Short Communication

First detection of equine coronavirus (ECoV) in Europe

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ABSTRACT

Equine coronavirus (ECoV) is involved mainly in enteric infections. Following the recent description of ECoV in 2000, this study reports for the first time the presence of ECoV in France and, on a wider scale, in Europe. ECoV was molecularly detected from diarrheic and respiratory specimens. Sequencing and phylogenetic analyses demonstrated that European strains are most closely related to the reference North American strain (ECoV-NC99) than the Asian strain (ECoV-Tokachi09).

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1. Introduction

Coronaviruses are enveloped, positive-strand RNA viruses with exceptionally large and polycistronic genomes (27–31 kb) (Lai et al., 2006). They are divided in four distinct genera tagged alpha-, beta-, gamma-, and delta-coronaviruses. These viruses are characterized by a considerable plasticity of their genome, which gives them a strong evolutionary potential, especially with respect to the crossing of species barriers (Woo et al., 2009). The history of coronaviruses is often marked by some successful emergences like the human SARS coronavirus in 2002 (Drosten et al., 2003). In 2012, the emergence of a new human betacoronavirus named *Middle East Respirato-ry Syndrom Coronavirus* (MERS-CoV) has set off an international warning (Zaki et al., 2012).

Equine coronavirus (ECoV) belongs to the betacorona-virus-1 species in lineage A (betacoronavirus A1) within the genus betacoronavirus, along with human coronavirus OC43, bovine coronavirus, and porcine hemagglutinating encephalomyelitis virus. ECoV was first isolated in North Carolina (USA) from the feces of a diarrheic foal in 1999 (ECoV-NC99) (Guy et al., 2000). The full-length genome of the ECoV-NC99 strain was then sequenced and published in 2007 (Zhang et al., 2007). Recently in Japan, ECoV was detected from the feces of adult horses with pyrogenic and enteric disease (ECoV-Tokachi09), and from fecal samples collected in three outbreaks that occurred at Obihiro Racecourse in Hokkaido (Oue et al., 2011, 2013). In October 2012, 4 outbreaks were associated with ECoV in adult horses in 4 different states in the USA (Pusterla et al., 2013). Of 59 sick horses, 44 were tested for ECoV by PCR and 38 of them (86%) were positive in feces samples. Surprisingly,

the follow-up samples available for 7 sick horses showed that ECoV persisted only 3–9 days in feces, suggesting a potential difficulty in detecting ECoV in these specimens. In mammals, coronaviruses are mainly involved in enteric, respiratory, and neurological diseases. In horses, ECoV has been associated so far with enteric diseases and mainly sought out in feces specimens. However, the main clinical signs observed during these recently described outbreaks were anorexia, lethargy, leucopenia, and fever, *i.e.* unspecific and discrete symptoms that do not lend themselves to rapid diagnosis. Outside the USA and Japan, very little information about the circulation of ECoV in the equine population is currently available in literature.

2. Materials and methods

2.1. Samples collection

During the winter season 2011/2012, 395 feces and 200 respiratory specimens were received in a veterinary diagnosis laboratory for virological, bacteriological, and parasitological routine testing. Samples were collected from foals and adult horses suffering from respiratory or enteric mild diseases. They were collected in 58 French counties, mainly in two counties of breeding in the Northwest (Calvados) and in the Southeast (Gard) (Fig. 1). Fecal and respiratory specimens were collected from November to May, the normal coronavirus circulation period in human and bovine models.

2.2. Molecular detection

Virological investigations were performed in order to determine the presence of the coronavirus in the French equine population. Two quantitative real-time RT-PCRs (qRT-PCRs) assays were designed for ECoV screening in horses. The first assay targets the M gene encoding the membrane protein (ECoV-M-f: 5′-GGTGGAGTTTCAACC-CAGAA-3′; ECoV-M-r: 5′-AGGTGCGACACCTTAGCAAC-3′; ECoV-M-p: 5′-(6FAM)-CCACAATAATACGTGGCCACCTTTA-(BHQ1)-3′), and the second one targets the N gene encoding the nucleocapsid protein (ECoV-N-f: 5′-GCCAATTCCGGCACTAGAAC-3′; ECoV-N-r: 5′-ACCCCTT-TTTCCAAAGCACT-3′; ECoV-N-p: 5′-(6FAM)-GACTGCCAAA-GAAGTCAGGC-(BHQ1)-3′). The complete M and N genes of the ECoV-NC99 strain were amplified from RNA extracted from tissue culture fluid. PCR products were then cloned and transcribed *in vitro*. The RNA transcript was used as a positive control in both qRT-PCRs.
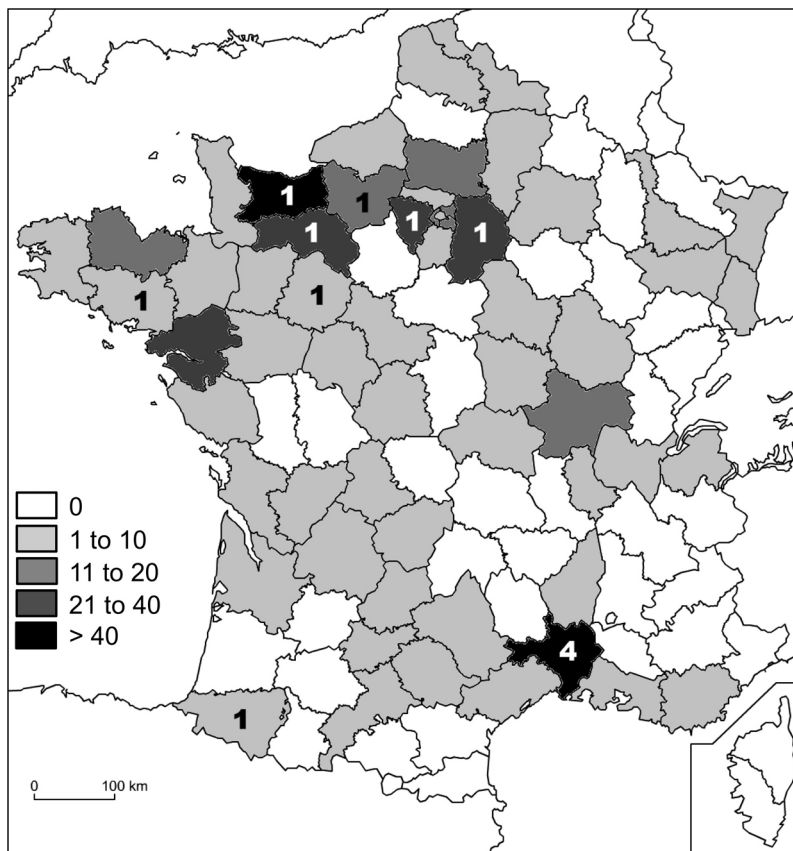

Fig. 1. Number of samples collected in French counties during winter season 2011/2012 and localization of ECoV positive samples. Number of collected samples, from 1 to more than 40, in each French county is indicated in gray-scale. The number of detected ECoV positive samples is indicated inside the county.

2.3. Sequencing and phylogenetic analysis

All ECoV positive samples detected by qRT-PCRs were sequenced on partial M and S1 genes with specific primers (M gene: ECoV-M-s: 5′-ACCTGGACTGCTGATGAAGC-3′ and ECoV-M-as: 5′-GTGTCCATGCCTGAACCTTT-3′; and S1 gene forECoV-S1-s: 5′-CAATGCCTTTATGGCTTGGT-3′ and ECoV-S1-as: 5′-AAACTCGGAAGGGATCTGAA-3′). Phylogenetic trees were constructed based on sequences obtained from partial M and S1 genes.

3. Results

A total of 12 samples (11 fecal samples and one respiratory specimen) out of 595 taken from 58 French counties, mainly in March/April, was positive for ECoV (Fig. 1). Two of the 12 ECoV positive samples were coinfected with cryptosporidium spp.; no other pathogen was associated with ECoV in these samples. The only positive respiratory sample (FRA_2011/2) was collected 3–4 days following the clinical signs from a 9-month-old foal suffering from respiratory infection. This is the first time that the detection of ECoV from a respiratory sample has been described; however, this aspect requires more examination since an etiological role of the agent was not conclusive. All other specimens were associated with enteric diseases in young foals. Four fecal samples were positive in the same county and were all related to an ECoV occurrence. The identification of the index case resulted in the collection of a number of samples in this area. During this occurrence, two ECoV positive fecal samples were collected at a five-week interval from the same adult horse who died subsequently with severe diarrhea. No other pathogen was detected in these samples.

Sensitivity of the two qRT-PCRs was evaluated on serial dilution of the extracted RNA from cell culture supernatant of the NC99 strain and with the RNA transcript. The comparison of the two "M and N qRT-PCRs" revealed a higher sensitivity of the "M qRT-PCR". Of the 12 ECoV positive samples detected by qRT-PCR, 12 were detected with the "M qRT-PCR" and 8 with the "N qRT-PCR". The "M qRT-PCR" produced a linear quantitative detection range of 9 logs with the correlation coefficient $R^2 = 0.999$ and a slope value of -3.822 (efficiency = 83%), with the lower limit of ECoV detection (RNA transcript) of 3×10^5 copies/ml. Ct values ranged from 20.1 to 39.6 cycles from fecal samples and had a Ct value of 35.4 cycles in the respiratory specimen. The "N qRT-PCR" had a dynamic range of 7 logs with a sensitivity of 3×10^7 copies/ml of RNA transcript, a correlation coefficient (R^2) of 0.983, and a slope value of -4.209 (efficiency = 73%).

Sequences of partial M and S1 genes were obtained for 5 out of the 12 ECoV positive samples (GenBank accession numbers KC178696–KC178705). Analysis of the partial S1 sequence revealed that nucleotide identities of the French strains ranged from 98.7% to 99.6% compared to the ECoV-NC99 sequence and from 97.2% to 98.1% compared to the Tokachi09 sequence. Phylogenetic trees constructed from partial M and S1 sequences showed that French molecular isolates are phylogenetically closely related to the ECoV-NC99 strain (Fig. 2). The molecular analysis revealed that none of the French strains had the 185-nucleotide deletion inside the S gene observed in the Tokachi09 strain (7).

4. Discussion

Since the 1999 identification of ECoV in North Carolina, only a few studies have demonstrated the presence of ECoV
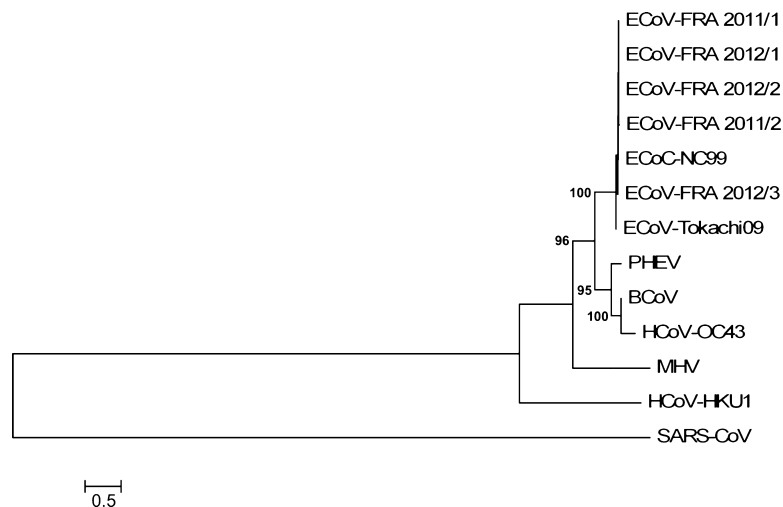

Fig. 2. Phylogenetic neighbor-joining tree of the partial S1 sequences (540 nt) of the French ECoV and other reference coronaviruses. The following sequences were used for phylogenetic analysis: French ECoV strains (GenBank accession no. KC178701–KC178705); ECoV NC99 (EF446615) and Tokachi09 (AB555560) strains; bovine coronavirus (BCoV) strain (AF220295); murine hepatitis virus (MHV) strain (NC006852); porcine hemagglutinating encephalomyelitis virus (PHEV) strain (NC007732); and human coronaviruses OC43 (AY585229), HKU1 isolate Caen1 (HM034837) and SARS (NC004718) strains. Phylogenetic tree was constructed using MEGA4.1 and values resulting from bootstrap analysis (1000 replicates) are indicated adjacent to the branching points (values > 50). The length of each pair of branches represents the distance between the sequence pairs. The scale bar represents the percentage of nucleotide differences (0.5 substitution per site).

in horses (Davis et al., 2000; Guy et al., 2000; Oue et al., 2011; Pusterla et al., 2013). The present study provides the first descriptive report of the presence of the virus in France, an important country for horse breeding in Europe.

To date, ECoV has only been identified in fecal samples from foals and adult horses having enteric disease or fever. The current study demonstrates the identification of ECoV in a respiratory specimen a few days after the onset of respiratory clinical signs. Prior to these results, the identification of ECoV in two sequential fecal samples at a 5-week interval contrasts with previously observed persistence of ECoV in horses for only 3–9 days (8). Our findings indicate a viral replication that is intense and persistent, hence the presence of potentially severe clinical forms.

Although isolation of ECoV strains by cell culture has been previously described in Guy et al. (2000), Zhang et al. (2007), Suzuki et al. (2008), and Oue et al. (2011), ECoV has proven difficult to isolate and propagate in cell culture as indicated in Balasuriya (2013). For this reason, ECoV isolation was not performed here, but is currently under development on epithelial cells in 3D cell culture in our laboratory.

In light of these findings, the "M qRT-PCR" seems to be a sensitive and useful tool for ECoV detection in field samples. However, this tool needs to be tested with an internal quality control for viral diagnosis in order to exclude false negatives due to possible inhibition. Sequencing and phylogenetic analyses have demonstrated that European strains are more closely related to the reference North American strain (NC99) than to the Asian strain (Tokachi09).

Despite the presence of ECoV in Europe – and more widely on 3 continents – clearly shows the ubiquitous nature of the virus, relatively little is known about ECoV strains circulating in the world. Indeed, much is to be learned about the circulation of the betacoronavirus A1 group inside coronaviruses, which will, in turn, play an important role in our understanding of the emergence of CoVs. A novel betacoronavirus A1 was discovered very recently in fecal samples from dromedary camels in Dubai (United Arab Emirates) – the dromedary camel coronavirus (DcCoV) UAE-HKU23 – as part of the continuous surveillance conducted to identify the potential animal reservoir of MERS-CoV. The DcCoV has a 126-nt sequence in the RdRp identical to that of equine CoV, but differs from the MERS-CoV also detected in dromedaries (Woo et al., 2014). Knowledge of ECoVs and closed coronavirus strains will make an important contribution to the comprehension of circulating strains in the world. Epidemiological studies on ECoV infections will contribute to determining the impact and the prevalence of the virus in the equine population.

Acknowledgements

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