus, depending on the life stage sampled

All the individually taken pig fecal samples in this study came from 39 different farms: 11 nursing farms, 14 nursery farms, and 14 fattening farms. At least one *Kobuvirus*-positive samples were found on 61.5% to 100% of the farms, with variable detection rates depending on the life stage of the pigs (Table 4). The results from the environmental samples revealed higher percentages of *Kobuvirus*-positive samples in nursery farms in comparison with all the other life stages (swabs: $p < 0.02$; composite samples: $p < 0.0001$). All swabs were positive for the MNV internal process control (142/142), suggesting that the extraction method was effective in yielding quality RNA that was free of PCR inhibitors.

Table 3
Number of occurences a *kobuvirus*-positive sample was found in the same pig.

Group	*Kobuvirus*-positive pig sample occurences				
	0	1	2	3	4
1	0/43	20/43	17/43	5/43	1/43
2	0/15	6/15	6/15	3/15	0/15
3	3/68	18/68	37/68	8/68	2/68
total	3/126	44/126	60/126	16/126	3/126

Group 1: piglets with diarrhea at < 3 weeks.
Group 2: healthy piglets < 3 weeks sampled in a pen where there was diarrhea.
Group 3: healthy piglets < 3 weeks sampled in a healthy pen.

Table 4
Kobuvirus detection in the environment.

Stage of life	*Kobuvirus*-positive environment samples (%)		
	Swabs samples	Composite samples	Positive farms*
< 3 weeks	45/70 (64.3)[a]	–	7/11 (63.6)
5 weeks	59/72 (81.9)[a]	75/76 (98.7)[b, c]	14/14 (100)
12 weeks	–	32/41 (78.1)[b, d]	13/14 (92.9)
20 weeks	–	7/25 (28.0)[c, d]	8/13 (61.5)
total	104/142 (73.2)	114/142 (80.3)	42/52 (80.8)

a: $p < 0.02$; b, c, and d: $p < 0.0001$.

* farms with at least one kobuvirus-positive pig sample.

3.4. Pigs from the same farm of origin shed similar Kobuvirus strains

A total of 135 samples were sequenced in this study. Of those samples, 58 were from nursing farms (Fig. 2), 48 were from nursery farms (data not shown), and 29 were from fattening farms (data not shown). Individual phylogenetic trees were generated from each of these farm types, and then a "consensus" phylogenetic tree was created using representative samples from each main "branch" for each nursing farm (Fig. 3). Nucleotide differences between samples and groups of samples originating from the same nursing farm were calculated for the four resulting phylogenetic trees (Table 5). Strains from piglets at the nursing farm (< 3 weeks of age) were clustered according to their farm of origin (Fig. 2). Interestingly, samples taken 5 months apart on the same farm clustered together (e.g. samples A-8-165 and A-8-84; Figs. 2 and 3). All strains originating from sows clustered with strains from their respective piglets. There was no clustering of strains according to whether they originated from diarrheic or healthy piglets. Strains from the same farms but from different life stages also tended to cluster together (e.g. A-11-146, B-14-146, and C-12-149; Fig. 3). However, three piglets were detected with different strains at different life stages (numbers 91, 92, and 96). Interestingly, four strains (C-11-144, B-9-93, C-6-91, and A-6-49; Fig. 3) were genetically divergent from all the other strains found from the same farm of origin. Overall, the vast majority of the strains were grouped according to their farm of origin. The nucleotide difference between the already published porcine kobuvirus sequences and the strains from this study varied from 90.0% to 94.8%; the lowest pairwise comparison being with the Chinese (KC204684) strain and to the highest with the Hungarian (EU787450.2) strain.

Furthermore, nucleotide difference averages calculated between all sequenced samples for a given life stage or the consensus tree (Table 5) were consistently higher than the sequence averages from the same farm of origin ($p < 0.03$). Consequently, *Kobuvirus* samples originating from the same nursing farm had a lower nucleotide difference average, resulting in a higher similarity percentage.

4. Discussion

In the present study, porcine kobuviruses were detected at every life stage, from the nursing farm to the end of the fattening period. To our knowledge, this is the first report of *Kobuvirus* detection in swine in Canada and the first study monitoring shedding of the virus in individual pigs throughout their life in a farrow-to-finish production system.

The number of piglets shedding kobuviruses increased with age during the early stage of life of piglets in nursing farms (Fig. 1). This observation is consistent with the normal maturation of the piglet intestine (Pohl et al., 2015; Moeser et al., 2017). In the first 3 weeks, the intestine undergoes rapid maturation involving its functions and permeability in addition to the development of the immune system, modulated by environmental, endocrine, and microbial signals. Higher shedding levels in piglets during late ages in nursing farms may also be due to the incubation phase of the virus or to a less mature immune

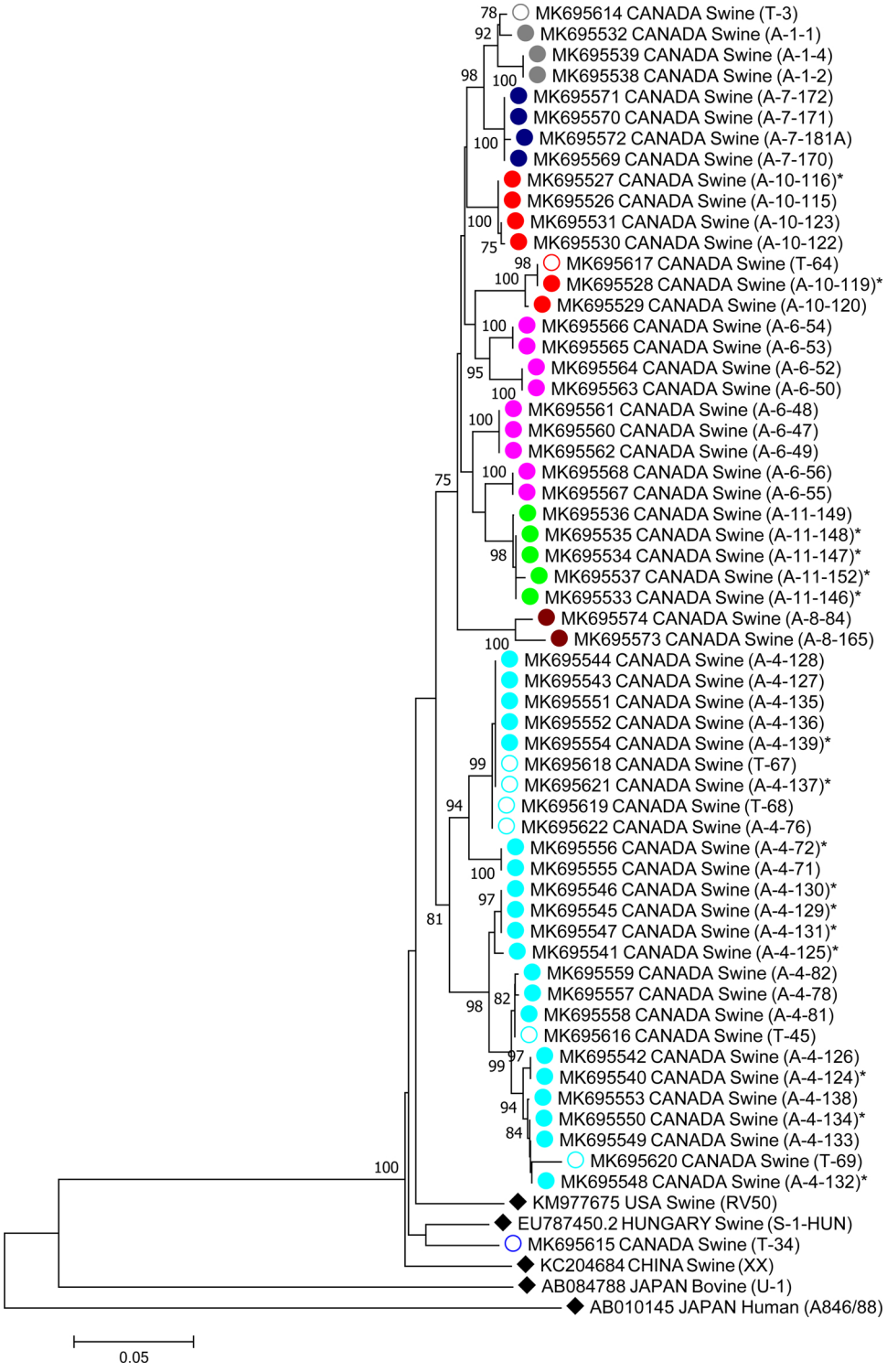

Fig. 2. Phylogenetic tree of porcine kobuvirus strains from nursing farms. Phylogenetic tree created by the neighbor-joining method, based on the 845-nt sequences in the conserved 3D region of the *Kobuvirus* genome from piglets at the nursing stage isolated in this study (solid circles), their sows (empty circles), and previously-published strains from human, bovine, and porcine hosts retrieved from GenBank (black diamonds). Different colours represent different nursing farms of origin. Bootstrap values (1000 replicates) higher than 75% are shown, and the bar represents 5% sequence divergence. Each entry is identified with its GenBank accession number, the country from which it was isolated, the species from which it was isolated, and the isolate name. Isolate names with an asterisk are samples taken from diarrheic piglets.

system. Furthermore, a higher percentage of piglets shedding *Kobuvirus* were found in pens where sows were also shedding the virus. During most of their time in the nursing farm, piglets and their sows live in the same environment (Moeser et al., 2017; Pluske et al., 2018), and piglets are therefore exposed to their mother's fecal microbiota. Piglets showing signs of diarrhea and their sows might shed *Kobuvirus*, infecting healthy individuals that are not yet shedding the virus at the time of sampling but might shed it later in their life, as shown in Table 2. In addition, environmental samples from the nursing farms were positive for *Kobuvirus* at a rate of more than 60%, indicating that the virus was disseminated throughout the farms' environment. A portion of these environmental samples were found on mobile objects that were used in multiple pens and chambers and might have been contamination vectors for *Kobuvirus*.

Studies from around the world have generally associated the presence of *Kobuvirus* in pig feces with diarrhea (Khamrin et al., 2009; Park et al., 2010; Van Dung et al., 2016; Almeida et al., 2018; Theuns et al., 2018). However, a number of studies have failed to reach similar conclusions (Dufkova et al., 2013; Goecke et al., 2017; Jackova et al., 2017). Although there is a correlation between *Kobuvirus* shedding and

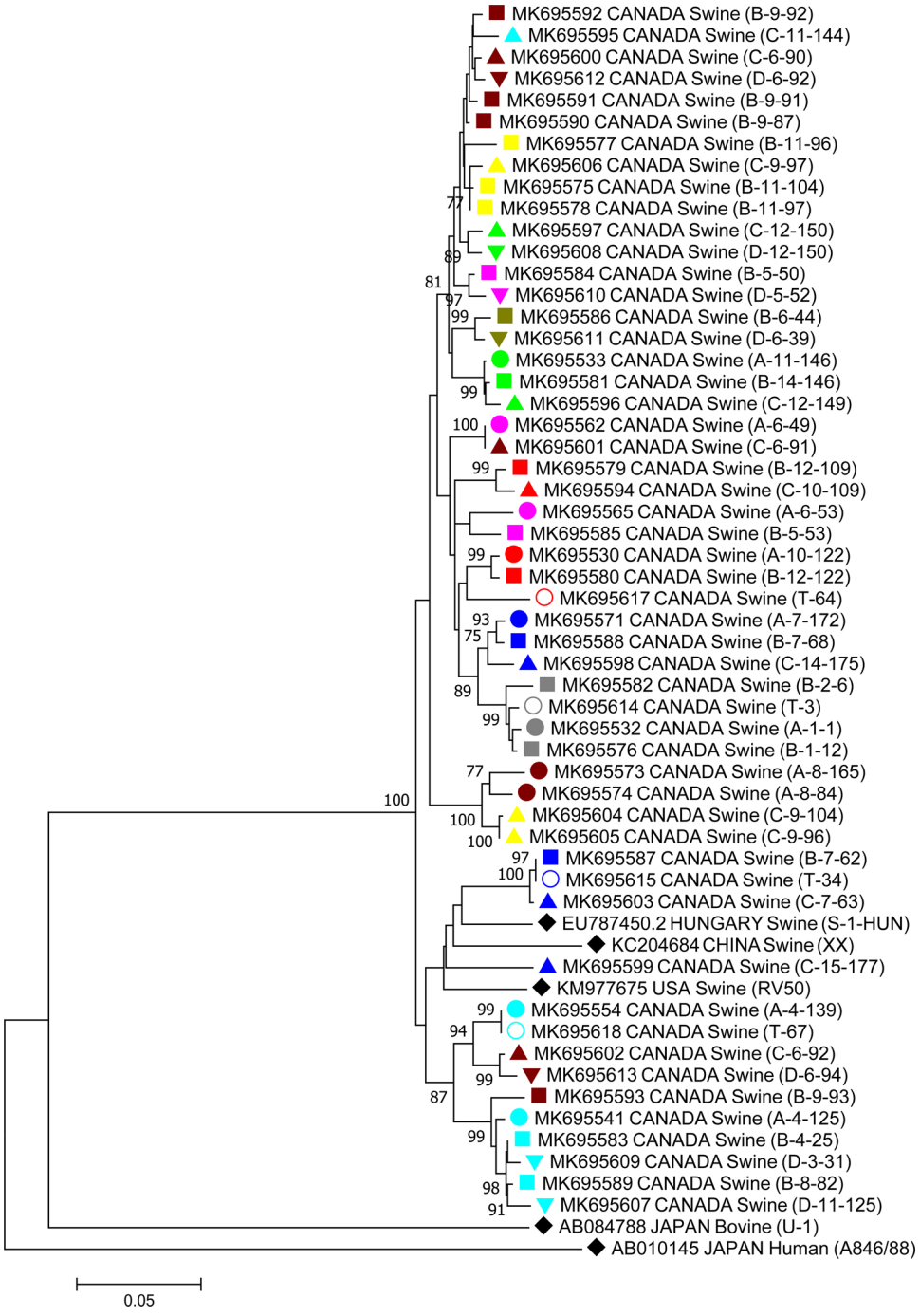

Fig. 3. Phylogenetic tree of porcine kobuvirus strains from nursing, nursery and fattening farms. Phylogenetic tree created by the neighbor-joining method, based on the 845-nt sequences in the conserved 3D region of the *Kobuvirus* genome from pigs isolated in this study and strains from human and bovine hosts. Different colours represent different nursing farms of origin. Different shapes represent the different pig life stages during which the samples were taken: circles indicate the nursing stage (empty circles are from sows), squares indicate the nursery stage, upward triangles indicate the early fattening stage, and downward triangles indicate the late fattening stage. Black diamonds represent previously-published sequences retrieved from GenBank. Bootstrap values (1000 replicates) higher than 75% are shown, and the bar represents 5% sequence divergence. Each entry is identified with its GenBank accession number, the country from which it was isolated, the species from which it was isolated, and the isolate name. Isolate names with the same ending number (e.g. –150) are samples taken from the same pig, sampled at different life stages.

diarrheic symptoms in older piglets in nursing farms in the present study, porcine kobuvirus cannot be designated as the sole cause in this context, since no other pathogens were tested here. Additional factors such as stress or the presence of a highly antigenic substance in the sow's milk can also contribute to diarrhea in the newborn piglet (Pohl et al., 2015; Jayaraman and Nyachoti, 2017). Future in vivo studies of *Kobuvirus* infection in piglets are warranted to better evaluate the etiological role of *Kobuvirus* in neonatal diarrhea.

Nearly 100% of the piglets shed *Kobuvirus* at least once in their lifetime (Table 3). In addition, 80% of the farms were positive for *Kobuvirus*, with that rate varying from 60% to 100%, depending on the age at which the pigs were sampled (Table 4). In this study, the highest detection rate in fecal samples was from piglets in nurseries at 5 weeks of age, which is similar to the findings of Di Bartolo et al. (2015) and Dufkova et al. (2013). However, Barry et al. (2011) found the highest *Kobuvirus* shedding rate in piglets younger than 3 weeks of age, and Jackova et al. (2017) reported no differences between any of the life stages. The shedding peak in the present study corresponds to the post-weaning period, when dietary, microbiological, and environmental stressors have the potential to disrupt gastrointestinal maturation, leading to inflammation, malabsorption, and decreased brush-border enzyme activity, all of which could have an influence on *Kobuvirus* shedding (Jayaraman and Nyachoti, 2017; Moeser et al., 2017). In nature, pig weaning does not happen abruptly at 3 weeks of age like in a commercial production system; instead, weaning is a gradual process that completes at around 10 to 12 weeks of age, when the gastrointestinal epithelium is mature (Moeser et al., 2017). Therefore, an early weaning, coinciding with declining passive immunity from the sow, could be the cause of the shedding peak observed in the pigs sampled at 5 weeks of age on the nursery farms. Most pigs followed

Table 5
Nucleotide differences and similarity percentages between *kobuvirus* PCR fragments from different life stages.

	Nucleotide differences			Similarity percentages		
Phylogenetic analysis	Average	S.D.*	(min-max)	Average	S.D.*	(min-max)
Between farms						
Sows and < 3 weeks piglets	23.1[a]	15.8	(1.6-26.2)	97.3%	1.9%	(96.7%-99.8%)
5 weeks	22.7[b]	21.0	(6.2-31.3)	97.3%	2.5%	(96.1%-99.2%)
12 and 20 weeks	33.3[c]	20.6	(4.0-46.7)	96.1%	2.4%	(94.1%-99.5%)
Consensus	31.5[d]	18.4	(9.5-40.4)	96.3%	2.2%	(95.2%-98.9%)
Between all samples						
Sows and < 3 weeks piglets	36.8[a]	14.1	(0-69)	95.6%	1.7%	(91.4%-100%)
5 weeks	33.7[b]	14.4	(0-67)	96.0%	1.7%	(91.6%-100%)
12 and 20 weeks	41.0[c]	17.2	(0-58)	95.1%	2.0%	(92.8%-100%)
Consensus	40.4[d]	14.3	(0-69)	95.2%	1.7%	(91.4%-100%)

a, b, d: $p < 0.0001$, c: $p = 0.008$.
* S.D.: Standard Deviation.

from this study (123/126) were exposed to *Kobuvirus* during their lifetime, and their acquired immunity might have been sufficient to prevent reinfection with a similar or different strain, resulting in much lower shedding rates in late fattening. Moreover, the porcine *Kobuvirus* strains or groups of strains infecting the piglets revealed higher nucleotide identity values within farms than between farms, even when the piglets were housed with other pigs at later life stages (Figs. 2 and 3 and Table 5). Hence, piglets infected at nursing farms will carry and shed a similar *Kobuvirus* strain or group of strains throughout their life. Shedding was detected mostly during the nursery stage (post weaning), and piglets are unlikely to be infected later in life by a different strain type. Cleaning and disinfecting procedures should be of greater concern to stakeholders working on nursing farms, as this particular life stage might be the point of entry of *Kobuvirus* into pig production systems.

Key areas in the farm environment were also sampled in this study to investigate the presence of *Kobuvirus* and its potential entry routes. The same detection peak as for the individual pig fecal samples on the nursery farms was found for both the composite and swab environmental samples collected in this study (Table 4). The lower detection rate of *Kobuvirus* in the fattening farms' environment suggests either that the virus did not survive the 2-months time gap between sampling dates or that standard in-farm biosecurity procedures are effective enough to remove the infected manure from the pen floors, which could explain the low detection rate in pigs at the late-fattening stage. Collectively, the individual and environmental samples revealed a drop in the *Kobuvirus* detection rate after the piglets were over 12 weeks of age. Environmental samples, although a good proxy to evaluate the dynamics of *Kobuvirus* shedding, tended to overestimate the detection rate in individual pigs at each of the four life stages during which samples were taken in this study. Environment samples represent a higher number of pigs per sample, which may have given different *Kobuvirus* RNA yields. There is also the question of whether the viruses found by molecular techniques in the environment (or in fresh fecal samples to a certain extent) are still viable and infectious, even though studies have shown that kobuviruses are very resistant in the environment and can withstand heat, high pressure, and chemical inactivation better than other viruses such as sapoviruses and noroviruses (Cromeans et al., 2014; Kingsley et al., 2014). The presence of *Kobuvirus* in environmental samples should thus be considered with caution but gives a good general overview of the pigs' actual *Kobuvirus* shedding dynamics in relation to the animals' life stage. Protocols for the infection of cell cultures with human kobuviruses have been developed and are complex (Richards and Watson, 2001; Kingsley et al., 2004). Since those cell cultures are only used to study human kobuviruses, a new cell culture protocol capable of isolating the porcine kobuvirus is needed to evaluate the viability of porcine kobuviruses.

To assess the phylogenetic differences found between the samples in this study, positive *Kobuvirus* samples identified using the "universal" primer pair were subjected to amplification tests with different primer pairs targeting the more variable VP1 segment, in the same manner as other studies in Europe and Asia (Yu et al., 2011; Di Profio et al., 2013). The unsuccessful attempts to amplify the VP1 with European and Asian primer pairs, as previously documented in a study conducted in Minnesota, USA (Verma et al., 2013), led to the design of a new set of primers amplifying a longer fragment. Multiple primer sets were tested, and the set recovering the most positive samples was kept; that set amplifies a 976-nt fragment in the polymerase region and was used to sequence the positive *Kobuvirus* samples. Phylogenetic analyses clustered all strains from this study with prototypical porcine kobuvirus (*Aichivirus C*), with nucleotide identity among them varying between 92% and 100%. However, they failed to show any correlation between strain type (Fig. 2) and increased virulence or a higher incidence of neonatal diarrhea, unlike what has been previously reported (Jin et al., 2015). This apparent lack of correlation may be due to the conserved genomic region analyzed in the present study or the geographical difference between the porcine kobuvirus strains analyzed. The strains reported by other authors (Jin et al., 2015) and allegedly associated with diarrhea were analyzed using the more genetically variable VP1 genomic region and came from pigs sampled in China.

In addition to the inability to amplify a variable region, the highest nucleotide identity with a previously published Asian kobuvirus sequence was lower than 91%, which goes against the globally distributed observation, where most of the strains published were phylogenetically undistinguishable from their country of origin (Di Profio et al., 2013; Akagami et al., 2017). The porcine kobuvirus strains in the present study appear phylogenetically unique, and therefore, to better document variations in the *Kobuvirus* genome, a further genetic characterization in a more variable region is required, if whole-genome approaches are unavailable or unpractical.

5. Conclusion

Although most of the piglets sampled in this study shed *Kobuvirus* at least once in their lifetime, shedding peaked post weaning at 5 weeks of age and was at its lowest at 22 weeks of age. On the nursing farms, shedding was found to be highest in the older piglets (6 to 21 days old) with diarrheic symptoms. *Kobuvirus* strains can infect sows and their piglets and can be maintained on a farm, as evidenced by shedding data from nursing to the fattening periods. Overall, the findings from this study throw light on *Kobuvirus* shedding dynamics and the potential implication of this virus in diarrhea in piglets in early life, a life stage that may possibly be the point of entry for *Kobuvirus* into swine production systems. In vivo infection studies may be necessary to evaluate whether porcine kobuvirus is a significant etiological agent in neonatal diarrhea, and that knowledge may be useful for further assessment of swine biosafety measures and husbandry practices.

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