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Review Article

Enteric coronavirus infection in adult horses

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ABSTRACT

A new enteric virus of adult horses, equine coronavirus (ECoV), has recently been recognized. It is associated with fever, lethargy, anorexia, and less frequently, colic and diarrhea. This enteric virus is transmitted via the feco-oral route and horses become infected by ingesting fecally contaminated feed and water. Various outbreaks have been reported since 2010 from Japan, Europe and the USA. While the clinical signs are fairly non-specific, lymphopenia and neutropenia are often seen. Specific diagnosis is made by the detection of ECoV in feces by either quantitative real-time PCR, electron microscopy or antigen-capture ELISA. Supportive treatment is usually required, as most infections are self-limiting. However, rare complications, such as endotoxemia, septicemia and hyperammonemia-associated encephalopathy, have been reported, and have been related to the loss of barrier function at the intestinal mucosa. This review article will focus on the latest information pertaining to the virus, epidemiology, clinical signs, diagnosis, pathology, treatment and prevention of ECoV infection in adult horses.

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Introduction

Equine coronavirus (ECoV) is a newly recognized enteric virus of adult horses that has been associated with fever, lethargy and anorexia, as well as colic and diarrhea. Outbreaks have been reported in Japan, Europe and the USA since 2010. This article reviews biology, epidemiology, clinical signs, diagnosis, pathology, treatment and prevention of ECoV infection in horses.

Etiology

Coronaviruses are single-stranded, positive sense, non-segmented, enveloped RNA viruses belonging to the family *Coronaviridae*. They are responsible for respiratory, enteric, hepatic and neurologic disease in mammals and birds (Wege et al., 1982). Within the *Coronaviridae* subfamily *Coronavirinae*, there are four genera defined on the basis of serological cross-reactivity and genetic homology: *Alphacoronavirus*, *Betacoronavirus*, *Deltacoronavirus* and *Gammacoronavirus* (Woo et al., 2012). ECoV is classified within the *Betacoronavirus 1* genus, along with human coronaviruses OC43, 4408 and HKU1, bovine coronavirus (BCoV), porcine hemagglutinating encephalomyelitis virus, canine respiratory

coronavirus, mouse hepatitis virus, bubaline coronavirus and sialodacryoadenitis rat coronavirus (Zhang et al., 2007). Coronaviruses have helical capsids made up of the nucleocapsid protein complexed with the genomic RNA. In the envelope, the spike protein trimerises to form peplomers, giving the virion its corona or crown-like morphology. ECoV also has smaller spikes on the membrane made from the hemagglutinin esterase protein. In addition, two structural proteins, designated the membrane and small membrane proteins, of the virion are fully trans-membranous (Weiss and Leibowitz, 2011). Full genome sequences are available for four ECoV isolates, one from the USA and three from Japan (Zhang et al., 2007; Nemoto et al., 2015a). The three recent isolates from Japan were closely related to the isolate from North Carolina (NC99), with sequence homology ranging from 98.2 to 98.7% (Nemoto et al., 2015a).

Epidemiology

Since 2010 (this is the first full-year data set after ECoV real-time-quantitative PCR (qPCR) testing was introduced by molecular diagnostic laboratories in December 2009), the compiled data from three veterinary diagnostic laboratories in the USA (IDEXX Laboratories, West Sacramento, USA; Animal Health Diagnostic Center, AHDC, Cornell University, Ithaca, USA; Real-time PCR Research and Diagnostics Core Facility, University of California, Davis, USA) shows that the total yearly number of ECoV qPCR

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Fig. 1. Relative proportion of equine coronavirus real-time quantitative PCR positive (black) and negative (white) samples by states submitted from January 2012 to December 2016 to three commercial veterinary diagnostic laboratories in the USA (IDEXX Laboratories, West Sacramento; Animal Health Diagnostic Center, Cornell University, Ithaca; Real-time PCR Research and Diagnostics Core Facility, University of California, Davis).

positive cases has steadily increased, while the frequency of positive cases has remained within the 2 to 6% range. The increase in overall number is driven by increased awareness and testing. The number of ECoV qPCR-positive cases is higher during the colder months of the year (October to April), similar to the seasonal disease pattern seen with BCoV. Samples from horses testing positive for ECoV by qPCR have come from all states of the USA except Hawaii and Alaska (Fig. 1).

Since 2010, clinical ECoV infections have predominantly been reported in adult horses (Oue et al., 2011, 2013; Pusterla et al., 2013; Miszczak et al., 2014; Fielding et al., 2015). As an example, in 2014, the age distribution of confirmed ECoV infections was 20.5% in foals (age 0–6 months), 25.3% in horses aged 6 months to 5 years and 54.2% in horses older than 5 years. Foals from Central Kentucky with clinical gastrointestinal disease were found to be only positive for ECoV if other co-infections were present (e.g. rotavirus or *Clostridium perfringens*) with the detection frequency of ECoV by qPCR being similar between healthy and clinically affected foals (Slovic et al., 2014). This is in sharp contrast to a similar study performed in Japan (Nemoto et al., 2015b), showing that ECoV was not detected in rectal swabs from 307 diarrheic foals in the Hidaka district of Hokkaido. Adult horses with clinical signs experience a mono-infection with ECoV (Pusterla et al., 2013). The closely-related BCoV also causes enteric disease in adult cows and mild respiratory disease in calves (Saif et al., 1986; Tsunemitsu et al., 1999; Trávně et al., 2001; O'Neill et al., 2014). ECoV outbreaks in adult horses have been reported in riding, racing and show horses, and less frequently in breeding animals. It is speculated that given the lack of large numbers of documented clinical cases at larger breeding farms, one hypothesis is that ECoV circulates between young and adult horses, conferring protection against clinical disease in adult horses.

A recent seroepidemiological study performed on 5250 healthy adult horses from 18 states in the USA showed various risk factors associated with ECoV seropositivity (Kooijman et al., 2017). The Midwest region displayed the highest odds ratio (OR) of seropositivity in horses (OR=1.80). Furthermore, draft horses showed the highest odds ratio of seroprevalence of the breeds tested (OR=1.95), while Thoroughbreds had the lowest odds ratio of seroprevalence (OR=0.48). Ranch/farm and breeding horses displayed the highest odds ratio of seroprevalence (OR=1.30, OR=1.60). The authors of this study speculated that factors contributing to the higher ECoV seroprevalence in the Midwest could be related to the higher numbers of ECoV seropositive draft horses used for farm/ranch work and breeding in that region.

While the determined risk factors highlighted by Kooijman et al. (2017) are very specific to the studied horse population, it is interesting to notice that draft horses have a higher infection rate compared to other breeds. This parallels with the first ECoV cases reported from Japan in racing draft horses (Oue et al., 2011, 2013).

A recent experimental study in which young Japanese draft horses were infected by naso-esophageal intubation using feces from a confirmed ECoV infected horse, supports a feco-oral route of transmission (Nemoto et al., 2014). Seventy-five percent of the horses developed clinical signs compatible with ECoV infection and ECoV was detected in their feces using qPCR. Nasal secretions were ECoV qPCR-positive during the time of peak fecal shedding. However, it could not be determined if this was due to nasal replication and shedding of the virus, from environmental contamination from the feces, or from both sources.

In a study of young and adult horses with either respiratory or gastrointestinal signs in France, 11/395 (2.8%) fecal samples and 1/200 (0.5%) nasal swabs were positive for ECoV by qPCR (Miszczak et al., 2014). Out of 2437 nasal secretions from horses with fever and respiratory signs tested for common respiratory viruses, 17 horses (0.7%) tested qPCR-positive for ECoV (Pusterla et al., 2015). Five of 315 (1.6%) rectal swabs and 0/306 nasal swabs from apparently healthy horses in Saudi Arabia and Oman were positive for ECoV by PCR (Hemida et al., 2017). Collectively, these studies show that ECoV is infrequently detected in nasal secretions from healthy horses and horses with respiratory infections.

In field outbreaks, the morbidity rates for ECoV infections have been reported to range from 10 to 83% (Oue et al., 2011, 2013; Pusterla et al., 2013; Fielding et al., 2015). Fatalities are rare, but have been associated with disruption of the gastrointestinal mucosal barrier leading to septicemia, endotoxemia and hyperammonemia-associated encephalopathy (Fielding et al., 2015; Giannitti et al., 2015). The incubation period for ECoV is short and clinical disease develops between 48 to 72 h after either natural exposure or experimental infection (Nemoto et al., 2014; Fielding et al., 2015). Clinical signs are present for a few days to 1 week and generally resolve with minimal supportive care.

Under natural conditions, fecal shedding of ECoV by qPCR has been reported to range from 3 to 25 days (Pusterla et al., 2013; Fielding et al., 2015; L.D. Mittel, unpublished data). It is not known how long ECoV persists in the environment and, if so, whether this can act as a source of infection. Human coronaviruses, such as severe acute respiratory syndrome (SARS)-CoV, have been shown to persist up to 2 days in waste water and dechlorinated tap water, 3 days in feces and 17 days in urine at room temperature (Wang et al., 2005). Survival may be longer at lower temperatures; the virus persists for up to 14 days in waste water and at least 17 days in feces or urine at 4 °C (Wang et al., 2005). Greater survival and viability of ECoV and BCoV in colder weather is one possible explanation for the apparent higher prevalence of virus-positive fecal samples and clinical disease during cooler weather.

Clinical presentation

When clinical information was pooled from 20 outbreaks of ECoV during the period of November 2011 to March 2017, 130/472 (27.5%) horses presented with clinical signs (Pusterla et al., 2013; N. Pusterla, unpublished data). The most frequent clinical signs reported were anorexia (97%), lethargy (88%) and fever (83%). Peak rectal temperature of febrile horses ranged from 38.6 to 41.0 °C (median 39.9 °C). Changes in fecal character, ranging from soft-formed to watery consistency, and colic were observed in 23% and 19% of clinically affected horses, respectively. Systemic signs (anorexia and fever) preceded gastrointestinal signs. Lethargy, anorexia and pyrexia were also the main clinical signs in recent outbreaks in adult draft horses from Japan, while specific

gastrointestinal signs were observed in about 10% of affected horses (Oue et al., 2011, 2013). Signs of encephalopathy, including circling, head pressing, ataxia, proprioceptive deficits, nystagmus, recumbency and seizures, have been reported in 3% of clinically infected horses (Pusterla et al., 2013; Fielding et al., 2015). Fielding et al. (2015) reported a case that had severe hyperammonemia ($677 \mu\text{mol/L}$; reference $\leq 60 \mu\text{mol/L}$) and died after exhibiting encephalopathic signs. Hyperammonemia associated with ECoV infection may be caused by increased ammonia production within or absorption from the gastrointestinal tract as a consequence of gastrointestinal barrier breakdown. Enteric ammonia production could increase as a result of bacterial microbiome changes associated with ECoV infection.

During various outbreaks, 4–83% of horses have remained asymptomatic (inapparent infection), even when ECoV was detected in their feces (Pusterla et al. 2013; N. Pusterla, unpublished data). The wide range of inapparent infections likely relates to a combination of viral, host and environmental factors modulating disease expression.

Diagnostic evaluation

A diagnosis of ECoV infection relies on the presence of clinical signs compatible with ECoV infection, hematological changes (neutropenia and/or lymphopenia), the exclusion of other infectious agents and the detection of ECoV in feces.

The majority of horses infected with ECoV develop leukopenia due to neutropenia and/or lymphopenia. In 37 clinical cases of ECoV infection, the total nucleated cell count ranged from 520 to $6890/\mu\text{L}$ (median $3210/\mu\text{L}$; reference interval $5000\text{--}11,600/\mu\text{L}$; Fig. 2). The blood white cell count showed leukopenia in 73%, neutropenia in 65% and lymphopenia in 81% of the horses. The neutrophil and lymphocyte counts in these horses ranged from 250 to $4814/\mu\text{L}$ (median $1643/\mu\text{L}$; reference interval $2600\text{--}6800/\mu\text{L}$) and $80\text{--}3510/\mu\text{L}$ (median $950/\mu\text{L}$; reference interval $1600\text{--}5800/\mu\text{L}$), respectively. Some animals showed less consistent hematological abnormalities, including the presence of band neutrophils and shifts in monocyte counts (usually changing from

a low or a normal count to a high-normal or rebound monocytosis). If no complications associated with the disruption of the gastrointestinal barrier occurred, the hematological abnormalities generally resolved in 5–7 days. However, a normal blood cell count and white cell differential did not rule out an ECoV infection, as rather 11% of ECoV infected horses showed no hematological abnormalities.

Usually, biochemical parameters are unremarkable, but elevation of total and indirect bilirubin due to partial or complete anorexia, electrolyte changes consistent with enterocolitis, transient elevation of liver enzymes and renal parameters suggesting pre-renal azotemia have been observed in some cases. Blood ammonia should be measured in any horse with signs of encephalopathy and suspected ECoV infection.

Detection of coronavirus in feces historically was based on negative-stain electron microscopy and antigen-capture ELISA (Davis et al., 2000; Guy et al., 2000). These types of tests have not subsequently been evaluated for ECoV detection as analytical sensitivity and limits of detection are likely to be poorer than the more sensitive molecular tests. Similarly, isolation of ECoV in cell culture is difficult and time consuming, and is not recommended for diagnostic purposes (Guy et al., 2000; Oue et al., 2011).

Sensitive laboratory diagnosis of ECoV is possible through fecal reverse transcription qPCR. The kinetics of viral shedding in feces from experimentally infected horses showed onset at 3–4 days, continuing until 12–14 days post-infection (Nemoto et al., 2014); peak shedding followed 3–4 days after the development of clinical signs (Fig. 3). In naturally-infected horses, detectable ECoV shedding by qPCR can last for 3–25 days (Pusterla et al., 2013; Fielding et al., 2015).

A correlation between high viral load and mortality has recently been reported for ECoV (Fielding et al., 2015). In SARS-CoV infections, patient survival during acute disease is correlated with viral load (Hung et al., 2009). One factor affecting viral load is the ability of individuals to express genes involved in inflammation and innate immunity (Chen et al., 2006). Variations in viral load can also be influenced by the coronavirus strain. The replication capability of feline coronavirus (FCoV) provides good evidence of its effect on virulence (Chang et al., 2012). The benign feline enteric coronavirus (FECV) genotype has low replication competency in enterocytes, but FCoV also has a highly virulent feline infectious peritonitis (FIP) virus (FIPV) genotype with an enhanced ability to replicate in different cell populations. A single point mutation on the spike gene of the different FCoV genotypes has been linked to the changes in cell tropism, replication potential and clinical manifestations (Chang et al., 2012).

Post-mortem diagnosis of ECoV can be achieved by qPCR on feces or small intestinal contents collected at post-mortem examination. Small intestinal tissue can also be tested for the presence of ECoV by immunochemistry and direct fluorescent antibody testing using BCoV reagents (Giannitti et al., 2015).

Pathogenesis

ECoV may be detected in both healthy foals and foals with diarrhea; however, of the two major studies documenting this, there is a difference in results from two different geographic regions. Surprisingly, in the Hidaka district of Hokkaido, all 337 rectal swabs from foals with diarrhea were negative for ECoV by qPCR, while 3/120 (2.5%) fecal swabs from healthy foals were positive for ECoV. This study is in contrast to a study performed in Central Kentucky, where ECoV was detected at a similar frequency in feces of both healthy and diarrheic foals (Slovits et al., 2014). The difference between healthy and clinically-affected animals was that, from the panel of infectious agents tested, healthy foals usually (except for one) only showed infection with ECoV, whilst

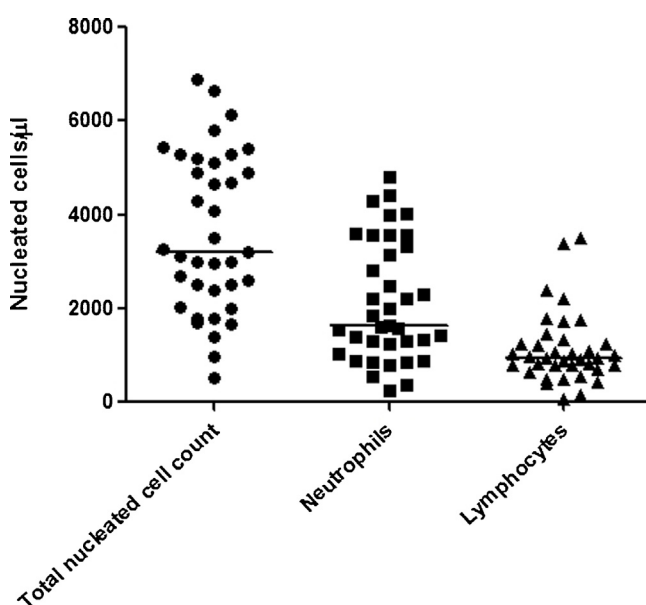


Fig. 2. Hematological findings in 37 adult horses with laboratory confirmation of equine coronavirus infection. The results are expressed as individual values, with the median indicated by a horizontal bar.

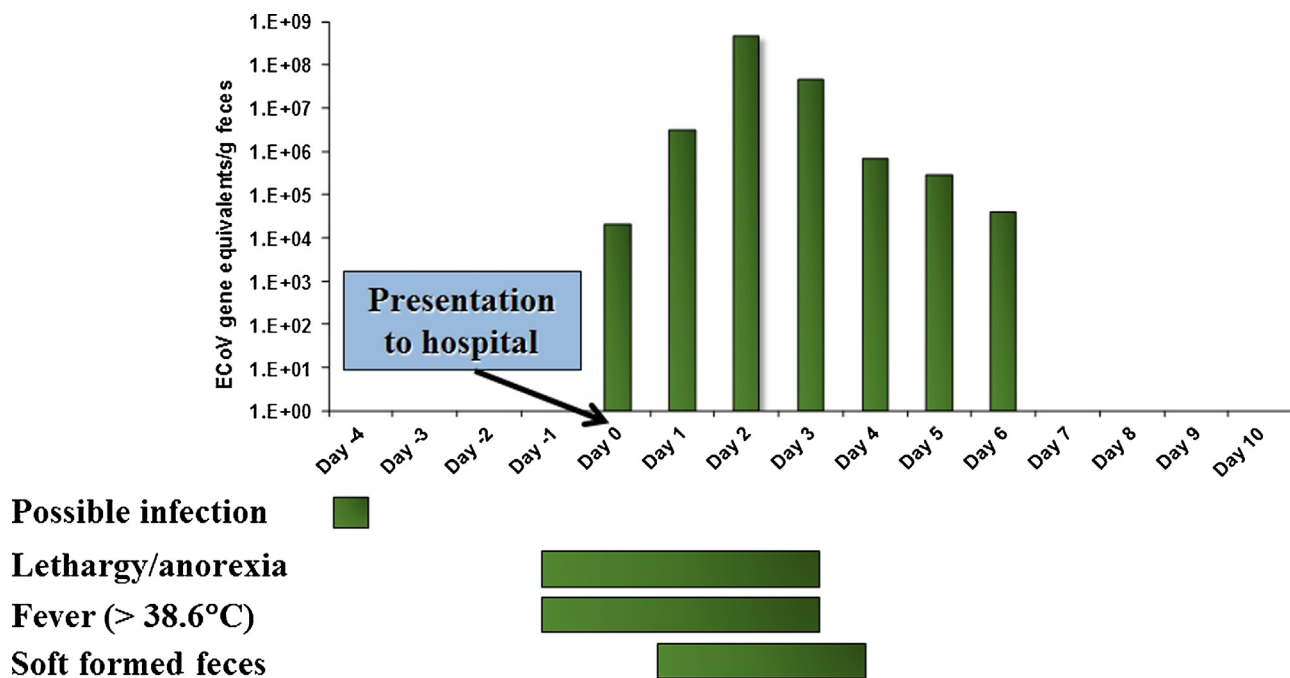


Fig. 3. Diagram showing temporal clinical signs and fecal shedding of equine coronavirus (ECoV) in an adult horse presented to a referring hospital because of anorexia, lethargy and fever.

ECoV positivity in foals with gastrointestinal disease was associated with co-infections (Slovic et al., 2014). An ECoV infection in a foal may predispose the animal to a secondary infection with other enteric pathogens, as shown for other coronaviruses (Pakpinyo et al., 2003; Srikumaran et al., 2007; Brockmeier et al., 2008).

However, in clinically-affected adult horses, the predominant infection pattern is of ECoV alone. This suggests that the infected animal's age influences the pathogenicity of ECoV, perhaps due to differences in immunological responses between foals and adult horses, or the presence of protective maternally-derived antibodies in foals. Age-related patterns of disease have been reported for FCoV, where most FIP cases occur in cats younger than 2 years of age and protracted dry cases tend to occur in older cats. The clinical outcome of the infection is affected by the development of humoral, cell-mediated or mixed immune responses against the

highly virulent FIPV genotype of FCoV. Interestingly, severe suppression of natural killer cells and T regulatory cells is correlated with clinical outcome, confirming reports that fatal FIPV infection is associated with a weakened cell-mediated immune response (Vermeulen et al., 2013).

Giannitti et al. (2015) has recently described the pathology of ECoV in three adult equids. These infected animals displayed severe diffuse necrotizing enteritis, with marked villus attenuation, epithelial cell necrosis in the tips of the villi, neutrophilic and fibrin extravasation into the small intestinal lumen (pseudomembrane formation), as well as crypt necrosis, microthrombosis and hemorrhage (Fig. 4a). ECoV was detected by RT-qPCR in small intestinal tissue, gastrointestinal contents and/or feces, and coronavirus antigen was detected by immunohistochemistry and/or direct fluorescent antibody testing in the small intestine of all cases (Fig. 4b).

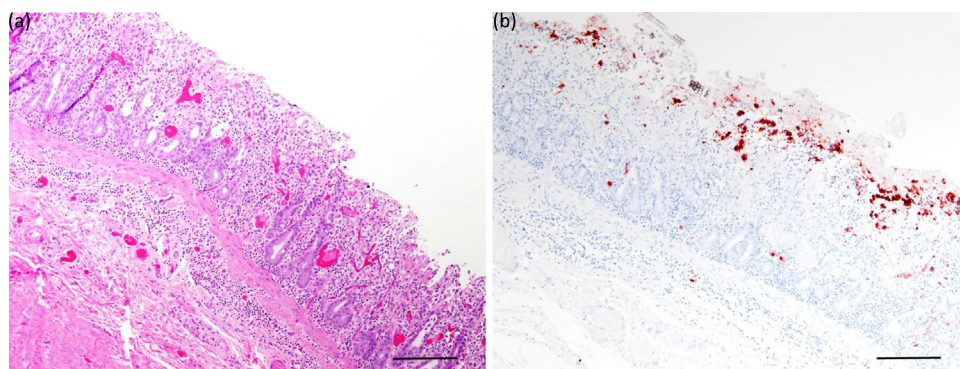


Fig. 4. (a) Histological section of jejunum from an adult horse with equine coronavirus (ECoV) infection. There is loss of crypts and the few remaining crypts are dilated, lined by attenuated epithelium and contain sloughed necrotic enterocytes. The lamina propria and superficial submucosa are expanded by inflammatory infiltrates. Capillaries and venules in the mucosa and submucosa are occluded by fibrin thrombi. Haematoxylin and eosin. (b) Immunohistochemistry for bovine coronavirus with hematoxylin counterstain on a histological section of jejunum from an adult horse with ECoV infection. Strong granular/globular immunoreactivity is seen in the cytoplasm of enterocytes deep in the crypts. Scale bar = 200 µm.

Treatment and prevention

Most horses with ECoV infections require no to minimal supportive treatment, as they are self-limiting. However, when clinical signs persist for longer than 24 h, affected horses should be treated with non-steroidal anti-inflammatory drugs (NSAIDs), as long as their hydration status is believed to be normal. NSAIDs are known to have adverse effects on the equine intestine and this should be taken into consideration before these drugs are used. Horses with more severe signs, such as diarrhea and/or colic, should be treated more intensively with parenteral or enteral fluids and electrolytes. In horses developing signs of endotoxemia and/or septicemia secondary to disruption of the gastrointestinal barrier, antimicrobial agents and gastrointestinal protectants should be considered. While hyperammonemia-associated encephalopathy only occurs in a small percentage of horses with ECoV infection, early recognition and treatment with oral lactulose or neomycin sulfate is associated with a positive outcome (N. Pusterla, unpublished data).

There is no licensed vaccine against ECoV, although the virus is closely related to BCoV, against which modified-live vaccines are available that are routinely used in cattle for the prevention of winter dysentery (Welter, 1998). The use of BCoV vaccines for the prevention of ECoV in horses has, to the authors' knowledge, not been investigated for safety or efficacy.

Biosecurity recommendations

Routine management practices aimed at reducing the chances of introducing and disseminating ECoV into premises (boarding facilities, showground or veterinary hospitals) should be implemented. Any horse developing or presenting with fever ($\geq 38.5^{\circ}\text{C}$), anorexia and lethargy, with or without enteric signs (colic, diarrhea), should be strictly isolated until a laboratory diagnosis is made. If ECoV infection is confirmed by qPCR, isolation procedures should continue and secondary quarantine of the source stable should be put in place. Post-infection testing of clinical cases should be undertaken to prevent viral spread to other horses. All new arrivals onto stables and farms should ideally be isolated for at least 3 weeks.

Strict biosecurity measures include the use of foot-baths, and personal protective equipment, which must be adequately maintained for sanitary purposes. Grooms and other personnel should work with the infected/possibly infected animals last in the course of their daily routine. Infected horses should be exercised when other horses are not present in the training areas, and riders should wear protective clothing, and clean and disinfect their boots, tack and hands after contact with these animals. Depending on specific circumstances, horses returning from shows or extended traveling events should be isolated. All horse vans and trailers should be thoroughly cleaned and disinfected after use. Common disinfectants inactivate ECoV. The most effective tool in determining possible sources of virus introduction remains the examination of at-risk horses for clinical signs of disease, including twice daily assessment of rectal temperature.

Conclusions

Equine coronavirus (ECoV) has been recognized recently in Japan, Europe and the USA as a new, clinically important, enteric virus of adult horses. There are increasing reports of the disease, arising from increased awareness in the field and the availability of diagnostic tests for detecting ECoV in feces of affected horses. Clinical signs are relatively non-specific; however hematological findings are consistent with a viral infection and generally are characterized by lymphopenia and neutropenia. Laboratory

diagnosis of ECoV infection is now made by the detection of ECoV in feces by qPCR. The epidemiology, pathogenesis and prevention of ECoV infection in horses is now being actively investigated.

Conflict of interest statement

Drs Vin and Leutenegger work for IDEXX Laboratories and Dr Mittel works for the Animal Health Diagnostic Center at Cornell University. Drs Pusterla, and Divers have no conflicts of interest to declare.

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