Journal of Equine Veterinary Science 167 (2026) 106141

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

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

Original Research

Limited evidence for exposure of horses in the United Kingdom to influenza D virus

Ge Wu [a], Freya Norman [a], Meshach M. Maina [a], Suresh V. Kuchipudi [b], Ruth H. Nissly [b], Simon D. Scott [c], Nigel Temperton [c], Stephen P. Dunham [a], Janet M. Daly [a,*]

[a] One Virology, Wolfson Centre for Global Virus Research, School of Veterinary Medicine and Science, University of Nottingham, Landcroft Lane, Sutton Bonington, Leicestershire, LE12 5RD United Kingdom
[b] Animal Diagnostic Laboratory, Department of Veterinary and Biomedical Sciences, The Pennsylvania State University, University Park, PA 16802, USA
[c] Viral Pseudotype Unit, Medway School of Pharmacy, University of Kent and University of Greenwich, Central Avenue, Chatham Maritime ME4 4TB United Kingdom

ARTICLE INFO

Keywords:
Antibody
Haemagglutination inhibition
Prevalence
Pseudotyped virus
RT-qPCR

ABSTRACT

Background: Since it was first detected in 2011, antibodies to influenza D virus (IDV) have been found in several species, although cattle are the major host. In cattle, IDV is typically associated with mild respiratory signs, but it is clinically significant as a component of bovine respiratory disease complex. Although antibodies to IDV have been detected in equids, no association of IDV with naturally occurring respiratory disease in horses has yet been demonstrated.

Aims/objectives: The aim of this study was to determine whether IDV may be a causal agent of equine respiratory disease in the UK.

Methods: Nasopharyngeal swab extracts from horses with respiratory signs were screened by RT-qPCR for the presence of IDV RNA. Equine serum samples from apparently healthy horses were screened by haemagglutination inhibition (HI) test for the presence of IDV antibodies. Cattle and HI positive equine serum samples were screened by neutralisation test using an IDV pseudotyped virus (PVNT)

Results: None of the 264 equine respiratory samples were positive for IDV RNA. Of the 330 serum samples from 169 Thoroughbred brood mares and 105 Thoroughbreds in training yards, only one (from a 5-year-old brood mare) was positive for HI antibodies. This single positive sample was confirmed in the PVNT. In contrast, 103 of 145 bovine serum samples (71%) were positive by PVNT, confirming widespread circulation of IDV in cattle in Great Britain and Ireland.

Conclusion: This study found a low risk of horses being exposed to IDV in the UK, with no evidence that IDV contributes to equine respiratory disease.

1. Introduction

Influenza D virus (IDV) or *Deltainfluenzavirus influenzae* [1] is the type species of the genus *Deltainfluenzavirus* in the family *Orthomyxoviridae*. It is most closely related to influenza C virus (ICV or *Gammainfluenza influenzae*), having seven genome segments. In ICV and IDV, the functions of the haemagglutinin (HA) and neuraminidase (NA) membrane glycoproteins found in *Alphainfluenzavirus influenzae* (IAV) and *Betainfluenzavirus influenzae* (IBV), which are encoded by separate genome segments, are combined in the haemagglutinin esterase fusion (HEF) protein. Unlike ICV, which is an almost exclusively human infection, IDV appears to have a broader host range. Influenza D virus was first isolated from pigs with respiratory disease in the USA (D/swine/Oklahoma/1334/2011) [2]. However, cattle are thought to be the reservoir host. In cattle, IDV is typically associated with at most mild respiratory signs, but it is clinically significant as a component of the bovine respiratory disease complex.

Antibodies to IDV have been detected in several species, including horses [3–5]. Seropositivity determined by the haemagglutination inhibition (HI) test ranged from 5% in Italian equine serum samples [3] to 57% in Ukrainian equids [4]. However, no association of IDV with naturally occurring respiratory disease in horses has yet been demonstrated. The aim of this study was to determine whether IDV may be a causal agent of equine respiratory disease in the UK. Therefore,

* Corresponding author.
E-mail address: janet.daly@nottingham.ac.uk (J.M. Daly).

https://doi.org/10.1016/j.jevs.2026.106141
Received 5 April 2026; Received in revised form 22 August 2026; Accepted 9 September 2026
Available online 10 September 2026

nasopharyngeal swab extracts from horses with clinical signs of respiratory disease were screened for IDV RNA. Due to the potential for IDV to circulate in the equine population without causing clinical signs, we also screened serum samples from apparently healthy horses for IDV-specific antibodies by HI test. To confirm circulation of IDV in UK cattle, which had not been reported at the time of the equine sampling, archival bovine serum samples were screened for antibodies using an IDV pseudotyped virus neutralisation test (PVNT) [6].

2. Materials and methods

2.1. Ethical approval

The study protocol was approved by the University of Nottingham School of Veterinary Medicine and Science's Committee for Animal Research & Ethics (Approval number, 2633; Approval date, 9 November 2018).

2.2. Samples

Nasopharyngeal swab extracts were obtained from 264 horses with respiratory disease submitted to the Animal Health Trust diagnostic service in 2018 ($n = 42$) and 2019 ($n = 222$). The samples had all been screened and tested negative for IAV and some also tested negative for other respiratory pathogens. Convenience serum samples ($n = 330$) from Thoroughbred horses housed on training yards or stud farms that would have otherwise been discarded after laboratory testing were obtained from Newmarket Equine Hospital, UK during 2018 and 2019 (Table 1). Convenience bovine serum samples ($n = 145$) were submitted to the Nottingham University Veterinary Nutritional Analysis service (NUVetNA), during 2012 and 2013.

2.3. Detection of viral RNA by RT-qPCR

RNA was extracted using a MagMAX-96 Viral RNA isolation kit and a KingFisher Sample Purification System (Thermo Fisher Scientific). RT-qPCR was performed using primers and probes published in Faccini, De Mattia [7] and SensiFAST Probe No-ROX One-Step Kit (Bioline). Positive control RNA was generated from IDV PB1 gene bases 1200 to 1600 cloned into a T7 plasmid vector using T7 DNA polymerase (Promega) and a dilution series of 10^1 to 10^7 copies generated.

2.4. Haemagglutination inhibition (HI) test

Influenza D/bovine/France/5920/2014 was cultured in embryonated hens' eggs. To remove non-specific inhibitors of haemagglutination, 25 μl of each serum sample was incubated at 37°C with 75 μl of receptor destroying enzyme (RDE; Sigma-Aldrich, catalogue number C8772) overnight. The RDE was then inactivated at 56°C for 30 min. The serum samples were titrated in two-fold dilutions in a V-bottomed microtitre plate and incubated with an equal volume (25 μl) of D/bovine/France/5920/2014 at 4 haemagglutinating units (HAU) for 30 min at room temperature. Finally, 50 μl of 1% equine or 0.5% chicken red blood cells (RBCs) were added and left at 4°C for 1 h. Inhibition of agglutination at a serum dilution of ≥1:10 was regarded as a positive result, which although an arbitrary cut-off was informed by published work [8].

Table 1
Demographic data for Thoroughbred serum samples.

	Training yards (46 premises)	Stud farms (28 premises)	Total
Sex			
Male	56	0	56
Female	105	169	274
Total	161	169	330
Age category (years)			
2–3	61	1	62
4–5	48	35	83
6–7	19	35	54
8–9	10	35	45
10–11	6	22	28
12–13	5	14	19
14–15	1	7	8
≥16	11	20	31
Total	161	169	330

2.5. Pseudotyped virus neutralization test (PVNT)

For screening by PVNT, lentiviral pseudotyped virus (PV) expressing the HEF from D/swine/Italy/199724-3/2015 was produced as previously described [6]. Initial screening of serum samples was performed by diluting the PV to produce a signal of 10^6 relative light units (RLUs) and incubating with duplicate serum samples diluted 1:50 for 1 h at 37°C before adding 1×10^4 swine testicular (ST) cells, which are IDV permissive, per well. The plates were incubated for 48 h and luciferase expression was quantified after addition of reagent (SteadyGlo, Promega) with a microplate reader (GloMax Discover, Promega). The antibody value was calculated as the percentage reduction in RLUs compared to virus-only control wells. Confirmation of the positive-testing equine serum sample, a negative equine sample and a positive-testing bovine serum was carried out using a method described for quantification of SARS-CoV-2 neutralising antibodies [9], using ST cells with the 50% effective concentration (EC50) calculated using the Excel spreadsheet provided in the supplementary materials of the paper by Nie et al. [9].

3. Results

All 264 equine nasopharyngeal swab extracts were negative for presence of IDV RNA.

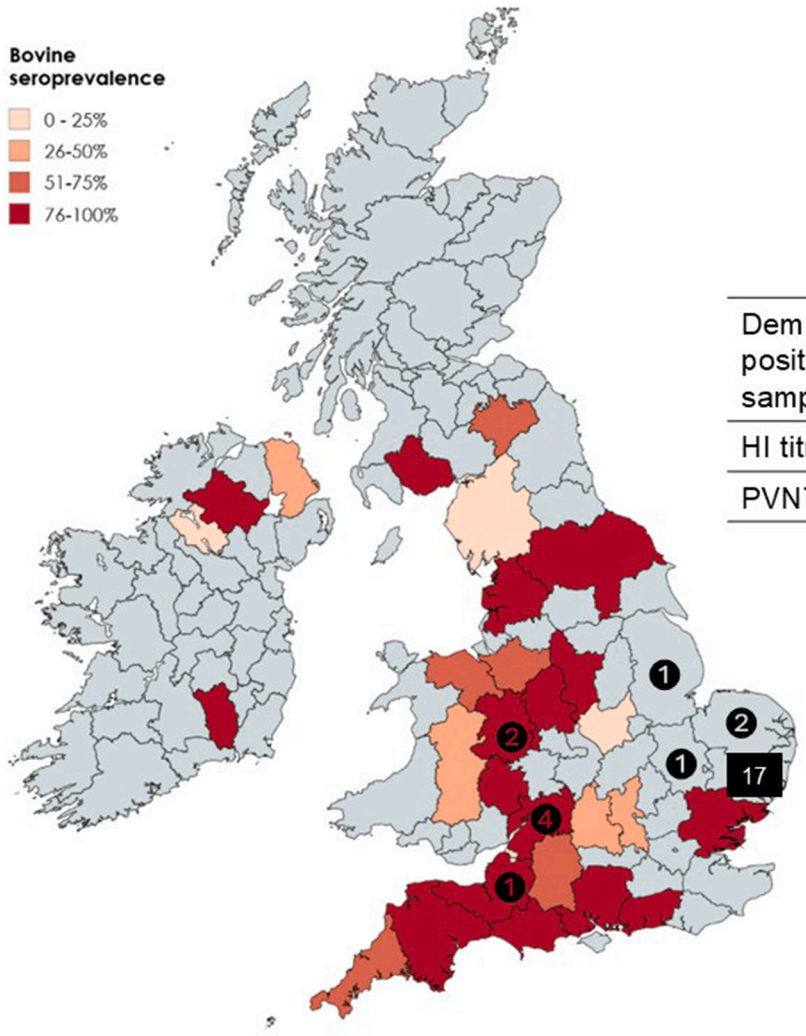
In initial screening of 330 equine serum samples (169 stud animals and 105 Thoroughbreds in training) by HI test using 1% equine RBCs, one sample (from a 5-year-old brood mare) was positive. The positive sample was confirmed by performing an HI test using 0.5% chicken red blood cells, giving an HI titre of 1:40.

Of the 145 bovine serum samples tested in the PVNT, 103 were positive giving an apparent seroprevalence of 71% (95% CI [63, 78]). The seroprevalence in different UK counties varied from 0 to 100% (Fig. 1). The single HI-positive equine serum sample was confirmed to be positive in the PVNT with a mean inhibition of 88% at a 1:50 dilution. One of the positive-testing bovine serum samples was used as a positive control during screening of the equine serum samples and had a mean inhibition of 97% at a 1:50 dilution. On titration, the positive equine serum had an EC50 of 114, a negative sample had an EC50 of <30 and the positive bovine serum had an EC50 of 253.

4. Discussion

The primary aim of this study was to determine whether infection with IDV was associated with respiratory disease in horses in the field. None of the nasopharyngeal swabs obtained from 264 horses with respiratory disease were positive for viral RNA. Furthermore, only 1 of 330 equine serum samples (0.3%) screened for IDV antibodies using a traditional HI test was positive.

Haemagglutination inhibition (HI) tests for antibodies to IAV in equine serum are known to be subject to false positive results if non-specific serum inhibitors are not efficiently removed by trypsin-periodate or RDE treatment [10]. Using RDE, we did not identify any evidence of non-specific inhibition during test development. Therefore, in the absence of a known-positive equine serum sample, we adopted the HI positivity cut-off titre of 1:10 established for screening cattle sera using 1% horse RBCs [8]. The only HI-positive equine serum sample had



Demographic data of positive equine sample:	5-year-old brood mare in Suffolk
HI titre	1:40
PVNT EC_{50}	114

Fig.1. Distribution of cattle and brood mare serum samples by county of Great Britain and Ireland. Cattle serum samples were screened for antibodies to influenza D virus by pseudotyped virus neutralization test. Grey shading indicates no samples were obtained from that county. Red shading represents different levels of seroprevalence from 0 to 100% as indicated in the key. Serum samples from brood mares were obtained from 28 studs in seven counties: Cambridgeshire (1), Lincolnshire (1), Somerset (1), Norfolk (2), Shropshire (2), Gloucestershire (4) and Suffolk (17, including the positive-testing equine serum sample for which demographic data is summarised). The map was generated using MapChart.

a titre of 1:40, which was the cut-off value adopted by Nedland, Wollman [5] for screening equine serum samples in the USA. We also confirmed the positive result in the PVNT. However, the prevalence of antibodies in horses in this study was much lower than previously reported [3–5].

Since IDV was first identified in Oklahoma in 2011, the virus has been classified into five lineages (D/OK, D/660, D/Yama2016, D/Yama2019 and D/CA2019) [11]. Therefore, a potential limitation of our study was that the viruses used for the HI test and PVNT were both from the originally isolated D/OK lineage. However, only D/OK and D/660 lineage viruses have thus far been identified in Europe, and D/OK lineage viruses continue to predominate in Europe [11]. In the US, Nedland, Wollman [5] found 44 of 364 (12%) of equine samples were positive using D/OK virus in the HI test and similarly, 39/364 (11%) were positive using D/660, suggesting that using either lineage would have the same outcome. Falsini, Coppola [3] detected antibodies in 5 of 98 Italian equine serum samples (5%) using a D/660 lineage virus in HI tests, but only 2 had titres ≥1:40 and were confirmed positive (titres ≥1:10–<1:40) in a neutralisation test using authentic virus. Using the same D/OK virus that was used in our PVNT, they only detected one positive sample (titre ≥1:10–<1:40). Interestingly, only 2/98 (2%) of samples were positive in a neutralisation test using either the D/OK or the D/660 virus. More recently, seroprevalence of 57% (79 of 138 samples) was reported for Ukrainian equids by HI testing with a D/660 virus [4]. In contrast, only one sample (1%) was positive to the D/OK virus. The HI protocol was the same as that used to screen the Italian equine serum samples [3], but the results were not confirmed in a neutralisation test.

In contrast to the equine serum samples, 103 of 145 bovine serum samples (71%) were positive in the PVNT. This identified a positive serum sample for use as a control in screening equine serum samples and confirmed the presence of IDV in the UK cattle population, which had not yet been reported. Due to the use of convenience samples, there is a discrepancy in the timing of collection of the bovine sera in our study (2012–2013) and collection of the equine sera (2018–2019). However, the high seroprevalence found in cattle (71%) and similar seroprevalence (65%) reported for sera collected from Irish cattle in January 2017 [12] suggests that IDV is endemic in cattle in the UK and Ireland such that there is likely to be a consistent risk of exposure in horses. The PVNT provided a convenient high throughput method for screening cattle samples for the presence of neutralising antibodies to IDV and confirmed the HI test results obtained with the equine serum samples. At first, six false positive results were obtained due to the 'edge' effect in the PVNT [13]. This was subsequently avoided by adding phosphate-buffered

saline rather than adding samples to the outside wells of the microtitre plate.

The bovine serum samples were from across Great Britain and Ireland but were predominantly from the wetter western regions where grazing cattle are primarily concentrated. As around half of the equine serum samples were obtained from Thoroughbreds in training yards in the east of England and these animals would be mostly stabled, there may have been fewer opportunities for transmission of IDV from the putative reservoir host to the sampled equids. On the other hand, cattle and breeding horses are often co-grazed to reduce worm burden on pastures [14]. It is therefore noteworthy that the single positive sample was from a 5-year-old brood mare. Most of the horses housed on training yards were 3- or 4-year-olds (data not shown), although there were horses up to 26 years old housed on training yards. In contrast, only one horse sampled from a stud farm was <4 years old and most were between 4 and 9 years old with some up to 22 years old. Testing of more samples from each population would be required to accurately estimate seroprevalence; it would also be interesting to focus on horse populations with known contact with cattle.

The primary aim of the study was to determine whether there was any evidence for involvement of IDV in equine respiratory disease. The nasopharyngeal swab extracts screened were submitted for respiratory disease diagnosis and had tested negative for IAV. Although the sampling locations of these samples were anonymised, they were not restricted to a specific population or location, unlike the serum samples. The lack of IDV-positive samples supports other research that suggests that infection of horses is restricted by receptor availability. The HEF of IDV binds 9-O-acetylated sialic acid receptors (reviewed in [15]). Studies have shown that these receptors are abundant across the entire bovine respiratory tract and IDV attaches to tissue sections of the nasal turbinate, submucosal glands, pharynx, trachea and lungs [16,17]. In contrast, the virus only attaches to equine respiratory tract tissue from the submucosal glands. Nonetheless, Sreenivasan, Uprety [18] demonstrated that horses can be experimentally infected with IDV by aerosol delivery of 6.25×10^7 50% tissue culture infectious doses per animal of D/bovine/California/0363/2019 to four young horses. Virus shedding was detected by RT-qPCR testing of nasopharyngeal swabs and all four horses seroconverted with neutralizing antibody titres using authentic virus from around 1:16 to 1:160. However, none of the horses had any clinical signs of disease. This suggests that, with sufficient exposure, horses can be asymptomatically infected with IDV.

5. Conclusion

In conclusion, this study found a low risk of IDV exposure in the UK with no evidence for a role for IDV in causing equine respiratory disease.

Ethics statement

Animal subject. This study was conducted in accordance with the following guidelines for animal welfare and/or reporting: Samples from non-experimental animals. This study was approved by the University of Nottingham School of Veterinary Medicine and Science's Committee for Animal Research & Ethics. (Approval No 2633). Statement for studies in animals. The study protocol was approved by the University of Nottingham School of Veterinary Medicine and Science's Committee for Animal Research & Ethics (Approval number, 2633; Approval date, 9 November 2018).

CRediT authorship contribution statement

Ge Wu: Writing – review & editing, Investigation, Formal analysis. **Freya Norman:** Investigation, Formal analysis. **Meshach M. Maina:** Writing – review & editing, Investigation. **Suresh V. Kuchipudi:** Writing – review & editing, Resources. **Ruth H. Nissly:** Writing – review & editing, Resources. **Simon D. Scott:** Writing – review & editing, Resources. **Nigel Temperton:** Writing – review & editing, Resources. **Stephen P. Dunham:** Supervision, Methodology. **Janet M. Daly:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, 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.

Acknowledgements

This research was funded by the Horserace Betting Levy Board (project reference SPrj:036). The authors are grateful to former colleagues at the Animal Health Trust Dr Debra Elton and Dr Adam Rash for their support of this project. Portions of this work were previously presented at the 2021 International Equine Infectious Diseases Conference (IEIDC) held online.

References

[1] ICTV. Virus taxonomy: 2024 release. 2024.
[2] Hause BM, Ducatez M, Collin EA, Ran Z, Liu R, Sheng Z, et al. Isolation of a novel swine influenza virus from Oklahoma in 2011 which is distantly related to human influenza C viruses. PLoS Pathog 2013;9:e1003176.
[3] Falsini A, Coppola C, Fiori A, Buonavoglia D, Marchi S, Montomoli E, et al. Influenza D virus circulation among bovines, swine, equines, and wild boars in Italy: a sero-epidemiological study. Pathogens 2025;14:891.
[4] Falsini A, Fiori A, Montomoli E, Muzyka N, Muzyka D, Trombetta CM. Circulation of influenza D virus in Ukraine: a multi-species serological study. Acta Trop 2026;274:107980.
[5] Nedland H, Wollman J, Sreenivasan C, Quast M, Singrey A, Fawcett L, et al. Serological evidence for the co-circulation of two lineages of influenza D viruses in equine populations of the Midwest United States. Zoonoses Public Heal 2018;65:e148–ee54.
[6] Marotta MG, Neto MM, Daly J, Maina M, van Diemen P, Everett H, et al. Development and optimisation of influenza C and influenza D pseudotyped viruses. J Virol Methods 2026;339:115243.
[7] Faccini S, De Mattia A, Chiapponi C, Barbieri I, Boniotti MB, Rosignoli C, et al. Development and evaluation of a new real-time RT-PCR assay for detection of proposed influenza D virus. J Virol Methods 2017;243:31–4.
[8] Saegerman C, Salem E, Ait Lbacha H, Alali S, Zouagui Z, Meyer G, et al. Formal estimation of the seropositivity cut-off of the hemagglutination inhibition assay in field diagnosis of influenza D virus in cattle and estimation of the associated true prevalence in Morocco. Transbound Emerg Dis 2021;68:1392–9.
[9] Nie J, Li Q, Wu J, Zhao C, Hao H, Liu H, et al. Quantification of SARS-CoV-2 neutralizing antibody by a pseudotyped virus-based assay. Nat Protoc 2020;15:3699–715.
[10] Boliar S, Stanislawek W, Chambers TM. Inability of Kaolin treatment to remove nonspecific inhibitors from equine serum for the hemagglutination inhibition test against Equine H7N7 influenza virus. J Vet Diagn Investig 2006;18:264–70.
[11] Limaye S, Lohar T, Dube H, Ramasamy S, Kale M, Kulkarni-Kale U, et al. Rapid evolution leads to extensive genetic diversification of cattle flu Influenza D virus. Commun Biol 2024;7:1276.
[12] O'Donovan T, Donohoe L, Ducatez MF, Meyer G, Ryan E. Seroprevalence of influenza D virus in selected sample groups of Irish cattle, sheep and pigs. Ir Vet J 2019;72:11.
[13] Mansoury M, Hamed M, Karmustaji R, Al Hannan F, Safrany ST. The edge effect: a global problem. The trouble with culturing cells in 96-well plates. Biochem Biophys Rep 2021;26:100987.
[14] Forteau L, Dumont B, Sallé G, Bigot G, Fleurance G. Horses grazing with cattle have reduced strongyle egg count due to the dilution effect and increased reliance on macrocyclic lactones in mixed farms. Animal 2020;14:1076–82.
[15] Liu R, Sheng Z, Huang C, Wang D, Li F. Influenza D virus Curr Opin Virol 2020;44:154–61.
[16] Nemanichvili N, Berends AJ, Wubbolts RW, Gröne A, Rijks JM, de Vries RP, et al. Tissue microarrays to visualize Influenza D attachment to host receptors in the Respiratory tract of farm animals. Viruses 2021;13:586.
[17] Mazzetto E, Bortolami A, Fusaro A, Mazzacan E, Maniero S, Vascellari M, et al. Replication of Influenza D viruses of bovine and swine origin in ovine Respiratory explants and their attachment to the Respiratory tract of Bovine. Sheep Goat Horse Swine Front Microbiol 2020;11:1136.
[18] Sreenivasan CC, Uprety T, Reedy SE, Temeeyasen G, Hause BM, Wang D, et al. Experimental infection of horses with Influenza D virus. Viruses 2022;14:661.