Original Article

Evaluation of safety, humoral immune response and faecal shedding in horses inoculated with a modified-live bovine coronavirus vaccination

J. S. W. Prutton* [iD], S. Barnum and N. Pusterla [iD]

Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, California, USA
Corresponding author email: Jamie.Prutton@theleh.co.uk

Dr Prutton's current address: Liphook Equine Hospital Forest Mere, Liphook, Hampshire, GU30 7JG, UK

Summary

Equine coronavirus (ECoV) is considered an emerging enteric virus with reported morbidity rates ranging from 10 to 83% and fatality rates ranging from 7 to 27% in adult horses; a vaccine for ECoV is currently not available. This study investigated the safety, humoral response and viral shedding in horses inoculated with a commercially available modified-live bovine coronavirus (BCoV) vaccine. Twelve healthy adult horses were vaccinated twice, 3 weeks apart, either orally, intranasally or intrarectally. Two healthy unvaccinated horses served as sentinel controls. Following each vaccine administration, horses were monitored daily for physical abnormalities whilst the onset and duration of BCoV shedding was determined by quantitative PCR (qPCR) in nasal secretions and faeces. Whole blood was collected every 3 weeks to determine BCoV-specific antibody response. With the exception of transient and self-limiting changes in faecal character observed in seven vaccinated and one control horse, no additional abnormal clinical findings were found in the study horses. Following the first and second vaccine administration, two and one horse, respectively, tested qPCR-positive for BCoV in nasal secretions 1-day post intranasal vaccination. No vaccinated horses tested qPCR-positive for BCoV in faeces following each vaccine administration. One of the two horses that shed BCoV seroconverted to BCoV after the first vaccine administration and an additional two vaccinated horses (oral and intrarectal) seroconverted to BCoV after the second vaccine administration. In conclusion, the results show that the modified-live BCoV is safe to administer to horses via various routes, causes minimal virus shedding and results in detectable antibodies to BCoV in 27% of the vaccinates.

Introduction

Equine coronavirus (ECoV) is considered an emerging enteric virus with reported morbidity rates ranging from 10 to 83% and a fatality rates ranging from 7 to 27% in adult horses (Oue *et al.* 2011; Pusterla *et al.* 2013; Fielding *et al.* 2015). Clinical signs include pyrexia, anorexia, lethargy, colic and diarrhoea accompanied by leucopoenia secondary to neutropenia and lymphopenia. Recent studies reported the detection of ECoV worldwide in faeces and respiratory secretions of adult horses with pyrogenic and enteric disease (Oue *et al.* 2013; Pusterla *et al.* 2013; Miszczak *et al.* 2014). Coronaviruses are grouped into three different genera: alphacoronavirus, betacoronavirus

and gammacoronavirus (Woo *et al.* 2009). ECoV shares the betacoronavirus-1 genera together with bovine coronavirus (BCoV) and is therefore closely related (Woo *et al.* 2009). Given the strong homology between BCoV and ECoV, the former could therefore act as a surrogate for ECoV vaccination (Guy *et al.* 2000; Oue *et al.* 2011).

Vaccination is the cornerstone of reducing clinical signs of viral disease but at present a vaccine for ECoV is not available. An inactivated BCoV vaccine was recently shown to lead to a measurable BCoV and ECoV antibody response (Nemoto *et al.* 2017). The recent study used an inactivated adjuvanted BCoV vaccination which probably would lead to a more measurable immune response than a modified-live vaccine but the effect on mucosal immunity is still undetermined in horses. Therefore, investigating a modified-live vaccine could, if safe, change the mucosal immunity and response to infection.

A modified-live bivalent vaccine containing bovine rotavirus and coronavirus is commercially available for the reduction of enteric disease in cattle. Field efficacy studies in calves have shown a significant reduction in the incidence and death from neonatal calf diarrhoea following the use of the modified-live BCoV vaccine (Waltner-Toews *et al.* 1985).

Based on the close genetic and antigenic relationship between ECoV and BCoV, it was hypothesised that the modified-live BCoV vaccine would be safe to administer to adult healthy horses, would induce a short duration of viral shedding based on the vaccine administration route and probably trigger a measurable humoral immune response in vaccinated horses.

Materials and methods

Fourteen adult, healthy horses housed individually at the Center for Equine Health, School of Veterinary Medicine, University of California at Davis were enrolled in the study. Animal use was approved by the Institutional Animal Care and Use Committee at the University of California at Davis. The study horses were deemed healthy based on normal physical examination and haematological parameters. Further, nasal secretions and faeces from every study horse were determined to be qPCR-negative for BCoV and ECoV and all horses tested seronegative against BCoV and ECoV prior to study commencement. Four study horses per group

were vaccinated either orally, intranasally or intrarectally using a commercially available modified-live bovine rota-coronavirus vaccine (CalfGuard).[1] The vaccine vials were reconstituted according to the manufacturer's recommendations and given either orally, intranasally using a small 6″ nasal cannula or intrarectally using a 10 French 10″ rubber catheter. To increase the vaccine volume and increase mucosal contact, the oral and intrarectal vaccines were diluted each in 50 mL of PBS prior to administration. Vaccinated horses were given a primary vaccination followed by a booster vaccination 3 weeks later (as per the manufacturer's label). Two additional horses remained unvaccinated and served as environmental sentinels. Following the first and second vaccination period, all horses were monitored daily, for 7 days, by means of a physical examination. Further, faeces and nasal secretions were collected daily from each of the study horses for the molecular detection of BCoV via qPCR. Briefly, the qPCR assay used was based on the detection of a specific 93 base-pair product of the *N* gene of BCoV (GenBank accession number EU401980.1; oligonucleotides: forward primer BCoV-826f CCCAATAAACAATGCACTGTTCA, reverse primer BCoV-919r CACTAGTTCCAAGTTTTAACATTTCTCC, probe UPL #119 TTGGTGGT).

The samples were amplified in a combined thermocycler/fluorometer (7900 HT Fast),[2] with the standard thermal cycling protocol: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 60 s at 60°C. Furthermore, a qPCR assay targeting a universal sequence of the bacterial *16S rRNA* gene (faeces) and a qPCR assay targeting the housekeeping gene eGAPDH (nasal secretions) were used as quality control (i.e. efficiency of DNA purification and amplification) and as an indicator of inhibition (Mapes *et al.* 2007). A standard curve was run for the assay using BCoV plasmids and the amplification efficiency calculated from the slope using the formula $E = 10^{1/-s} - 1$. The amplification efficiency was 98.3% for the *N* gene of ECoV indicating a very high analytical sensitivity. The qPCR assay was able to detect the modified-live BCoV vaccine but unable to detect field sample positive for ECoV.

Whole blood was collected prior to each vaccination and every 3 weeks up to 21 days following the second vaccine administration for the detection of specific antibodies to BCoV. A commercial ELISA for BCoV[3] was utilised to detect specific antibodies to BCoV. Briefly, blood samples were centrifuged for 1 h after collection and serum was aliquoted and stored at −80°C until analysed. The BCoV antibody test manufacturer's instructions were followed except the secondary antibody was replaced with horseradish peroxidase conjugated antiequine IgG antibody[4] at a dilution of 1:110,000 and 5 positive and 5 negative equine coronavirus serum samples were used to determine OD cut-off titre. Each serum sample was diluted 1:25 with saline and was added to both the viral antigen and noninfectious BCoV antigen wells. After one-hour incubation and plate washing, the secondary antibody was added and incubated for one additional hour. The plate was washed again, and substrate solution was added for exactly 10 min and then stopped. The plate was read within 10 min of stopping at an OD of 450 nm. The ELISA was able to reliably and repeatedly classify negative and positive equine control serum samples using an OD cut-off of 0.233.

Results

The study group consisted of seven geldings and seven mares with an age range of 11–22 years (median 17 years). One of the oral vaccinated horses was lost to follow-up shortly before the second vaccine administration due to an unrelated disease. Physical examination showed no abnormalities with the exception of transient and self-limiting changes in faecal character ("cow-pie" faeces) in eight horses (**Table 1**). During the primary vaccination, 2 intranasally vaccinated horses (Horse 1: Days 1–6; Horse 4: Days 6–7), 3 intrarectally vaccinated horses (Horse 5: Days 6–7; Horse 6: Days 6–7; Horse 8: Days 5–7) and one orally vaccinated horse (Horse 10: Day 5) demonstrated "cow-pie" faeces. Following booster vaccination, all the same horses showed "cow-pie" faeces (Horse 1: Days 2, 6 and 7; Horse 4: Day 3;

TABLE 1: Specific clinical, molecular and serological findings determined for each study horse during the two vaccination periods

Vaccination group	Horse	Vaccination period 1 (Days 1–7)			Vaccination period 2 (Days 21–27)			Day 42
		Soft formed faeces (number of days)	BCoV PCR (time post-vaccination)	BCoV Serology	Soft formed faeces (number of days)	BCoV PCR (time post-vaccination	BCoV Serology	BCoV Serology
Nasal	1	Positive (6)	Negative	Negative	Positive (4)	Negative	Negative	Negative
	2	None	Positive nasal secretions (2nd day)	Negative	None	Negative	Negative	Negative
	3	None	Negative	Negative	Positive (2)	Negative	Negative	Negative
	4	Positive (2)	Positive nasal secretions (2nd day)	Negative	Positive (1)	Positive nasal secretions (2nd day)	Positive	Positive
Rectal	5	Positive (2)	Negative	Negative	Positive (1)	Negative	Negative	Negative
	6	Positive (2)	Negative	Negative	Positive (1)	Negative	Negative	Negative
	7	None	Negative	Negative	None	Negative	Negative	Positive
	8	Positive (3)	Negative	Negative	Positive (4)	Negative	Negative	Negative
Oral	9	None	Negative	Negative	None	Negative	Negative	Negative
	10	Positive (1)	Negative	Negative	Positive (2)	Negative	Negative	Negative
	11	None	Negative	Negative	None	Negative	Negative	Positive
Control	13	None	Negative	Negative	Positive (1)	Negative	Negative	Negative
	14	None	Negative	Negative	None	Negative	Negative	Negative

Horse 5: Day 3; Horse 6: Day 1; Horse 8: Days 1, 5, 6 and 8 and Horse 10: Days 6–7) with the addition of one intranasally vaccinated horse (Horse 3: Days 1 and 7) and one control horse (Horse 13: Day 7). One of the intranasally vaccinated horses had soft formed faeces prior to vaccination and throughout the study (Horse 1).

Following primary nasal vaccination, two horses tested qPCR-positive for BCoV 24 h post-vaccine administration (**Table 1**). One of the two horses tested qPCR-positive for BCoV 24 h post-vaccine administration in nasal secretions following the second vaccination. All other nasal swabs tested qPCR-negative for BCoV. The faecal samples from all vaccinated and control horses tested qPCR-negative for BCoV following both vaccine administration periods.

A total of three horses seroconverted to BCoV (**Table 1**). One intranasally vaccinated horse seroconverted after the first vaccine and remained seropositive following the second vaccine administration. This horse was not one of the two horses that tested qPCR-positive in nasal secretions. Further, one orally and one intrarectally vaccinated horse seroconverted after the second vaccine administration.

One horse had a normocytic, normochromic anaemia (28.8%; ref: 30–46%), one horse was lymphopaenic (926/μL; ref: 1600–5800/μL) prior to the second vaccination and three horses had a mild eosinophilia 204–340/μL (ref: 0–200/μL). All other haematological parameters were within reference ranges for the laboratory.

Discussion

The results from this study showed that the modified-live BCoV vaccine was safe to administer via various routes, as none of the vaccinates developed clinical signs associated with enteric coronavirus infection (lethargy, anorexia, fever, diarrhoea or colic). This is in agreement with various studies evaluating the safety of BCoV vaccination in cattle (Thurber *et al.* 1977; Waltner-Toews *et al.* 1985). The only physical abnormal findings noticed during the study period was mild, transient changes in faecal character in eight study horses. Of those horses affected by changes in faecal character, three were in the intranasal group, three in the intrarectal group, one in the oral group and one control. Without evidence of BCoV faecal shedding in all horses with "cow-pie" faeces, and the inclusion of a control, it is probable that the transient faecal character change was related to dietary or environmental factors. During the study protocol, the faecal samples were not analysed for bovine rotavirus which can have a genetic homology with equine rotavirus (Ghosh *et al.* 2013). Rotavirus can cause severe diarrhoea in foals but does not cause clinical disease in adult horses and therefore was not deemed necessary to test during the study. It is not possible to definitively rule out the bovine rotavirus as a cause of the soft faeces but given the inclusion of a control horse it seems unlikely.

BCoV shedding was only found in nasal secretions of two intranasally vaccinated horses. The detection of BCoV by qPCR in the two horses 24 h after vaccine administration was attributed to persistence of vaccine virus rather than viral replication in respiratory epithelial cells.

Overall, 27% of vaccinated horses had a measurable serological immune response after two vaccine administrations. Seroconversion to BCoV was determined in one horse from each of the three vaccination routes, indicating antigen detection by the immune system. The study investigated alternative vaccine administration routes in order to determine if a serological response could be achieved whilst bypassing the stomach, known to negatively impact avirulent pathogens due to its low pH. Intrarectal vaccine administration of *Lawsonia intracellularis* in foals has been previously shown to be more effective than oral vaccination, as this route triggered a weak humoral and long-lasting cell-mediated response (Pusterla *et al.* 2009). The limited number of horses showing a measurable serological response for the various administration routes could relate to the vaccine antigen mass, inability of the vaccine BCoV strain to replicate in equine epithelial cells or pre-existing neutralising cross-reactive antibodies to ECoV. Unfortunately, the study did not measure mucosal or cell-mediated immunity and detection of peripheral antibodies to BCoV alone is not a measure of protection.

During the study period, no serological diagnostics existed to confirm the seropositive, or seronegative, ECoV status of a horse. An ELISA has since been created, and the samples were retrospectively tested for ECoV antigens (Kooijman *et al.* 2016). All samples were shown to be negative for antigens against ECoV, and therefore, there should not have been alteration in the response to the vaccine due to previous exposure to ECoV. Therefore, the changes in BCoV serological values are probably due to the administration of the vaccine.

A previous study looked at the response to a BCoV vaccination which was based on an inactivated vaccine rather than the modified-live vaccine used in this study (Nemoto *et al.* 2017). Various studies have assessed the response in equine cases to both inactivated and modified-live vaccines with the latter showing an improved reduction in clinical signs in Equine Herpes Virus-1 infections (Goodman *et al.* 2006) whilst in humans a modified-live vaccine leads to an increased IgA concentration on mucosal surfaces (Cox *et al.* 2004). Overall modified-live vaccinations show a significantly higher immunogenicity than inactivated vaccines (Kollaritsch and Rendi-Wagner 2013) but killed-adjuvanted vaccines will trigger a more reliable, measurable immune response, although, mucosal immunity response is still undetermined. Therefore, use of the modified-live vaccine may have represented a more efficacious route of vaccination in ECoV.

The limitations of the current study included the small number of horses, the unknown viability of the virus within the oral and nasal cavity and stomach and the unknown naivety of the horses to coronavirus in the study.

Conclusions

As the threat of ECoV amongst adult horses increases, there is a need to develop a means of effective protection. The modified-live BCoV vaccine appears safe in adult horses; however, it triggers a limited seroconversion response. At this time the vaccine cannot be recommended for the prevention of ECoV. Future studies will investigate mucosal and cell-mediated immunity and investigate the efficacy of the BCoV in the prevention of ECoV infection under experimental conditions.

Authors' declaration of interests

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of this paper.

Ethical animal research

Source of funding

Acknowledgements

Authorship

Manufacturers' addresses

[1]Zoetis, Parsippany, New Jersey, USA.
[2]Applied Biosystems, Foster City, California, USA.
[3]Svanovir, Uppsala, Sweden.
[4]Sigma-Aldrich, St Louis, Missouri, USA.

References

Cox, R.J., Brokstad, K.A. and Ogra, P.L. (2004) Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. *Scand. J. Immunol.* **59**, 1-15.

Fielding, C.L., Higgins, J.K., Higgins, J.C., McIntosh, S., Scott, E., Giannitti, F., Mete, A. and Pusterla, N. (2015) Disease associated with equine coronavirus infection and high case fatality rate. *J. Vet. Intern. Med.* **29**, 307-310.

Ghosh, S., Taniguchi, K., Aida, S., Ganesh, B. and Kobayashi, N. (2013) Whole genomic analyses of equine group A rotaviruses from Japan: evidence for bovine-to-equine interspecies transmission and reassortment events. *Vet. Microbiol.* **166**, 474-485.

Goodman, L.B., Wagner, B., Flaminio, M.J.B.F., Sussman, K.H., Metzger, S.M., Holland, R. and Osterrieder, N. (2006) Comparison of the efficacy of inactivated combination and modified-live virus vaccines against challenge infection with neuropathogenic equine herpesvirus type 1 (EHV-1). *Vaccine* **24**, 3636-3645.

Guy, J.S., Breslin, J.J., Breuhaus, B., Vivrette, S. and Smith, L.G. (2000) Characterization of a coronavirus isolated from a diarrheic foal. *J. Clin. Microbiol.* **38**, 4523-4526.

Kollaritsch, H. and Rendi-Wagner, P. (2013) Principles of Immunization. In: *Travel Medicine*, 3rd edn., Eds: Jay S. Keystone, Phyllis E. Kozarsky, David O. Freedman, Hans D. Nothdruft and Bradley A. Connor, Elsevier, Philadelphia, PA. pp 67-76.

Kooijman, L.J., Mapes, S.M. and Pusterla, N. (2016) Development of an equine coronavirus–specific enzyme-linked immunosorbent assay to determine serologic responses in naturally infected horses. *J. Vet. Diagn. Invest.* **28**, 414-418.

Mapes, S., Rhodes, D.M., Wilson, W.D., Leutenegger, C.M. and Pusterla, N. (2007) Papers & articles. *Vet. Rec.* **161**, 716-718.

Miszczak, F., Tesson, V., Kin, N., Dina, J., Balasuriya, U.B.R., Pronost, S. and Vabret, A. (2014) First detection of equine coronavirus (ECoV) in Europe. *Vet. Microbiol.* **171**, 206-209.

Nemoto, M., Kanno, T., Bannai, H., Tsujimura, K., Yamanaka, T. and Kokado, H. (2017) Antibody response to equine coronavirus in horses inoculated with a bovine coronavirus vaccine. *J. Vet. Med. Sci.* **79**, 1889-1891.

Oue, Y., Ishihara, R., Edamatsu, H., Morita, Y., Yoshida, M., Yoshima, M., Hatama, S., Murakami, K. and Kanno, T. (2011) Isolation of an equine coronavirus from adult horses with pyrogenic and enteric disease and its antigenic and genomic characterization in comparison with the NC99 strain. *Vet. Microbiol.* **150**, 41-48.

Oue, Y., Morita, Y., Kondo, T. and Nemoto, M. (2013) Epidemic of equine coronavirus at Obihiro Racecourse, Hokkaido, Japan in 2012. *J. Vet. Med. Sci.* **75**, 1261-1265.

Pusterla, N., Hilton, H., Wattanaphansak, S., Collier, J.R., Mapes, S.M., Stenbom, R.M. and Gebhart, C. (2009) Evaluation of the humoral immune response and fecal shedding in weanling foals following oral and intra-rectal administration of an avirulent live vaccine of Lawsonia intracellularis. *Vet. J.* **182**, 458-462.

Pusterla, N., Mapes, S., Wademan, C., White, A., Ball, R., Sapp, K., Burns, P., Ormond, C., Butterworth, K. and Bartol, J. (2013) Emerging outbreaks associated with equine coronavirus in adult horses. *Vet. Microbiol.* **162**, 228-231.

Thurber, E.T., Bass, E.P. and Beckenhauer, W.H. (1977) Field trial evaluation of a reo-coronavirus calf diarrhea vaccine. *Can. J. Comp. Med.* **41**, 131.

Waltner-Toews, D., Martin, S.W., Meek, A.H., McMillan, I. and Crouch, C.F. (1985) A field trial to evaluate the efficacy of a combined rotavirus-coronavirus/*Escherichia coli* vaccine in dairy cattle. *Can. J. Comp. Med.* **49**, 1.

Woo, P.C.Y., Lau, S.K.P., Huang, Y. and Yuen, K.-Y. (2009) Coronavirus diversity, phylogeny and interspecies jumping. *Exp. Biol. Med.* **234**, 1117-1127.