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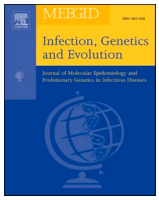
Infection, Genetics and Evolution 113 (2023) 105483

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

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



Short communication

Molecular and genetic characterization of bovine parainfluenza type 3 European field and vaccine strains

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ARTICLE INFO

Keywords:
Bovine parainfluenza type 3
Cattle
Bovine respiratory disease
Vaccines
Phylogeny

ABSTRACT

Bovine Parainfluenza Type 3 virus (BPIV-3) is an enveloped, non-segmented single-stranded, negative-sense RNA virus belonging to the *Paramyxoviridae* family (genus *Respirovirus*) with a well-known role in Bovine Respiratory Disease (BRD) onset. Being isolated for the first time in 1959, BPIV-3 currently circulates worldwide in cattle herds and is routinely tested in suspected BRD cases. Different commercial vaccines are available to prevent infection and/or to reduce the clinical signs associated with BPIV-3 infection, which are essential to prevent secondary infections. Despite years of molecular surveillance, a very limited number of complete genome sequences were made publicly available, preventing thus the understanding of the genetic diversity of the circulating strains in the field. In addition, no data about the genetic identity between field and vaccine strains is currently available. In this study, we sequenced the full-genome and genetically characterized BPIV-3 strains isolated from animals displaying respiratory illness in France and Sweden, as well as the vaccine strains contained in three different commercialized vaccines. Our results show that the sequences from France and Sweden belong to genotype C. However, a third sequence from Sweden from 2017 clustered within genotype A. The sequencing of vaccine strains revealed that two of the vaccine strains clustered within genotype C, whereas the third vaccine strain belonged to genotype A. Altogether, our findings suggest that both genotypes A and C circulate in Europe and that BPIV-3 field and vaccine strains are genetically divergent. Our sequencing results could be useful to better understand the genetic differences between the circulating field and vaccine BPIV-3 strains. This is crucial for a correct interpretation of diagnostic findings and for the assessment of BPIV-3 prevalence in cattle population.

1. Introduction

Bovine parainfluenza type 3 virus (BPIV-3), officially known as Bovine Respirovirus 3, is an enveloped, non-segmented single-stranded, negative-sense RNA virus belonging to the *Paramyxoviridae* family (genus *Respirovirus*) (ICTV Virus Taxonomy Profile) (Rima et al., 2019). Its genome encodes for six viral proteins: the nucleocapsid (N), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the hemagglutinin-neuraminidase (HN) and the large polymerase (L) (Sakai et al., 1987; Suzu et al., 1987). BPIV-3 was isolated for the first time in 1959, and today it is a worldwide diffused enzootic pathogen associated with respiratory outbreaks in cattle farms (Reisinger RC; Heddleston KL; Manthei CA, 1959). Its predisposing role in Bovine Respiratory Disease (BRD) onset is well recognized, as shown in different experimental studies (Baldwin et al., 1967; Hamdy et al., 1963; Saunders and Berman, 1964). BPIV-3 infection in cattle induces moderate respiratory signs, including nasal and ocular discharge, fever, dry cough and increased respiratory rate (Ellis, 2010). BPIV-3 is often co-detected with other respiratory pathogens and a primary infection creates an opportunity for secondary bacterial superinfections (Gaudino et al., 2022).

So far, three circulating BPIV-3 genotypes have been described, named A, B and C (Neill et al., 2015). Despite its worldwide prevalence, the complete genome sequences on public databases are available for few countries, including Egypt, Turkey, China, Japan, South Korea,

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https://doi.org/10.1016/j.meegid.2023.105483
Received 16 February 2023; Received in revised form 8 June 2023; Accepted 20 July 2023
Available online 22 July 2023

Australia and the USA (Albayrak et al., 2019; Horwood et al., 2008; Kumagai et al., 2020; Maidana et al., 2012; Neill et al., 2015; Oem et al., 2013; Zhu et al., 2011). In Europe, BPIV-3 circulation was described in recent surveillance studies (Flynn et al., 2018; Kudirkiene et al., 2021; Studer et al., 2021), however no complete genomic sequences have been generated and made publicly available so far for the continent. Although vaccines against BPIV-3 infection have been commercially available for several years (Makoschey and Berge, 2021), no information about the genetic sequences of the strains that are routinely used for vaccination in cattle herds is currently available, limiting thus the comprehension of the adequacy of prophylactic measures in this geographic area. In this study, we isolated and sequenced BPIV-3 from 6 RT-qPCR positive samples collected in three herds of South-West region of France from calves with respiratory signs (Salem et al., 2020), and in two Swedish herds in the county of Uppland. In addition, we sequenced three commercially available live-attenuated vaccines used in Europe and we conducted phylogenetic analyses in order to assess their genetic similarity with the circulating BPIV-3 field strains.

2. Materials and methods

2.1. Clinical samples and molecular testing

Clinical samples ($n = 93$) for BPIV-3 testing were retrieved from a previous study conducted in the South-West of France (Occitanie region) during respiratory outbreaks in veal calves' herds (Salem et al., 2020). Briefly, nasal swabs (NS) and bronchoalveolar lavages (BAL) were collected from young calves (two to five months of age) displaying respiratory signs in 23 different herds during the winter seasons between 2013 and 2017. The samples were collected only from animals that were either not vaccinated or at least three weeks following the last vaccination. For each farm, the samples were pooled (5 samples/pool) and the RNA was purified using the QIAamp viral RNA minikit (Qiagen). The samples were screened by RT-qPCR using the VetMAX Screening pack–Ruminant Respiratory Pathogens real-time PCR kit (Thermo Fischer). The RT-qPCR experiments were performed on a LightCycler 96 real-time PCR system (Roche) and the results were analysed using the LightCycler® 96 Software v1.1.01320 (Roche).

Two nasal swabs were collected from calves with signs of respiratory disease in one Swedish herd in the county of Uppland in 2016 and in 2017. In addition, between two and ten nasal swabs were collected from 21 herds in 2020 and 355 nasal swabs were collected from 216 farms across the country in 2021. The swabs were frozen at −75 °C until analysis for BPIV-3-RNA by Taqman RT-qPCR using LSI VetMaxTM Screening Pack – Ruminants Respiratory Pathogens (Life technologies, France), after extraction by using the RNAeasy® Mini kit (Qiagen, Sweden) according to the manufacturers' instructions.

2.2. Viral isolation

BPIV-3 positive samples collected in France were filtered (0.22 μm) and inoculated on confluent monolayers of Madin-Darby Bovine Kidney (MDBK) cells. The inoculum was removed 1-h post-infection and the cell monolayers were washed twice with a phosphate-buffered saline solution. The cells were then cultivated and daily observed for the appearance of visible cytopathic effects (CPEs) using Opti-MEM (Thermo Fischer Scientific) supplemented with amphotericin B (2.5 μg/mL, Sigma-Aldrich) and 1% of penicillin-streptomycin (10,000 U/10 mg/mL, Pan Biotech). To confirm viral isolation, the RNA from infected supernatants was purified using the QIAamp viral RNA minikit (Qiagen) and tested using the VetMAX Screening pack–Ruminant Respiratory Pathogens real-time PCR kit (Thermo Fischer). Swedish samples positive for BPIV-3 by RT-PCR (Ct <25) were inoculated on Foetal bovine turbinate (FBT) cells propagated in Dulbecco's modified Eagle medium (DMEM, Lonza, Belgium), supplemented per litre with 20 mL 1 M Hepes buffer (VWR, BioWhittaker BE17-737F), 10 mL 200 mM L-glutamine (VWR, BioWhittaker BE17-605F) and 10 mL 0.9% NaCl solution containing 60 mg benzyl penicillin sodium (Sigma-Aldrich, P3032-10MU) and 100 mg streptomycin sulfate salt (Sigma-Aldrich, S6501-5G), as well as gamma-irradiated foetal calf serum (Sigma-Aldrich, F3885) to a final concentration of 2%. Cultures of inoculated cells were examined daily for visible cytopathic effects.

2.3. Metagenomic Illumina sequencing of clinical samples and vaccines

Three commercially available BPIV-3 intranasal live-attenuated vaccines (strains RLB 103, INT2–2013 and Bio/23A) were purchased and the lyophilised strains were resuspended following the manufacturer's instructions. The viral RNA of vaccine strains was extracted from 150 μL of sample using the QIAamp viral RNA minikit (Qiagen). The quality and yield of the extracted RNA was assessed by spectrophotometry using the CLARIOstar Plus plate reader (BMG Labtech). Random primed cDNA synthesis was prepared on fragmented mRNA and the samples were sequenced by Eurofins Genomics (Germany) on a Genome Sequencer Illumina NovaSeq by generating 10 million of 2 × 150 bp reads per sample. The reads were mapped on a reference genome using Burrows-Wheeler Alignment tool v.0.7.12-r1039 implemented on Galaxy workbench (Giardine et al., 2005). For clinical samples, the RNA purified from infected cell culture supernatants was converted into cDNA by using Superscript IV First-Strand Synthesis Kit (Invitrogen). The concentration of each sample was determined using High Sensitivity reagents with Qubit 2.0 fluorometer (Invitrogen). DNA libraries were prepared using the NexteraXT kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Each DNA was initially analysed by Bioanalyzer (Agilent Technologies, using DNA1000 chips) to ascertain library quality and average size distribution. DNA libraries were sequenced on the Illumina MiSeq in paired-end mode using the NextSeq 600 cycle v3 kit (Illumina). Raw sequence data were analysed and mapped using the CLC-Genomics software package, workbench 21 (CLC Bio, Aarhus, Denmark). Raw reads were trimmed, and subsets of each trimmed dataset were assembled de novo to generate reference sequences for each data set. In the CLC workflow, the following steps are run automatically: Basic trimming, the mapping of raw sequence data to the reference genome, calling new consensus sequences, and finally outputting the genome consensus sequence. Consensus sequences were generated and annotated. The data processing workflow has in-build defaults for k-mer and coverage cut-off for de novo assembly and the e-value cut-off for BLAST. The complete genome of BPIV-3 obtained from clinical samples was deposited in GenBank under the accession numbers: OQ302282, OQ302283, OQ302284 (sequences from France), OQ349372, OQ349373, OQ349374 (sequences from Sweden). BPIV-3 complete genome sequences of the commercial vaccines are available upon request.

2.4. Electron microscopy

The cell supernatant of the infected FBT cells was processed for electron microscopy (EM) by standard methods. Briefly, the cell supernatant was centrifuged at 8000 X g to remove cell debris. The sample was afterward negatively stained with 2% phosphotungstic acid (Sigma, USA) for 30 s and were applied onto 400-mesh Formvar-coated copper grids. Grids were viewed using a transmission electron microscope (FEI Tecnai Spirit BioTwin, Netherlands).

2.5. Molecular characterization and phylogenetic analysis

All publicly available BPIV-3 complete genome sequences were downloaded from the National Center for Biotechnology Information GenBank. The sequences were aligned using Clustal Omega implemented on the on European Molecular Biology Laboratory (EMBL)-EBI search and sequence analysis tools (Madeira et al., 2019; Sievers et al., 2011). Genetic analyses were carried out using MEGA-X v10.1.7 (Kumar

et al., 2018). Pairwise distances were calculated on the complete genome coding sequences using the nucleotide and amino acid substitution model with the lowest BIC scores (Bayesian Information Criterion) identified for the alignment (Tamura-3 parameter for the nucleotide and Jones-Taylor-Thornton for the amino acid alignment). Maximum-Likelihood phylogenetic trees were generated using the and General Time Reversible model, which was also selected based on the lowest BIC score, and the tree robustness was assessed by 1000 bootstrap replicates.

3. Results and discussion

Out of the 23 calf herds in France, 3 tested positive for BPIV-3 by RT-qPCR (named ICSA-4, ICSA-6 and ICSA-11). The ICSA-4 ($n = 5$) and ICSA-6 samples ($n = 4$) were collected in farms with no vaccination against respiratory pathogens. For ICSA-11 samples ($n = 3$), information was not available. However, the inclusion criteria for the study included the absence of intranasal vaccination with a live vaccine within 3 weeks prior to collection. The pooled BAL samples were BPIV-3 positive for the three farms, whereas the nasal swab pools were positive only for the farm "ICSA-11".

Both samples collected from the same herd in Sweden in 2016 and

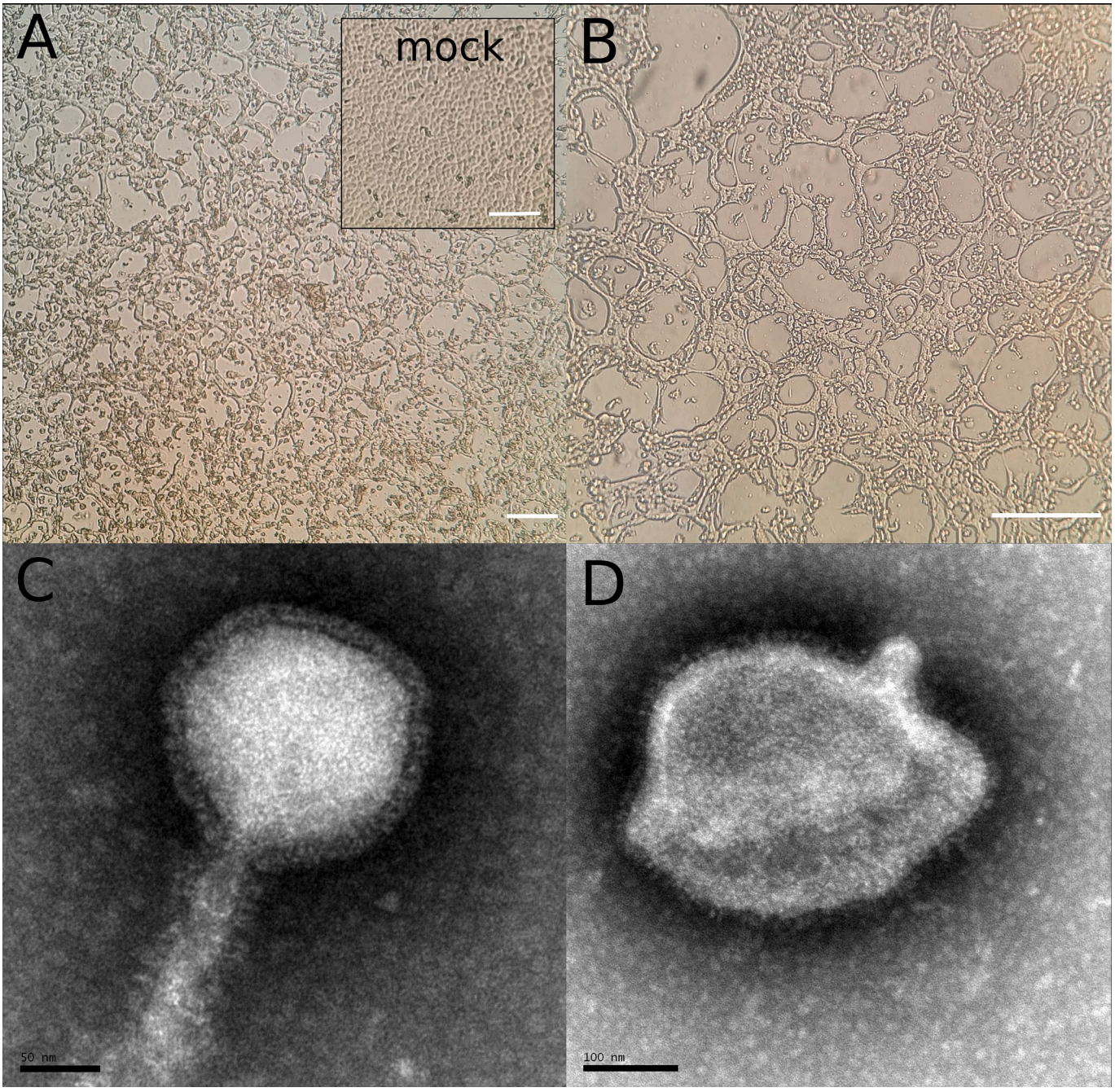

Fig. 1. Cytopathic effects induced by BIPV-3 replication from two different clinical samples from France on MDBK monolayers at five days' post-infection (Panels A and B). The upper part of panel A shows non-infected MDBK. The scale bar represents 200 μm. In panels C and D, BPIV-3 particles released from infected foetal bovine turbinate (FTB) cells are shown. The images were obtained by conventional transmission electron microscopy from samples collected in Sweden. Scale bar = 50 or 100 nm.

2017 tested positive for BPIV-3, however all nasal swabs collected in 2020 were negative. Out of the 355 nasal swabs collected in 2021, 39 tested positive for BPIV-3, coming from 21 different farms.

Upon isolation, CPEs on MDBK monolayers were observed at four to five days' post-infection, depending on the sample, as shown in Fig. 1. The CPEs consisted of cell rounding and detachment, with lysis and destruction of the cell monolayer during the course of the infection. Co-isolation of other bovine respiratory viruses was excluded by RT-qPCR for BCoV, BRSV, IDV and then by NGS analysis for others (Bovine Herpesvirus Type 1, Bovine Viral Diarrhea Virus, Bovine Adenovirus Type 3 etc). The electron micrograph of negatively stained BPIV-3 isolated in FBT cells is shown in Figs. 1 C and D. The sample contains a pool of morphologically differently shaped virions, which is in line with what has already been observed in alveolar macrophages from infected calves (Bryson et al., 1983).

The full genome sequences of three isolates from France and three isolates from Sweden (named SLU—K) and of three commercial live-attenuated vaccines were successfully obtained by Illumina sequencing by using a metagenomic approach, with a mean coverage ranging from 10× to 300×.

Phylogenetic analyses on the complete genome confirmed the presence of three existing genotypes and revealed that the sequences from France and the sequences from Sweden from 2016 and 2021 belonged to genotype C, whereas the sequence from Sweden from 2017 belonged to genotype A (Fig. 2). The vaccine strains RLB 103 and INT2–2013 clustered within genotype C, similarly to the isolates from France. On the other hand, the vaccine strain Bio23/A belonged to genotype A, similarly to the Japanese vaccine strain sequence BN-CE. The phylogenetic trees constructed for each gene individually displayed a similar tree topology to the one based on the complete genome (data not shown).

As shown in Table 1, a high genetic similarity among sequences from France was observed (99.98%), as well as between sequences from France and sequences from Sweden from 2016 (98.52%) and 2021 (98.30%). These sequences displayed high genetic similarity with the vaccine strains RLB 103 and INT2–2013, however they highly differed from the vaccine strain Bio23/A. The sequence from Sweden from 2017 (genotype A) was highly different from the vaccine strains RLB 103 and INT2–2013 (74.00 and 74.08% nucleotide (nt) identity, respectively) but it was genetically similar to the vaccine strain Bio23/A (90.90% nt identity).

Being a usual suspect of BRD, BPIV-3 is regularly included in routine diagnostic testing and surveillance together with other bovine respiratory pathogens. BPIV-3 was isolated for the first time in 1959 in the United States and since then its circulation was described worldwide in cattle herds (Reisinger et al., 1959). Only three circulating BPIV-3 genotypes were described to date, named A, B and C. The genotype A was previously described in China, Japan, Egypt and USA, whereas the genotype B was detected only in China, Australia and the United States so far (Horwood et al., 2008; Neill et al., 2015). Finally, the genotype C was detected only in Turkey, China, Japan, South Korea and USA (Albayrak et al., 2019; Neill et al., 2015; Oem et al., 2013; Ren et al., 2023). This could be attributed to a lack of sequencing of BPIV-3 positive samples worldwide. In this study, we isolated and obtained the full-genome sequences of different European BPIV-3 field strains, which were not publicly available so far. We observed the presence of BPIV-3 field strains with high genetic identity (>98%) in France and Sweden. The two countries have very limited live cattle import/export one with another (FAOStat), therefore similar BPIV-3 strains are likely circulating in other European countries and are then imported in France and Sweden through livestock trade (Gaudino et al., 2021). In our samples we did not detect BPIV-3 strains belonging to genotype B, however the number of tested samples was very limited and therefore the presence of the genotype B in Europe cannot be ruled out. The paucity of available genetic sequences currently limits the understanding of different aspects of BPIV-3 infection, such as the genetic diversity of the circulating field strains but also its evolution in presence and absence of vaccination, which induces selection pressure. An increase in complete genome sequencing could provide further insight into current circulating BPIV-3

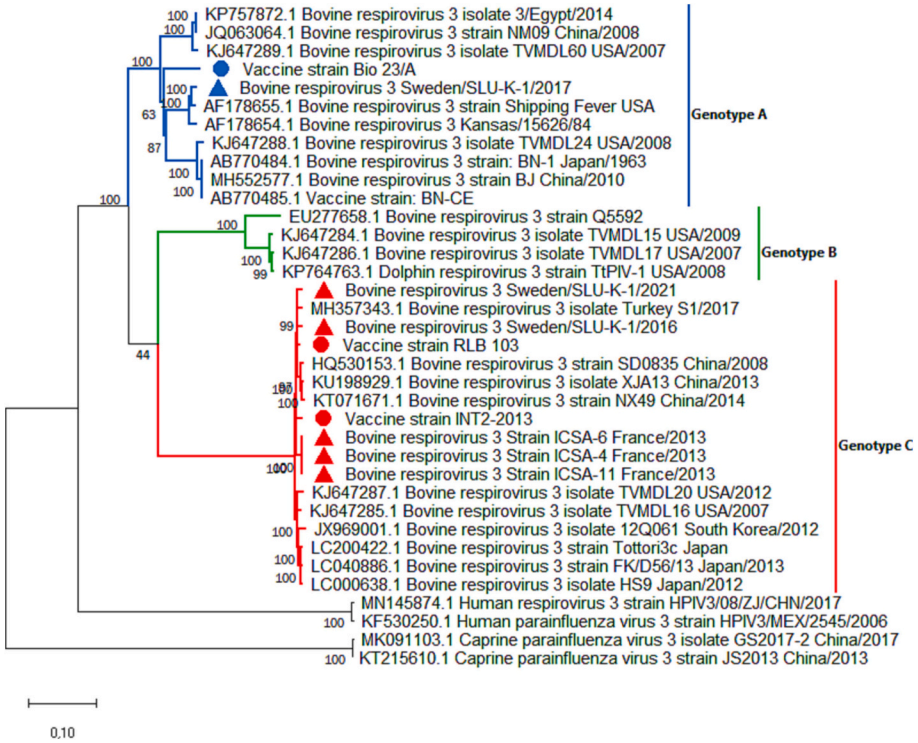

Fig. 2. Maximum-Likelihood phylogenetic tree based on the complete genome of BPIV-3 field strains and vaccine strains. The triangles indicate the field strains, whereas the circles indicate the vaccine strains. The tree was constructed using 1000 bootstrap replicates. The scale bar represents the number of nucleotide substitutions/site/year.

Table 1
Nucleotide (nt) and amino acid (aa) identities (based on the complete genome) between different BPIV-3 field strains and vaccine strains. The distance matrices were generated using Tamura-3 parameter and Jones-Taylor-Thornton models, respectively.

BPIV-3 strains		Genetic nt identity (%)	Genetic aa identity (%)
ICSA-4/France/2013	ICSA-11/France/2014	99.98	99.99
ICSA-6/France/2013	ICSA-4/France/2013	99.98	99.99
Sweden/SLU-K-1/2021	Vaccine RLB 103	98.91	99.24
ICSA-4/France/2013	Vaccine RLB 103	98.72	98.97
Sweden/SLU-K-1/2021	Sweden/SLU-K-1/2016	98.72	99.13
ICSA-6/France/2013	Sweden/SLU-K-1/2016	98.52	98.76
Sweden/SLU-K-1/2016	Vaccine INT2–2013	98.49	98.69
ICSA-11/France/2014	Vaccine INT2–2013	98.43	99.03
Sweden/SLU-K-1/2021	Vaccine INT2–2013	98.36	98.91
Sweden/SLU-K-1/2021	ICSA-4/France/2013	98.30	99.03
Sweden/SLU-K-1/2017	Vaccine Bio/23A	90.90	95.99
Sweden/SLU-K-1/2016	Vaccine Bio/23A	74.36	87.78
ICSA-11/France/2014	Vaccine Bio/23A	74.29	87.94
Sweden/SLU-K-1/2021	Vaccine RLB 103	74.15	87.77
Sweden/SLU-K-1/2017	Vaccine INT2–2013	74.08	88.05
Sweden/SLU-K-1/2017	Vaccine RLB 103	74.00	87.69
Human HPIV3/08/ZJ/CHN/2017	ICSA-6/France/2013	61.55	76.53
Caprine GS2017–2 China/2017	ICSA-11/France/2014	58.11	73.05

genotypes in cattle populations and could also help the detection of the putative new emergent and/or virulent strains.

Vaccination against BPIV-3 has been carried out since the last century and commercial vaccines (inactivated for injection or intranasal live-attenuated) are used to counteract the infection in cattle herds, often in combination with vaccine strains against BRSV (Makoschey and Berge, 2021). In our study, we observed a high genetic similarity (>98%) between field and some vaccine strains (i.e. sequences from France and INT2–2013 vaccine strain or RBL 103 vaccine strain). This could be due to the detection of vaccine strain in cattle farms, however our protocol included sampling collection in breeding farms without animal exchange and from calves that were either not vaccinated (ICSA-4 and ICSA-6) or the samples were collected at least three weeks following the vaccination (ICSA-11). Live-attenuated BPIV-3 shedding was detected in nasal swabs up to 8/10 days following the vaccine administration (Nuijten et al., 2022; Socha et al., 2013) with a peak at 5 days post-administration and with lower shedding compared to the wild-type strain. Therefore, the circulation of a vaccine strain among different farms in the same region is unlikely. In addition, the genetic similarity between BPIV-3 isolated from different French farms in different timeframes is very high (99.98%), suggesting the circulation of an actively replicating strain and not a live-attenuated BPIV-3. Although it cannot be totally ruled out, the probability of detecting vaccine strains in our samples is very low. Currently, there are no genetic markers that allow the discrimination between vaccine and field strains. This is due to the lack of publicly available BPIV-3 sequences. Our sequencing results could be useful to better understand the genetic differences between the circulating field and vaccine BPIV-3 strains. This is crucial for a correct interpretation of diagnostic findings and for the assessment of BPIV-3 prevalence in cattle population. In addition, the mutations in BPIV-3 attenuated vaccine strain genomes that prevent the virus from transmission among vaccinated animals are currently unknown and would be interesting to investigate in further studies.

Our genetic analyses indicated a heterogeneity between field strains and vaccine strains, with a nucleotide identity of <75% at a full-genome level for certain isolates (i.e. the sequence from Sweden from 2017 and vaccine strains INT2–2013 or RBL 103). In this study, we did not investigate the cross-reactivity between different BPIV-3 field strains. A serosurvey suggested a cross-reactivity between the genotypes A and C, despite the high genetic diversity (Muftuoglu et al., 2021). Therefore, the cross-reactivity also between genotypes A and B and between B and C has yet to be tested and cannot be excluded. In addition, neutralizing epitopes are known to be shared between human and bovine PIV-3 (Greenberg et al., 2005), despite the two sharing only a mean of 61% of nucleotide identity. To evaluate the real necessity of an update of vaccine strains, cross-neutralization of sera raised against all existing BPIV-3 genotypes should be carried out in future studies. More globally a study looking at cross-protection between BPIV-3 genotypes is warranted to better understand the protectotypes.

Our results provide the first full-genomes sequences of BPIV-3 in France and Sweden, and in Europe in general. More virological surveillance would be needed in order to assess if other genotypes are currently present on the continent. In addition, full genome sequences of circulating strains would help a better understanding of the adequacy of current vaccine strains compared to the strains that are detected during respiratory outbreaks.

CRediT authorship contribution statement

Maria Gaudino: Conceptualization, Investigation, Formal analysis, Writing – original draft. **Jean-François Valarcher:** Writing – review & editing, Funding acquisition. **Sara Hägglund:** Writing – review & editing, Funding acquisition. **Katarina Näslund:** Investigation, Writing – review & editing. **Siamak Zohari:** Investigation, Writing – review & editing, Funding acquisition. **Mariette F. Ducatez:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Gilles Meyer:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The whole genome sequence data obtained in this study were submitted to the National Center for Biotechnology Information Database (NCBI, accession numbers: OQ302282, OQ302283, OQ302284 (sequences from France), OQ349372, OQ349373, OQ349374 (sequences from Sweden). BPIV-3 complete genome sequences of the commercial vaccines are available upon request.

Acknowledgments

We thank E. Salem and H. Cassard for their precious help with samples collection. We are grateful to G. Croville for the valuable NGS support and to the Genotoul bioinformatics platform Toulouse Midi-Pyrenées and the Sigenae group for providing help and/or computing and/or storage resources thanks to Galaxy instance "http://sigenae workbench.toulouse.inra.fr". Swedish samples were collected through funding from the Swedish Research Council (Formas, Sweden), grant no. 2016–00463. This work was funded by the by the French National Agency for Research, project ANR-15-CE35-0005 'FLUD'. M. Gaudino is

supported by a PhD scholarship funded by the Departement Santé Animale (INRAE Toulouse) and the Région Occitanie. We also would like to thank the reviewers for their valuable suggestions that improved the quality of our manuscript.

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