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Borna disease virus and mental health: a cross-sectional study

D.Rh. THOMAS[1], R.M. CHALMERS[1], B. CROOK[2], S. STAGG[2], H.V. THOMAS[3], G. LEWIS[3], R.L. SALMON[1], E.O. CAUL[4], K.L. MORGAN[5], T.J. COLEMAN[6], P. MORGAN-CAPNER[7], M. SILLIST[8], S.M. KENCH[6], D. MEADOWS[9] and P. SOFTLEY[8]

From the [1]NPHS Communicable Disease Surveillance Centre, Cardiff, [2]Biomedical Sciences Group, Health and Safety Laboratory, Buxton, [3]Department of Psychological Medicine, University of Wales College of Medicine, Cardiff, [4]Bristol Public Health Laboratory, Bristol, [5]Department of Veterinary Medicine, University of Liverpool, South Wirral, [6]Hereford Public Health Laboratory, County Hospital, Hereford, [7]Chorley and South Ribble NHS Trust, Chorley and South Ribble District General Hospital, Chorley, [8]Norwich Public Health Laboratory, Norwich, and [9]Preston Public Health Laboratory, Royal Preston Hospital, Preston, UK

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Summary

Background: Borna disease is an infectious neurological disease of horses, sheep and possibly other animals. A role for Borna disease virus (BDV) in human neurological and psychiatric illness has been proposed, but this hypothesis remains controversial.
Aim: To investigate the epidemiology of BDV in UK farming communities.
Design: Retrospective cohort study.
Methods: We measured the seroprevalence of BDV in the PHLS Farm Cohort, a representative sample of those employed in agriculture in the UK, and investigated the clinical significance of our findings by comparing the prevalence of symptoms of neurotic psychopathology in those found seropositive and seronegative.
Results: Seroprevalence was 2.3% (95%CI 1.3–4.0%) in 1994, 3.1% in 1996 (95%CI 1.9–5.0%) and 2.6% in 1999 (95%CI 1.5%–4.6%). Those living or working on livestock farms had higher seroprevalence (2.6%) than those on mixed (2.3%) or arable (1.6%) farms, but this was not statistically significant. Exposure to horses, sheep and cats did not increase risk of seropositivity. Seropositives were no more likely to report symptoms of psychiatric morbidity.
Discussion: UK farming populations appear to be exposed to Borna disease virus. However, we found no evidence that exposure to BDV was associated with morbidity in this healthy occupational cohort.

Introduction

Farmers and their families are at increased risk of suicide,[1] which may reflect a higher than average burden of psychiatric morbidity. One explanation that has been advanced for the increased

Address correspondence to Dr D.Rh. Thomas, NPHS Communicable Disease Surveillance Centre, Abton House, Wedal Road, Cardiff CF14 3QX. email: daniel.thomas@nphs.wales.nhs.uk

prevalence of psychiatric morbidity in farming communities is exposure to Borna disease virus, an infectious cause of neurological disease of horses and sheep and a potential zoonosis.[2,3]

There have been few studies of BDV in animal or human populations in the UK.[4] We investigated the occupational significance of Borna disease virus in those employed in agriculture in the UK by: (i) developing an ELISA test for detection of Borna disease virus antibodies in human serum; (ii) using this ELISA to measure the seroprevalence of Borna disease virus in the PHLS Farm Cohort, a representative sample of farmers, farmworkers and their families; (iii) investigating associations between seropositivity and exposure to farm animals; and (iv) investigating associations between presence of antibodies to BDV and mental health in the same sample, by administering a mental health questionnaire to examine in more detail the clinical significance of this potential zoonosis.

Methods

Development of ELISA

A BDV enzyme-linked immuno-absorbance assay (ELISA) was adapted by Health & Safety Laboratory (HSL), from an assay developed at the Department of Clinical Veterinary Science, University of Bristol, to screen cat sera,[5] using a recombinant BDV p24 protein antigen supplied by the authors (C.R. Helps and D.A. Harbour). The assay developed for use in this study differed from the original ELISA, in that sera were not pre-absorbed with *E. coli* proteins. In preliminary assays, using *E. coli* proteins at a wide range of serum dilutions, no affect was observed on the performance of the assay and, following discussion with the originators of the assay who supplied the antigen, it was decided for simplicity to eliminate this step. In order to establish a sensitive ELISA, the optimal antigen concentration was determined by checker board titration of positive and negative sera, at dilutions ranging from 1:10 to 1:320 vs. various antigen dilutions. Thirteen control sera, determined negative or positive by indirect immunofluorescence on persistently BDV-infected MDCK cells (six negative, two positive at 1:40 titre, one at 1:80 and four at 1:160), were received as a gift from Dr S. Herzog, University of Giessen, Germany. One of those highly positive at 1:160 titre (ref. #7) was chosen as the positive control for use in all ELISA assays and run on each plate to detect and correct for any plate to plate variation. Serum samples (refs Q47, Q50, Q57, Q59) found early in the study series of assays to give high readings were periodically re-tested in subsequent assays as additional positive controls. Negative serum samples supplied by Dr Herzog (refs #4, #8, #9 and #10) were used as negative controls; also a sample of the study serum (B145) found early in the series of assays to give a low reading was included in subsequent assays as an additional negative control on all plates.

In summary, the procedure used was as follows. Ninety-six-well plates were coated with a 1:400 dilution of recombinant BDV p24 protein overnight in carbonate–bicarbonate coating buffer and then washed five times in PBS Tween. The serum was diluted 1:80 in PBS Tween, added to the plates and incubated at 37°C for 30 min. After a further five washes, alkaline phosphatase conjugated goat anti-human IgG, Fc specific monoclonal antibody (Sigma) at 1:5000 dilution was added to the plates and incubated at 37°C for 30 min. After a further five washes, bound alkaline phosphatase was detected by 30 min incubation at room temperature with 1 mg/ml p-nitrophenol phosphate in coating buffer. The reaction was stopped by the addition of sulphuric acid and the A_{405} minus the A_{492} was determined for each well with a microplate reader.

Subjects

The PHLS Farm Cohort was recruited in 1991 and 1995 to investigate occupational zoonoses.[6] In 1991, a random sample was drawn from the Ministry of Agriculture, Fisheries and Food (MAFF) June Agricultural Census lists of agricultural holdings for five local government districts in two areas of England. Holdings were telephoned up to three times, and a farmer in each holding replying was asked to participate. That farmer was also requested to nominate a further adult (>16 years) member of the household or enterprise. The latter did not necessarily fulfil the study definition of a farmer. Between May 1995 and June 1996, a further sample was recruited for two local government districts in the East of England, using the same methods.[7]

Sampling

Since 1991, venous blood samples have been taken from participating subjects at approximately yearly intervals and separated sera stored at –20°C. Stored aliquots taken in 1994/1995 and 1996/1997 were made available for testing. Between March and July 1999, each participant still enrolled in the study was re-visited by a field worker and a further 10 ml blood sample taken. At the same time subjects were asked to complete a questionnaire asking about their contact with animals and their mental health.

The Revised Clinical Interview Schedule (CIS-R)

The Revised Clinical Interview Schedule (CIS-R) was used to assess the prevalence of symptoms of neurotic psychopathology in the previous week.[8] The CIS-R is made up of fourteen sections, each covering a particular area of neurotic symptoms. The areas covered are: fatigue, sleep problems, irritability, worry, depression, depressive ideas, anxiety, obsessions, concentration and forgetfulness, somatic symptoms, compulsions, phobias, worry about physical health and panic.

Each section of the CIS-R starts with the establishment of the existence of a particular symptom in the past month. A positive response leads to a more detailed assessment of the symptom in the last week regarding frequency, duration, severity and time since onset. The answers to these questions determine the informant's score on each section. The minimum score on each section is zero where the symptom was either not present in the past week or was present only in mild degree. The maximum score on each section is four (five for the section on depressive ideas). Summed scores from all sections range between 0 and 57, the overall threshold for clinically significant psychiatric morbidity is 12. Individual symptoms are regarded as clinically relevant if they have a score of $\geqslant 2$.

Administration of the CIS-R

The CIS-R was administered using a laptop computer by the research fieldworkers between March and July 1999. Informants were directed through the questionnaire by instructions on the monitor. Progress through the questionnaire required one-finger typing of numbers on the keyboard. The time taken to complete the questionnaire ranged from 10 to 30 min, due to the filtering nature of the questions.

In addition to the 14 sections of the CIS-R, the questionnaire gathered information on general health, socio-demographic characteristics, recent life events and alcohol consumption. Up to date information regarding types of livestock that the informant came into contact with, and the frequency of this contact, was also ascertained.

Statistical analyses

Absorbance data from the ELISA antibody assay were analysed at HSL to define a cut-off for distinguishing reactive and non-reactive subjects. Firstly, the distribution of absorbance values was plotted as box and whisker plots, using a normal and log scale. Then, to attempt to identify reactive and non-reactive sub-populations, reiterative best fitting of log-normal distributions was done, where the ELISA absorbance distributions were characterized by means and standard deviations. Where the lines that best characterized the distributions of the reactive and non-reactive sub-populations crossed, was identified as the putative cut-off point.

Using this putative cut-off point, seroprevalence was calculated using STATA 6,[9] and 95%CIs were calculated using CIA 2.[10] Odds ratios were calculated for associations between occupational exposures and presence of antibodies to BDV by logistic regression using STATA 6.

Development of immunofluorescence test

An immunofluorescence assay (IFA) microscopy test was developed at HSL to validate results from the ELISA. The test was based on standard IFA assay methodology used by one of the co-authors (E.O. Caul, Bristol Public Health Laboratory). BDV positive rat glial cells (refs C6 and C6TL, kindly supplied by Professor H. Ludwig, Robert Koch Institute, Berlin) were grown to a confluent monolayer. The cells were then trypsinized with 0.2% trypsin and washed from the surface of the flask with 10 ml sterile PBS. The cells were centrifuged for 5 min at 1500 rpm and the resulting pellet resuspended in 5 ml PBS. Aliquots of the cell suspension (20 µl) were spotted on to Hendley-Essex PTFE-coated slides, and the slides dried on a hot plate at 50°C. The cells were then fixed by immersion in acetone at room temperature for 10 min. Sera were added to the cells at a 1:20 dilution and they were incubated at 37°C in a moist chamber for 60 min. This dilution of serum had been found in preliminary assay development to be the optimum dilution to give clear discrimination between positive and negative controls. Positive control serum was #7 and negative control was #9 from the ELISA assays. The slides were then rinsed off in water, washed in PBS for 5 min on a rotating platform and dried with a hot air blower. A 1:60 dilution of sheep anti-human IgGAM (H&L) FITC (The Binding Site, Birmingham, UK) was added to the slides and incubated and washed as above. Cover slips were then mounted using a 10% PBS with 90% glycerol and examined by fluorescence microscopy for evidence of granular nuclear fluorescence. This method was then repeated using uninfected glial cells to eliminate any false positives.

Validation of ELISA test results by immunofluorescence microscopy

All samples found positive by ELISA (absorbance value $\geqslant 0.85$), a 10% random sample of those

found negative (absorbance <0.85) and borderline values (⩾0.50, <0.85) were tested blind at HSL using the immunofluorescence test. Mantel-Haenzel χ^2 and the kappa score for inter-rater agreement were calculated using STATA 6. The kappa-statistic measure of agreement is 0 when the amount of agreement is that which would be expected by chance alone, and 1 when there is perfect agreement.[11]

Results

Subjects

Sera were available for 525 subjects in the PHLS Farm Cohort in 1994 (sample 1) (87% of the 606 originally recruited) and 489 in 1996 (sample 2) (81% of those recruited). In 1999, 422 provided a blood sample (sample 3), representing 91% of those subjects who provided a blood sample in the previous sampling round and 70% of the original recruits.

The majority of those sampled in 1999 (sample 3) were male, employed full-time in agriculture and had been in their current job for 16 years or more. Mean age was 51.5 years, range 20–83 years. Seventy-four percent of the farmers interviewed were the principal farmers of the farm holding. Livestock farmers were represented in the largest proportion, and of those, most were either dairy farmers or reared both cattle and sheep. Just over half of the farmers worked on holdings between 50 and 200 hectares in size.

Determination of ELISA cut-off

Results from all 3 sampling rounds were pooled to investigate the distribution of absorbance values. The distribution of ELISA absorbance values was skewed to the right. When the distribution was plotted on a log scale, a large proportion of the results were contained within one log-distribution, with a smaller number of results within a tail or a second log-distribution, shown as 'outside points' (upper quartile plus 1.5 times interquartile) at values >0.95. This plot suggested that there were two populations, one much larger in size than the other, and that log-normal distributions were appropriate descriptions. Using reiterative best fitting of the two log-normal distributions, an absorbance value of 0.85 was suggested as the point where the two fitted distributions crossed. Thus a cut-off of 0.85 was used to identify putative positives and to calculate seroprevalence. The control sera gave absorbance values as follows: #7 (positive at 1:160 titre) = 1.14; #4 (negative) = 0.20; #8 (negative) = 0.16; #9 (negative) = 0.13; #10 (negative) = 0.17.

Table 1 Seroprevalence of BDV in the PHLS Farm Cohort

Sample	Year	Seroprevalence	95%CI
1	1994	12/525 (2.3%)	1.3–4.0%
2	1996	15/489 (3.1%)	1.9–5.0%
3	1999	11/422 (2.6%)	1.5–4.6%

Prevalence of anti-BDV antibodies

Using a cut-off absorbance value of 0.85, seroprevalence was 2.3% (95%CI 1.3–4.0%) for sample 1, 3.1% for sample 2 (95%CI 1.9–5.0%) and 2.6% for sample 3 (95%CI 1.5%–4.6%) (Table 1). Seroprevalence did not appear to increase over time.

Four subjects were seropositive by ELISA for all three samples, indicating either that the longevity of the antibody response measured is >3 years, or that these individuals had been repeatedly exposed to a BDV-like organism. A further two subjects were reactive for two out of two possible samples, and one subject was reactive for one out of one sample. Four subjects were reactive for two out of three samples (two –/+/+, two +/+/–) and one for one out of two. Twelve subjects were positive once only out of the 3 samples (five –/–/+, four –/+/–, three +/–/–).

While a number of subjects went from being seronegative (absorbance value <0.85) to seropositive (absorbance >0.85), in most cases the seronegative absorbance values were close to the cut-off (>0.4) and located in the right hand tail of the distribution.

When sample 3 was examined in more detail, seroprevalence was higher in females than male subjects and increased with age (Table 2), but neither of these observations was statistically significant. Seroprevalence in subjects recruited from the local authority areas surrounding Preston Public Health Laboratory (0.8%) was lower than that for the other two sites (3.4%). Subjects living or working on livestock farms had higher seroprevalence (2.6%) than those on mixed (2.3%) or arable (1.6%) farms.

Occupational animal exposure (Table 3)

Associations between current animal exposures and BDV seropositivity were investigated. Of those reporting exposure to horses, 1.6% were seropositive compared to 3.1% of those reporting no contact with horses (OR 0.53, 95%CI 0.11–2.47). Of those

Table 2 Seroprevalence of BDV by sex, age, study site and type of farm where subject works or lives

Characteristic	Value	Seroprevalence	OR	95%CI
Sex	Male	6/299 (2.01%)	1	–
	Female	5/119 (4.20%)	2.14	0.64–7.15
Age	20–39	1/69 (1.45%)	1	–
	40–59	6/234 (2.56%)	1.79	0.21–15.12
	60+	4/115 (3.48%)	2.45	0.27–22.38
Study site	Hereford	5/146 (3.42%)	1	–
	Norwich	5/144 (3.52%)	1.03	0.29–3.63
	Preston	1/130 (0.77%)	0.22	0.03–1.90
Farm type	Arable	1/61 (1.6%)	1	–
	Mixed	2/88 (2.3%)	1.39	0.12–15.74
	Livestock	5/196 (2.6%)	1.57	0.18–13.70

Table 3 Associations between specific animal exposure and BDV seropositivity (OD > 0.85)

Exposure	Seroprevalence		OR	95%CI
	Not exposed	Exposed		
Cats	8/172 (4.7%)	3/246 (1.2%)	0.25	0.07–0.98
Cattle	5/137 (3.7%)	6/281 (2.1%)	0.58	0.17–1.92
Chickens	8/291 (2.8%)	3/127 (2.4%)	0.86	0.22–3.28
Deer	11/395 (2.7%)	0/12 (0%)	–	–
Goats	0/25 (0%)	11/393 (2.8%)	–	–
Horses	9/295 (3.1%)	2/123 (1.6%)	0.53	0.11–2.47
Pigs	10/343 (2.9%)	1/75 (1.3%)	0.45	0.06–3.57
Rodents	10/357 (2.8%)	1/61 (1.6%)	0.58	0.07–4.60
Sheep	6/181 (3.3%)	5/237 (2.1%)	0.63	0.19–2.09

reporting exposure to cats, 1.2% were seropositive, compared to 4.7% of those reporting no exposure to cats (OR 0.25, 95%CI 0.07–0.98). Exposure to cattle, chickens, deer, goats, pigs, rodents and sheep was also negatively associated with seropositivity, although these results were not statistically significant.

Validation of ELISA test results by immunofluorescence microscopy

Seven of the 11 samples positive by ELISA (64%) tested positive by immunofluorescence compared to 3/34 negatives (8.8%) ($p < 0.001$). When the level of agreement between the two tests was measured, a kappa statistic of 0.57 was obtained, representing moderate agreement.

Ten of the 68 samples giving a borderline result by ELISA tested positive by IF (14.7%). A kappa statistic was re-calculated, including the ELISA borderline results as negatives. As expected, the level of agreement between the tests was reduced, as sampling was biased towards equivocal results. However agreement was still found to be fair (kappa statistic 0.37).

Associations between exposure to BDV and mental health

Mental health questionnaire data were available for 425 farmers. When the summary score for the symptoms of mental illness (CIS-R) was plotted against ELISA absorbance value (OD) (Figure 1), no correlation was observed. None of the 11 seropositive subjects had CIS-R scores greater than 12, the cut-point previously determined for significant psychiatric morbidity.

When specific symptoms of mental illness were examined, two seropositive subjects reported fatigue, one reported irritability and two reported somatic symptoms. Two of 11 (18.2%) seropositive subjects reported somatic symptoms, compared to

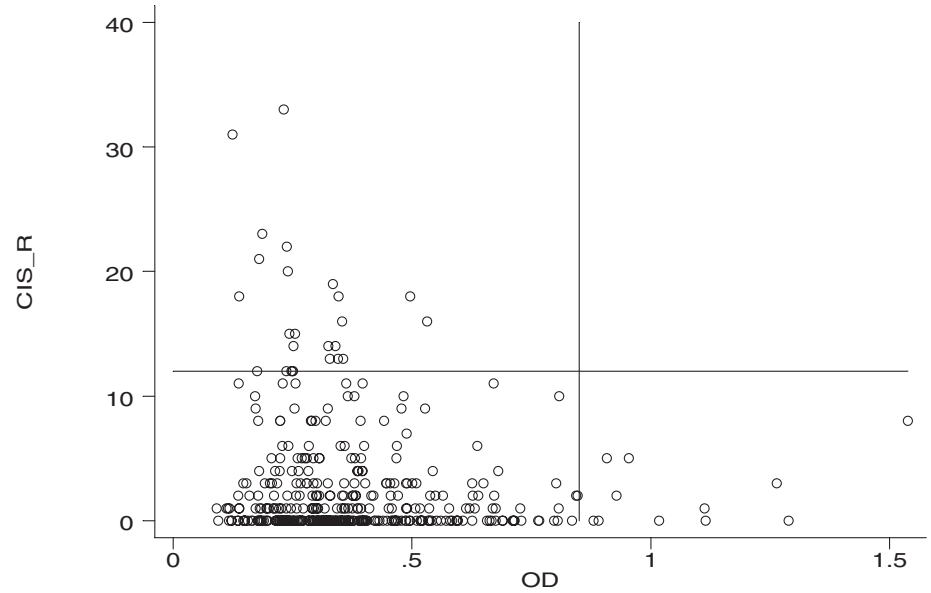

Figure 1. Scatter plot of BDV absorbance (OD) value by CIS-R score ($n = 418$), showing cut points for 'seropositivity' (OD 0.85) and significant morbidity (CIS-R score 12).

27/407 negatives (6.6%). However, this increased risk was not statistically significant. Clinical illness as defined by diagnostic algorithms from CIS-R was present in five subjects. Disorders identified were: mild depressive episode (2 subjects), moderate depressive episode (1 subject), agoraphobia (1 subject), social phobia (2 subjects), specific phobia (1 subject) and obsessive compulsive disorder (1 subject). One subject suffered from three illnesses: mild depressive episode, moderate depressive episode and social phobia. None of the five subjects with disorders specified by CIS-R had ELISA values >0.85 for any sample.

Discussion

We present evidence that farming communities in the UK are exposed to a BDV-like virus. Reactive antibodies were present in 2–3% of a representative farming cohort, with 95%CIs of 1–5%. While a recent study[12] provides evidence that BDV-reactive serum antibodies do indicate infection, rather than cross-reactivity with non-BDV proteins that might share common epitopes, the clinical significance of BDV seropositivity remains unclear.[13]

Controversy surrounding the validity of diagnostic tests has hampered progress in determining the epidemiology of BDV. In the absence of 'gold-standard' human positive controls, determining cut-offs for positive samples is problematic. In interpreting the ELISA results and in attempting to classify test results as 'negative' or 'positive', the assumption was made that there were two populations of test results present and that the log-normal distribution plotted reflects the real-life distributions. Wherever the cut-off is placed, there will be some misclassification of false-positives and false-negatives. Moving up from absorbance cut-off values, for example from 0.85 to 1.0, will reduce the number of false positives but increase the number of false negatives. The two log-normal distributions, plotted in log-space, that gave the best fit to the data, provided the cut-off used in this study. For the smaller 'positive' group, there are relatively large uncertainties in defining a log distribution with mean and standard deviation, whereas there is less uncertainty associated with the larger 'negative' population.

Ideally the putative cut-point could be tested by performing the ELISA on populations of known positives and known negatives. Unfortunately, in the absence of a causal link with a specific clinical syndrome, the classification of people into known positive or negatives can only really be done through modelling of the data.

If a 'gold-standard' test was available, another way of examining the ELISA data would be by Receiver Operator Curve (ROC) analysis.[11] This requires an independent means of ascribing subjects to categories outwith the antibody measurements, i.e. some clinical assessment or symptoms that can define subjects as positive cases or negative cases. ROC analysis of the antibody data would look at defining a cut-off absorbance value that gives

the best fit to subjects defined as positive or negative cases. Unfortunately, we do not have these independent data, so a formal ROC analysis is impossible.

Although a number of subjects had repeat test results that fluctuated above or below the putative cut-off, it is difficult to interpret these results as seroconversions or reversions. That some subjects had absorbance results consistently higher than the cut-off adds weight to the hypothesis that there are reactive subjects within the cohort. The antibody response measured may last more than 3 years, or may be the result of repeated exposure.

An attempt at validating the ELISA results by blind testing positive and negative sera using immunofluorescence gave moderate agreement between the tests. Given the lack of a gold-standard test, quantitative measures of sensitivity and specificity are difficult to obtain. It would appear however, from our data, that the IFA used was more sensitive than our ELISA. By extrapolating the results of the validation it would appear that if used as a screening test, the IFA would have given a significantly higher seroprevalence result. This further highlights the need for an internationally recognized, well-validated, diagnostic test for BDV.

Seroprevalence by ELISA was higher in livestock farmers. Livestock farmers in this cohort were also found to have higher levels of psychiatric morbidity.[14] However, psychiatric morbidity was not associated with the presence of antibodies. It was not possible within the scope of this study to compare seroprevalence of BDV in farming populations with that of non-farming rural populations or urban populations in the UK. However, a recent study of 25 000 plasma donations to the blood transfusion service in Scotland screened for BDV by reverse transcription-polymerase chain reaction failed to detect BDV RNA in any of the samples.[15]

Increased levels of morbidity in livestock farmers may therefore be due to factors other than exposure to BDV, affecting this sector of farming. Further, no positive associations were observed between specific animal exposures and BDV. Those reporting contact with horses had lower seroprevalence. Exposure to cats, another proposed zoonotic reservoir, had a statistically significant inverse association with BDV seropositivity. Whilst these findings might be interpreted as suggesting that BDV is not zoonotic, they should be interpreted with caution. The relationship between antibody response and active infection is by no means clear from the literature, with some studies reporting the detection of BDV RNA in patients in the absence of antibodies in serum.[16] Also, as no data are available on the natural history of BDV infection in humans, it is possible that, if it is a zoonosis, there might be a period of latency between infection and clinical symptoms. Those subjects previously exposed to BDV and who subsequently developed symptoms might have dropped out of the cohort either since recruitment or before recruitment ('healthy worker effect'). Given the rarity of significant psychiatric morbidity in this healthy population, it might be prudent to investigate further the zoonotic potential of BDV using case-control study designs. This might help determine if BDV indeed has an aetiological role in human mental illness.

In conclusion, we found evidence that UK farming populations are exposed to Borna disease virus, but no evidence that exposure to BDV was associated with poor mental health in this occupational cohort.

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