Investigation into an outbreak of Border disease virus in pigs in England

Akbar Dastjerdi1 | Rebecca Strong1 | S. Anna La Rocca1 | Mark Wessels2 | Julie Wessels3 | Kate Whitaker2 | Ben Strugnell4 | Susanna Williamson2

1Virology Department, Animal and Plant Health Agency (APHA)-Weybridge, Addlestone, Surrey, UK
2APHA-Preston, Animal Health Centre, Barton, Preston, UK
3APHA-Bury St. Edmunds, Suffolk, UK
4APHA-Thirsk, West House, Thirsk, UK

Correspondence
Akbar Dastjerdi, Virology Department, Animal and Plant Health Agency (APHA)-Weybridge, Addlestone, Surrey KT15 3NB, UK.
Email: akbar.dastjerdi@apha.gov.uk

Present address
Mark Wessels, Finn Pathologists, Mayflower Way, Harleston, Norfolk IP20 9 EB, UK
Julie Wessels, Crowshall Veterinary Services LLP, Crowshall Lane, Attleborough, Norfolk NR17 1AD, UK
Kate Whitaker, Town and Country Veterinary Group, Whalley Road, Clayton le Moors, Accrington, BB5 5ED, UK
Ben Strugnell, Farm Post Mortems Ltd, Hamsterley House, Hamsterley, Bishop Auckland, County Durham DL13 3QF, UK

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Department of Environment, Food and Rural Affairs

1 | INTRODUCTION

Pestiviruses, in the family Flaviviridae, infect pigs and ruminants, including cattle, sheep, goats and wild suids (Tautz et al., 2015) resulting in substantial economic loss. Moreover, pestivirus sequences have been detected in samples from bats and rats by next-generation sequencing (NGS), although no infectious pestivirus has yet been isolated from these host species (Firth et al., 2014; Wu et al., 2018). Virus species under the Pestivirus genus have been reclassified recently as Pestivirus A (Bovine viral diarrhea virus 1), Pestivirus B (Bovine viral diarrhea virus 2), Pestivirus C (Classical swine fever virus, CSF), Pestivirus D (Border disease virus) (BDV), Pestivirus E (pronghorn pestivirus), Pestivirus F (Bungowannah virus), Pestivirus G (giraffe pestivirus), Pestivirus H (Hobi-like pestivirus), Pestivirus I (Aydin-like pestivirus), Pestivirus J (rat pestivirus) and Pestivirus K (atypical porcine pestivirus) (Smith et al., 2017). A bat-derived virus and pestiviruses identified from sheep and...
We investigated a persistent problem of poor growth and anaemia in a small proportion of growing pigs on a mixed pig and sheep holding. Microarray, BDV-specific PCRs and pestivirus immunohistochemistry (IHC) confirmed presence of BDV, nearly 20 years after the first recorded case in the UK (Roehe et al., 1992). Clinical presentations of the progeny of sows showed similarity to those which might be seen in litters of breeding pigs infected during pregnancy with a CSFV of moderate to low virulence (Brown & Bevins, 2018).

2 MATERIALS AND METHODS

2.1 Animals

Six commercial hybrid pigs within three submissions were sent from a single pig farm to an APHA Veterinary Investigation Centre in England between September 2012 and March 2013 for post-mortem examination and full diagnostic investigation.

2.2 Sample processing and microarray analysis

Kidney, spleen and heparinised blood collected from one pig were processed for pan viral microarray analysis. TRIzol® Reagent (Fisher Scientific) and QIAamp viral RNA mini kit (Qiagen) were used to extract nucleic acid from various tissues. Approximately 25 mg of tissue was added to 1 ml of TRIzol Reagent inside an M tube (Milenyi Biotec) and homogenised using the gentleMACSTM Dissociator. The M tube was then centrifuged at 2000×g for 3 min and the content supernatant was transferred to an Ependorf tube and processed as per TRIzol manufacturer protocol. An aliquot (140 µl) of the aqueous phase or blood was used in the QIAamp viral RNA mini kit (Qiagen) to isolate the nucleic acid according to the kit protocol. A 16 µl volume of the extracted nucleic acid was treated with 2 µl DNase I (1 U/µl, Fisher Scientific) and prepared for analysis on a pan viral microarray (GEO accession number GPL8185) as described (Dastjerdi et al., 2016).

2.3 Molecular analysis and phylogeny

Extracted RNA was converted to cDNA using SuperScript III Reverse Transcriptase (Fisher Scientific) and random hexamers following the manufacturer’s protocol. A 285 bp DNA fragment of the 5’ UTR region was amplified from cDNA using primer 324 and primer 326 (Easton et al., 1994), corresponding to the positions of 98–118 and 362–382 on the sequence NC_003679.1. The PCR using the Taq Gold polymerase (Promega) was carried out at 95°C for 2 min, followed by 40 cycles of 95°C for 45 s, 52°C for 1 min and 72°C for 7 min. A 736 bp DNA fragment from Npro and a part of the C coding region was amplified using primer 320F (position in NC_003679.1, 308–328) and primer 320R (1021–1043) (Strong et al., 2010) using KOD Hot Start DNA polymerase (Sigma) at 95°C for 2 min, followed by 40 cycles of 95°C for 20 s, 52°C for 10 s, 72°C for 45 s and 72°C for 5 min. A 967 bp DNA fragment of the E2 gene was amplified from cDNA using primer
Other viral and bacterial tests

Spleen and/or lung samples from all six animals were collected and processed for BDV and BVDV RT-qPCRs. Ruminant pestivirus RT-qPCR assays were carried out as described by La Rocca and Sandvik (2009) and McGoldrick et al. (1999). The assays detect and differentiate between BDV and BVDV (BVDV1 and 2). A RT-qPCR was also used for the detection of PRRSV in blood and spleen or lung tissue samples (Kleiboeker et al., 2005). The ORF7 gene encoding the nucleocapsid protein of the virus is detected in this test. The swine influenza A virus real time RT-qPCR was carried out on a pool of tonsil, trachea and cranial lung from each pig as described (Hoffmann et al., 2010). Primary bacterial culture and identification, Mycoplasma species detection (including M. suis) and Salmonella culture were carried out using in-house standard operating procedures.

Histopathological findings and IHC

Histopathology revealed reactive lymphadenosis (four out of six pigs), thymic atrophy (two out of three), extra- medullary haematopoesis (in the spleen, lymph node, liver and kidney in six out of six), bone marrow hyperplasia with erythroid shift (six out of six), focal, segmental to global glomerulonephritis (five out of six), mild plasmalymphocytic gastritis (three out of three), minimal plasmalymphocytic colitis (one out of three) and multifocal, minimal, angiocentric, non-suppurative encephalitis (one out of three) (Figure 1).

Pestivirus antigen was detected by IHC in neurons, glia and pericytes (Figure 2). Intense antigen labelling in the cerebrum and hippocampus was also observed in all three pigs (pigs 4–6). Labelling was noted particularly in the lacunar layer of the hippocampus and ependymal cells together with smaller numbers of glial cells with morphology suggestive of astrocytes, mostly in the hippocampus. Rare labelling of a similar population of cells was also seen in cerebral cortex.

The brain was not fixed for histopathology from pig 1. The spleen of this pig showed sparse to moderate punctate labelling of approximately half of the megakaryocyte population involving maturation stages 1–3 (counts were not performed). Kidney had extensive granular to diffuse labelling of many tubular epithelial cells, also punctate labelling of
TABLE 1  Clinical details and gross pathology in submitted pigs

| Pig no. | Age (weeks) | Weight (kg) | Sex | LN | Thymic atrophy | Haemorrhages | Other pathologies |
|---------|-------------|-------------|-----|----|----------------|--------------|------------------|
| 1       | 3           | 17          | M   | 3+ | Not noted      | Subcutis, kidney, LN, small intestine, liver, thymus, salivary glands |
| 2       | 16          | 10          | F   | 1+ | Not noted      | LN           |
| 3       | 16          | 13          | M   | 1+ | Not noted      | LN           | Cranioventral lung consolidation. |
| 4       | 14          | 14          | M   | 2+ | Yes           | LN           | Multifocal severe necrotising pleuropneumonia. Cranioventral lung consolidation. |
| 5       | 14          | 13          | M   | 2+ | Yes           | LN           | Cranioventral lung consolidation. |
| 6       | 14          | 18          | F   | 2+ | No            | LN           | Cranioventral lung consolidation. |

Wasting was the main sign observed in the six pigs.
M, male; F, female; LN, lymph nodes enlargement.
Lymph node enlargement scores: 0 = none, 1 = mild, 2 = moderate, 3 = marked.

TABLE 2  Haematological findings in the six pigs infected with BDV

| Blood indices | Ref. range | Pig 1 | Pig 2 | Pig 3 | Pig 4 | Pig 5 | Pig 6 |
|---------------|------------|-------|-------|-------|-------|-------|-------|
| Hb            | 11–17 g/dl | 2.7   | 2.5   | 2.3   | 6.3   | 3.0   | 6.2   |
| MCH           | 15–25 pg   | 15.8  | 14.5  | 17.0  | 15.9  | 14.6  | 18.5  |
| MCHC          | 28–36 g/dl | 20.8  | 20.5  | 23.7  | 23.4  | 21.9  | 26.1  |
| MCV           | 55–67 fl   | 76.0  | 70.5  | 71.9  | 67.9  | 66.8  | 70.8  |
| PCV           | 0.37–0.5 L/L| 0.13  | 0.12  | 0.10  | 0.27  | 0.14  | 0.24  |
| RBC           | 6–9 × 10¹²/L| 1.70  | 1.70  | 1.40  | 4.00  | 2.00  | 3.40  |
| WBC           | 10–23 × 10⁹/L| 5.7   | 12.7  | 2.1   | 12.6  | 7.4   | 11.2  |
| Neutrophils   | 3.1–10.3 × 10⁹/L| 0.5 (9%)| 5.6 (44%)| 0.5 (24%)| 6.3 (50%)| 1.4 (19%)| 2.1 (19%)|
| Band neutrophils | Rare × 10⁹/L | (0%) | (8%) | (4%) | (3%) | (0%) |
| Lymphocytes   | 4.3–13.6 × 10⁹/L| 5.2 (91%)| 7 (55%)| 1.6 (76%)| 6.3 (50%)| 6 (81%)| 9.1 (81%)|
| Eosinophils   | 0–0.4 × 10⁹/L | 0 (0%)| 0 (0%)| 0 (0%)| 0 (0%)| 0 (0%)| 0 (0%)|
| Monocytes     | 0.2–1.1 × 10⁹/L| 0 (0%)| 0.1 (1%)| 0 (0%)| 0 (0%)| 0 (0%)| 0 (0%)|
| Basophils     | 0–0.2 × 10⁹/L | 0 (0%)| 0 (0%)| 0 (0%)| 0 (0%)| 0 (0%)| 0 (0%)|

Hb, haemoglobin; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; PCV, packed cell volume; RBC, red blood cells; WBC, white blood cells.

FIGURE 1  Histopathology of formalin-fixed tissues stained with haematoxylin and eosin (H&E). (a) Kidney with segmental to global membranous glomerulonephritis (in five out of six pigs). (b and c) Liver and lymph node, respectively, with marked extramedullary haematopoiesis (in four out of six pigs). Bar = 100 µm
FIGURE 2  Representative images of BDV immunohistochemistry of the brain. Intense neuronal labelling (arrows) was particularly observed in lacunar layer of hippocampus together with smaller numbers of glial cells, morphology suggestive of astrocytes, mostly in hippocampus (a). Rare labelling of a similar population of cells was also seen in cerebral cortex (b). Similar pattern of immunostaining was observed in the brain of other two piglets examined from this submission and occasional cells in Bowmans capsules as well as moderate labelling of vascular smooth muscle and connective tissue cells. Glomerular tuft labelling was not detected. Liver had extensive labelling of sinusoidal lining cells and moderately extensive labelling of portal cells including connective tissue/fixed tissue macrophage-like cells. Lung presented intense labelling of chondrocytes and in a reticular pattern in germinal centres in BALT, moderate labelling of bronchiolar epithelium, together with labelling of varying intensity of occasional clusters of macrophages. Small intestine (ileum) also showed intense labelling in a reticular pattern in germinal centres in GALT, and moderate labelling of villus cores including lacteals.

3.3  Microarray and pestivirus RT-qPCRs

Analysis of kidney, spleen and heparinised blood from one pig by the microarray indicated presence of a BDV RNA in the three samples. This microarray result was confirmed by the ruminant pestivirus RT-qPCR assays with only BDV detected in kidney and spleen samples. The presence of BDV nucleic acid in heparin blood was not confirmed, possibly due to the inhibitory property of heparin in the RT-qPCR (Schrader et al., 2012). BVDV1 and 2 were not detected. The virus was also isolated in cell culture from kidney, spleen and blood.

Spleen and blood samples and, in some, lung from the five other pigs were tested by the pestiviruses RT-qPCR. BDV RNA was detected in all pigs (though not all samples in any one pig) except pig 2 and all were negative for BVDV1 and 2 RNA.

3.4  Sequence similarity and phylogenetic analysis

The BDV detected from pig 1 was subjected to genomic characterisation through sequencing of 5′ UTR, Npro and E2 genes. For the three genes, 243, 662 and 866 nucleotide sequences, respectively, were compared with representative of other BDV sequences from GenBank. The BDV sequences shared the highest nucleotide identity with viruses in the sub-genotype BDV1b especially those detected in the UK from ovine and bovine species, an identity of 99.6% to both the bovine strain 1505744 and the ovine strain K1729/3 for the 5′UTR, 98.4% to the bovine strain 1505744 for Npro and 94.8% to ovine strain V2536/2 for E2. In the phylogenetic analysis, the virus also clustered with viruses in the sub-genotype 1b (Figure 3 and Supplementary figure). The virus from this outbreak was therefore classified as a BDV1b sub-genotype.

4  DISCUSSION

This is the first outbreak of BD in commercial pigs diagnosed in England since the 1990s. A sheep flock on the same site was suspected as the likely source of BDV infection. This serves as a reminder of the potential for cross species transmission of ruminant pestiviruses to pigs which is especially relevant for small-holder situations where direct or indirect contact between mixed species is more likely. In this case, the pig and sheep enterprises involved on the farm were commercial in nature with biosecurity gaps which provided opportunity for indirect contact and introduction of BDV infection from the sheep into pregnant sows. The farm was visited as part of the investigation. Sheep handling facilities were found to be immediately adjacent to the dry sow yard with no separation of air space and shared staff. This would have provided the opportunity for transmission of BDV between sheep and pregnant sows. Several management practices were identified which were likely to have favoured continued transmission and propagation of BDV in the pig herd without further introduction of infection from sheep being necessary. These practices included keeping in-pig gilts in the fattener shed with a shared scrape-through dung channel and using empty boar pens in the service house for growing pigs with ill thrift. Advice was given to change these practices, as well as preventing direct or indirect contact between pigs and sheep. Further diagnostic investigations were recommended, but no diagnostic submissions associated with the BD outbreak were subsequently received from the premises.
The clinical presentation of disease investigated here was of poor growth, pigs being small for their age and anaemic (seen on farm as pale pigs) and affected pigs were reported to either die or require euthanasia on welfare grounds. Of the six typical cases submitted for investigation, one (pig 1) had haemorrhagic lesions similar to those seen in cases of mucosal disease in cattle PI with BVDV, possibly representing a similar terminal event. This disease presentation might also occur in the progeny of breeding pigs infected during gestation with CSFV in countries where CSF is endemic, or elsewhere if the CSFV is of low virulence and wider disease is not apparent. When the first case was submitted, disease had been ongoing for several weeks on farm, the possibility of swine fever was considered and ruled out on clinical grounds. Ruminant pestivirus infection should be borne in mind as a differential diagnosis of low morbidity ill thrift and anaemia in pigs, where there is potential contact with sheep or cattle. Where haemorrhagic disease occurs in pigs, the possibility of swine fever should be considered and, if suspected, such cases must be reported to the Animal Health authorities. Where swine fever is not suspected, differentials include vasculopathies and coagulopathies caused by viraeemia; (non-CSFV pestiviruses, PRRSV, PCV2-associated disease, PCV3-associated disease); bacterial septicaemia (disseminated intravascular coagulation, for example due to Streptococcus suis, Klebsiella, Erystipelothrix, Eschericia coli, Glaesserella (Haemophilus) parasuis); immune-mediated disease (porcine dermatitis and nephropathy syndrome, thrombocytopenia purpura, acquired megakaryocyte aplasia); neoplasia (e.g. lymphoma, myeloma); vitamin K deficiency (acquired or anticoagulant toxicity) and mycotoxicosis. In sporadic cases of haemorrhagic disease in anaemic individual pigs, in the absence of disease on other pigs, the main differentials include ruminant pestivirus infection, septicaemia, anticoagulant toxicity and acquired immune-mediated thrombocytopenia (Bidewell et al., 2013).

Natural infection of pigs with ruminant pestiviruses, BVDV, BDV and even with APPV, usually results in mild clinical or sub-clinical signs so long as in utero infection does not occur and thus usually causes minimal economic loss. Such infections, however, may interfere with serological surveillance and diagnosis of CSFV and other pestivirus infections, due to cross-reactivity among this group of viruses (Kawanishi et al., 2014). Therefore, in-depth characterisation of existing and emerging non-CSFV pestiviruses is important to ensure specificity of pestiviruses detection assays. This case also illustrates the usefulness of a pan viral microarray in investigation of unusual disease incidents. In this case, histopathology and IHC findings combined with the results of virological molecular assays confirmed infection with BDV. The pronounced neuronal tropism and pestivirus antigen distribution demonstrated by IHC in tissue sections were very similar to those observed in persistent pestivirus infection in immunotolerant ruminants and likely to indicate congenital infection of the pigs (Fernandez et al., 1989; Hewicker et al., 1990; Montgomery, 2007; Potts et al., 1985; Waldvogel et al., 1995; Wilhelmsen et al., 1991; Wöhrrman et al., 1992). Previous studies have also demonstrated that fetal neuronal tissues are a primary target of congenital pestiviral infection in cattle, sheep, goat, white-tailed deer and alpacas (Duncan et al., 2008; Hennonson et al., 2013; Hewicker et al., 1990; Hewicker-Trautwein et al., 1995; Passler et al., 2012, 2014). It also has been shown that experimentally infected pigs with BVDV at the age of 6–10 weeks lack pestivirus antigen labelling in the brain tissue (Makoschey et al., 2002) which further suggests congenital infection with BDV in these pigs.
Widespread haemorrhages were seen in one pig for which a platelet count was not possible, although platelet anisocytosis was noted at blood smear examination. Nucleated RBCs were seen on the blood film indicating a regenerative anaemia and histology showed the bone marrow was reactive. Haemorrhagic disease in BVDV1 infection in cattle is associated with thrombocytopenia (Colloff et al., 2012) and that may be the explanation in this pig.

In conclusion, using a pan viral microarray, BDV PCRs and pestivirus IHC, we have confirmed infection with BDV in this commercial pig herd as the cause of their chronic ill thrift and anaemia. The virus was classified in the BDV sub-genotype 1b. This investigation highlights potential interspecies transmission of pestiviruses and their impact on disease surveillance and eradication programs as well as on health, welfare and productivity.

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ETHICS STATEMENT
The authors confirm that the ethical policies of the journal, as noted on the journal’s author guidelines page, have been adhered to. All samples used for this study were collected for clinical purposes under the Veterinary Surgeons Act 1966, the United Kingdom.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are openly available in GenBank at https://www.ncbi.nlm.nih.gov/nuccore/, accession number MT432532-4.

ORCID
Akbar Dastjerdi https://orcid.org/0000-0001-6251-6588

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

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