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EXPERIMENTALLY INDUCED DISEASE

Differences in Virulence Between Bovine-Derived Clinical Isolates of *Pasteurella multocida* Serotype A from the UK and the USA in a Model of Bovine Pneumonic Pasteurellosis

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Summary

The time of onset and subsequent degree and progression of clinical signs, bacterial colonization and tissue pathology during experimental disease induced by intratracheal inoculation of either a UK or USA isolate of *Pasteurella multocida* serotype A recovered from clinical cases of bovine pneumonia were determined. Calves aged 8 weeks were challenged with 300 ml phosphate buffered saline (PBS) alone (group 1, $n = 3$, negative control) or containing 7.1×10^8 colony forming units (cfu) of UK isolate (group 2, $n = 8$) or 5.8×10^8 cfu of USA isolate (group 3, $n = 8$). Bronchoalveolar lavage (BAL) at 0, 1 and 4 days post challenge (dpc) and at the time of necropsy examination (7–8 dpc) showed no significant differences between groups 2 and 3 in bacterial numbers recovered. No *P. multocida* were recovered from group 1 animals. No clinical disease was present in group 1 calves and in group 3 was limited to scour in 1 calf at 1 dpc. All calves in group 2 had reduced food intake at 4–5 dpc, five had periods of dullness, three a mild nasal discharge at 1 dpc, four had mild to substantial respiratory stridor and one was killed at 6 dpc for humane reasons. Rectal temperatures remained about 39°C in group 1 calves, but increased in *P. multocida*-challenged calves to 40–41°C within 8–12 h of challenge. Significantly ($P = 0.01$) greater percentages of lung surface area were consolidated in group 2 (mean ± SD, 21 ± 10.1) compared with group 3 (7 ± 8.6) calves. Significantly more extensive and severe histological lesions were present in the lung lobes ($P = 0.006$) and lymph nodes ($P = 0.02$) of group 2 compared with group 3 calves. Pleurisy was present in group 2 calves only and no pathology was present in group 1. Pulsed-field gel electrophoresis (PFGE) produced 11 (group 2, UK isolate) or 10 (group 3, USA isolate) bands with differences in banding patterns. Results overall showed that two isolates, distinct geographically and genetically (by PFGE), caused pneumonic pasteurellosis in a single host with significantly different severity of pathology. This information is relevant to the development of novel vaccine control and interpretation of diagnostic results.

Keywords: bovine; *Pasteurella multocida*; pneumonia; virulence

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Introduction

Pasteurella multocida has been identified as a common pathogen involved in losses of cattle due to respiratory disease both in the UK and USA (Barrett, 2000; Anon, 2006; Dabo *et al*., 2008). Figures from Veterinary Investigation Diagnosis Analysis (DEFRA, 2013) for bovine pneumonic pasteurellosis in England and Wales and data from the USA (Taylor *et al*., 2010) are indicative of *P. multocida* as a consistently frequent cause of fatal pneumonia. Bacteria of the family Pasteurellaceae exist often as commensal organisms in the upper respiratory tract of cattle and sheep (Dabo *et al*., 2008) and may, under certain conditions that are not understood, become pathogenic and cause acute bronchopneumonia. The various different diseases caused and host specificities shown by *P. multocida* are linked with characteristic capsular and somatic formulations (Ewers *et al*., 2006). For example, bovine pneumonic pasteurellosis is caused mainly by *P. multocida* serotype A:3, while haemorrhagic septicaemia in cattle and buffalo is caused by *P. multocida* serotypes B:2 or E:2. However, within this classification, understanding of the molecular mechanisms of infection and virulence is incomplete (Wilkie *et al*., 2012) and there is much unexplained variability in the antigenicity and pathogenicity between different isolates that limits our ability to understand the molecular pathogenic mechanisms involved or to generate effective vaccines.

The current work investigated some of these issues by comparing the time of onset and subsequent progression of clinical signs, bacterial colonization of selected tissues and pathology during experimental disease induced with either a UK (671/90) or USA (P1933) isolate of *P. multocida* serogroup A. Both isolates were recovered from clinical field cases of bovine pneumonic pasteurellosis.

Materials and Methods

Animals

A standard bovine model of pneumonic pasteurellosis caused by *P. multocida* serogroup A (Dowling *et al*., 2002) was used to study disease progress at intervals after bacterial challenge. Nineteen Holstein-Friesian calves of either sex were used, the number restricted to the minimum likely to generate a statistically significant result. All experimental protocols were approved by the Experiments and Ethical Review Committee of the Moredun Research Institute (MRI) and authorized under the Animals (Scientific Procedures) Act 1986. Nasal swabs taken on farm from calves within 3 days of birth were streaked over agar plates containing 5% sheep blood and vancomycin (final concentration 10 μg/ml to exclude gram-positive bacteria) in agar base No.2 (Oxoid Limited, Hampshire, UK). Calves with cultures negative for *P. multocida* were transferred to the MRI, allowed to acclimatize to their surroundings and each other, and held in open pens for the duration of the experiment. At approximately 7 weeks of age, calves were weaned from a liquid milk diet on to hay and mixed pellets and allocated randomly to three groups, group 1 ($n = 3$) and groups 2 and 3 (both $n = 8$), with groups kept in separate air spaces. Access to veterinary care was available at all times and calf health and well-being was assessed at intervals each day so that any necessary treatment was given with minimal delay.

Sampling Procedures, Challenge and Clinical Monitoring

At approximately 8 weeks of age, and immediately before challenge (designated day 0), calves were restrained without anaesthesia and bronchoalveolar lavage (BAL) with 2 × 60 ml of phosphate buffered saline (PBS) at 39°C was performed via a fibreoptic bronchoscope (model VFS-2A; Kruuse UK Ltd., Sherburn in Elmet, North Yorkshire, UK) inserted via a nasal passage, larynx and trachea into a principal bronchus until wedged in a small bronchiole. The fluid was aspirated gently immediately after each aliquot to recover BAL fluid (BALF). The procedure was repeated at 1 and 4 days post challenge (dpc) yielding, on average, about 60 ml of BALF each time. To reduce the number of procedures, sampling and challenge (day 0) were combined so that after taking the baseline lung fluid sample, the bronchoscope was withdrawn to a point just above the tracheal bifurcation and 300 ml PBS at 39°C was given with or without *P. multocida*. Combined sampling and challenge procedures took about 5 min and were tolerated well by calves, which returned rapidly to normal behaviour once the procedures were over. Group 1 calves received PBS alone, group 2 calves received PBS containing a target dose of 10^9 colony forming units (cfu) of *P. multocida* A:3, UK isolate 671/90 and group 3 calves received PBS containing a target dose of 10^9 cfu *P. multocida* A:3/4, USA isolate P1933. Clinical examination was performed (Dowling *et al*., 2002) on each calf four times at 0 and 1 dpc, twice at 2, 3 and 4 dpc and daily thereafter with an option to kill humanely any calf with clinical signs of severe disease to prevent unnecessary suffering. The experiment was terminated 7 or 8 dpc and calves were killed by lethal injection in order to assess gross pathology and obtain tissues for bacteriology and histopathology.

Provenance and Preparation of Bacterial Challenge Doses

An isolate of *P. multocida* (MRI reference 619/90) was retrieved in 1990 from the lung of a calf challenged with a mixture of five *P. multocida* isolates recovered from field cases of bovine pneumonic pasteurellosis in the UK. The isolate was passaged through another calf to confirm its virulence and stored in aliquots at −80°C. This isolate (designated MRI reference 671/90) was confirmed by somatic typing (Veterinary Laboratories Agency [VLA], now Animal and Plant Health Agency [APHA], Edinburgh, UK) as *P. multocida* A3. A further isolate of *P. multocida* serotyped as A:3/4 (designated P1933) was kindly supplied by MSD Animal Health Ltd. (Milton Keynes, UK). This isolate was recovered from a terminal case of bovine clinical pasteurellosis in Texas, USA, in 1970 and passaged four times at Ames College of Veterinary Medicine, Iowa State University, USA, prior to being stored in freeze-dried form. The isolate was subsequently procured by MSD Animal Health Ltd., passaged using transmissible spongiform encephalopathy (TSE)-compliant media, lyophilized and stored at room temperature. It was subjected to static overnight culture in 10 ml tryptic soy broth supplemented with yeast extract (TSB/YE, prepared using 30 g/l Bacto Tryptic Soy Broth [Cat No: 211823, Becton Dickinson, Oxford, UK] plus 10 g/l Bacto Yeast Extract [Cat No: 211931, Becton Dickinson]) at 37°C followed by shaking culture in 78 ml TSB/YE at 37°C, which was stopped in the log phase of growth by addition of 20 ml sterile glycerol and stored as 1 ml aliquots at −80°C until required. Both isolates were confirmed as *P. multocida* by classical bacteriology (colony growth on selective media, morphology and biochemical characteristics) and by polymerase chain reaction (PCR; see below).

Bacterial inocula were dilutions of initial static overnight cultures of *P. multocida* (from glycerol stocks held at −80°C) in 50 ml nutrient broth (NB) at 37°C followed by sub-culture of 15 ml into 300 ml NB, incubated for 3.5 h at 37°C with shaking (170 rpm). Aliquots (50–60 ml) were centrifuged at 3,000 g for 20 min and the pellets resuspended in PBS. Densimat (bioMerieux, Basingstoke, Hampshire, UK) readings were used to estimate the volume (circa 1 ml) containing 10^9 cfu of bacteria and doses were prepared by adding this volume to 300 ml PBS at 39°C. Actual live dose counts, derived retrospectively by plating out diluted aliquots on sheep blood agar, were 7.1×10^8 and 5.8×10^8 cfu for UK isolate 671/90 and US isolate P1933, respectively.

Necropsy Examination, Post-Mortem Bronchoalveolar Lavage and Bacteriology

Calves subjected to post-mortem examination were killed by intravenous injection of pentobarbitone sodium BP (200 mg/ml, Rhone Merieux, Harlow, UK) and the heart and lungs were removed to allow assessment of gross lesions (i.e. consolidation and pleurisy) as described previously (Dowling *et al.*, 2002). Whole lung lavage was performed using 2 × 500 ml of PBS, massaging the lungs gently over all areas for a short time after each addition and harvesting the washes by gravity. Whole BALF was filtered through sterile gauze, centrifuged at 200 g for 10 min at 4°C and serial dilutions were plated out to 10^{-5} as 3 × 20 µl aliquots to recover pure bacterial cultures of *P. multocida*. Samples from gross pulmonary lesions and areas of unaffected tissue were taken (approximately 1 cm^3) and processed to determine bacterial counts of *P. multocida* per gram of tissue as described previously (Dagleish *et al.*, 2010). For histological examination, representative samples of tissue from the various lung lobes (i.e. right and left cranial, right middle, right and left caudal and accessory) were placed into 10% neutral buffered formalin. Intrathoracic lymph nodes, which were recognized consistently in all calves ($n = 4$; left and cranial bronchial and mid- and caudal mediastinal), were removed whole and dissected free from all adjacent material prior to being weighed and then fixed as above. All tissue samples were prepared for histopathological examination by standard techniques (dehydrated through graded alcohols, embedded in paraffin wax, sectioned [5 µm], mounted on glass microscope slides and stained with haematoxylin and eosin [HE]). Histological lesions were scored 0–5 (0, no significant lesions; 1, perceptible lesions; 2, mild; 3, moderate; 4, severe; and 5, very severe) for each lung lobe, which allowed each group of calves to be given an overall severity score (number of animals affected × individual lobe severity score). The overall group lymph node lesion severity score was determined in an identical manner to that of the lung tissue.

Bacteria and Protein Analysis

Bacterial cultures derived from stock solutions of strains 671/90 (UK) and P1933 (USA) showing the colony morphology of *P. multocida* were analyzed by PCR for the presence of species-specific sequence KMT1 (Townsend *et al.*, 1998) to confirm identification. *P. multocida* recovered from tissues and lung BALF in challenged calves, both live and at post-

mortem examination, were analyzed in a similar fashion. Small samples of tissue (approximately 1 cm cubes, about 1 g) were taken from four preselected lung sites (i.e. right cranial, caudal part of the left cranial, right middle and left diaphragmatic lobes), bronchial lymph nodes, caudal and mid-mediastinal lymph nodes, tonsils, spleen, liver, kidney and heart. The samples were homogenized in 9 ml peptone water and diluted in 10-fold steps to 10^{-6}. Aliquots (10 µl) of each dilution from 10^{-6} to 10^{-2} were applied to SBA plates and incubated at 37°C for 16–20 h. Viable counts of colonies phenotypically resembling *P. multocida* were determined and expressed as cfu/g of tissue. For PCR, colonies were suspended in 500 µl of nuclease-free water, incubated at 95°C for 10 min and the resultant lysates stored at −20°C. Template (20 µl) was added to a reaction mixture (total volume 50 µl) containing 1 × PCR buffer, 1.5 mM $MgCl_2$ and 1 unit DNA polymerase (Platinum Taq DNA polymerase; Invitrogen, Paisley, UK), in addition to 0.2 mM of each dNTP (dNTP mix; Invitrogen) and 50 pmol of each primer KMT1sP6 and KMT1T7 (Townsend *et al.*, 1998). Samples were run on a thermal cycler (G-storm Gs1; Gene Technologies, Fitzroy, Victoria. Australia), with initial denaturation at 95°C for 2 min, followed by 35 cycles at 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, and a final extension at 72°C for 5 min. PCR products underwent agarose gel electrophoresis on 1% gels in 1 × tris-acetate-EDTA buffer before UV transillumination.

Pulsed-Field Gel Electrophoresis

Both *P. multocida* strains from stock solutions were subjected to pulsed-field gel electrophoresis (PFGE) for comparison of DNA using conditions described by Lainson *et al.* (2002) and separations were performed using contour-clamped homogeneous electric field (CHEF) equipment (Bio-Rad, Hemel Hempstead, UK). Briefly, 1.5 ml of bacterial suspension in PBS prepared from a blood agar plate culture and containing at least 10^8 cfu/ml were centrifuged at 15,500 g for 4 min and the pellet washed three times in Pett IV buffer (10 mM Tris–HCl, pH 7.6, 1 M NaCl). After resuspending the final pellet in 0.5 ml of Pett IV buffer and warming to 30–40°C, 0.5 ml of low gelling temperature (LGT) agarose was added and the mixture poured into a plug mould. Once set, LGT agarose plugs were incubated in EC lysis solution (Tris–HCl pH 7.6, NaCl, EDTA pH 8.0, Triton 100×, N-lauroylsarcosine, lysozyme and RNAse) overnight at 37°C followed by incubation in 2 ml of ESP buffer (0.5 M EDTA [pH9-9.5], 1% N-lauroylsarcosine and 1 mg/ml proteinase-K) at 50°C. Protease activity was stopped after 2 days by adding 5 ml tris–EDTA buffer and 50 µl 0.1 M phenylmethylsulphonyl fluoride per sample, shaken gently at room temperature for 30 min and washed with 3 × 5 ml tris–EDTA buffer alone. Trimmed plugs were digested at 30°C for 48 h using ApaI restriction enzyme (100 units per sample; Boehringer Mannheim, Lewes, UK), washed in Tris–EDTA buffer and loaded into 1% agarose gel, run for 23 h at 6 V/cm with a 1 sec initial switch time and a 40 sec final switch time. Gels were stained with ethidium bromide prior to washing in ultrapure water and viewed under UV transillumination. Isolates of *P. multocida* recovered from pre- and post-mortem collected BALF, post-mortem lung tissue and tonsil swabs from challenged calves were processed identically and compared by PFGE with those from stock solutions.

Statistical Analyses

Cattle weights were compared using the Kruskal–Wallace test due to the small number in the negative control group. Gross pathological lesion score differences between groups were compared using the *t*-test and the severity of histological lesions in lungs and lymph nodes by Fisher's exact test for count data using R software, version 2.15.2 (R Development Core Team, 2012). Overall differences in pulmonary lymph node weights in the three groups was compared by one-way ANOVA on the pooled weights of all four lymph nodes from each individual animal and pair-wise comparisons by Tukey's simultaneous confidence intervals. Numbers of bacteria recovered from BALF fluid and tissue samples from animals in groups 2 and 3 were compared using the Mann–Whitney test. Samples were considered significant when $P \leq 0.05$.

Results

Clinical and Pathological Outcome

Rectal temperatures increased in all calves challenged with *P. multocida* to a maximum of 41°C (mean ± SD; 40.2 ± 0.46°C) within 24 ± 10.6 h of challenge. Clinical signs of disease among calves given the USA strain of *P. multocida* A3 (group 3) were limited to scour in 1 calf at 1 dpc. By contrast, all calves given the UK strain became anorexic for 4–5d dpc, five showed occasional periods of dullness, three produced a mild nasal discharge at 1 dpc and mild to substantial respiratory problems occurred in four calves throughout the period of observation. One calf (number 4) infected with the UK strain was killed at 6 dpc for humane reasons due to the severity of clinical signs. Median body weights

(range) of calves on the day of post-mortem examination were 82.4 (78.8–84.4), 78.6 (67.7–88.4) and 84.4 (68.2–96.8) kg for groups 1, 2 and 3, respectively, and no significant difference was present between groups ($P = 0.37$).

Gross lung lesions, of variable size and severity, were present in calves of both of the challenged groups (groups 2 and 3) and comprised congestion and consolidation of lung lobes (Fig. 1). However, calves infected with the UK strain had significantly ($P = 0.01$) more extensive gross lesions of pneumonia, including necrosis and abscess formation, compared with cattle infected with the USA strain (Table 1). Additionally, six of the eight calves infected with the UK strain had pleurisy indicated by thickening of, and fibrinous adhesions between, the visceral and parietal pleura.

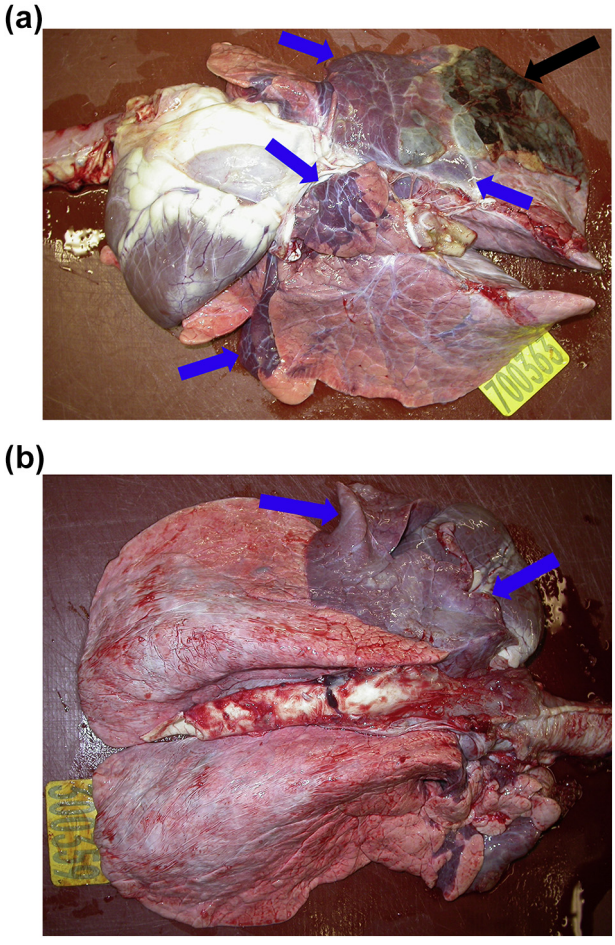

Fig. 1. Gross pulmonary lesions in cattle infected with different isolates of *Pasteurella multocida* A. (a) Group 2 (UK isolate), ventral aspect of most severely affected (calf 4). Note marked consolidation of virtually the entire lung field (blue arrows) and deep red–black discolouration of the left caudal lung lobe indicative of necrosis (black arrow). (b) Group 3 (USA isolate), dorsal aspect of most severely affected (calf 11). Note consolidated cranial lung lobes, especially the left cranial lobe (blue arrows).

Table 1
Extent of consolidation (percentage of dorsal and ventral surfaces) in calves infected with different strains of *Pasteurella multocida* A

Infected with UK strain 671/90					*Infected with USA strain P1933*				
Calf ID	*Dorsal*	*Ventral*	*Average*	*Pleurisy*	*Calf ID*	*Dorsal*	*Ventral*	*Average*	*Pleurisy*
1	17	21	19	1	9	8	10	9	0
2	18	24	21	0	10	4	5	4.5	0
3	14	24	19	1	11	22	30	26	0
4	34	45	39.5	3	12	6	11	8.5	0
5	29	23	26	1	13	0	0	0	0
6	13	13	13	0	14	0	0	0	0
7	22	30	26	1	15	0	0	0	0
8	3	7	5	1	16	6	9	7.5	0
Mean	18.8	23.4	21.1*		Mean	5.8	8.1	6.9*	

Severity of pleurisy (scored between 0 and 3, where 0 is no pleurisy and 3 is severe). *$P = 0.01$ (*t*-test).

No pleurisy was present in the calves infected with the USA strain (Table 1). The group 2 calf killed at 6 dpc for humane reasons had severe pleurisy and necrosuppurative pneumonia. Histological examination of the lung tissues from *P. multocida*-challenged animals showed suppurative bronchopneumonia with or without fibrin deposits and multiple abscess formation (Fig. 2) relating to the areas of gross lesions (areas of consolidation), consistent with an airway-derived bacterial infection, and was in agreement with our previously published findings for this experimental model (Dowling *et al.*, 2002; Dagleish *et al.*, 2010). There was a significant ($P = 6.2 \times 10^{-5}$) difference in the severity of lung lesions between the three groups, with calves challenged with the UK strain of *P. multocida* having significantly ($P = 0.006$) greater severity of lesions compared with those challenged with the USA strain (Table 2).

There was a significant difference ($P = 0.005$) in the pooled lymph node weights (weights of all four pulmonary lymph nodes from each individual animal, data not shown) between the three groups, reflecting the greater weight of the lymph nodes from the animals in the infected groups compared with the negative control group (mean ± SD; group 1 = 10.8 ± 5.4, group 2 = 29.5 ± 7.6, group 3 = 22.3 ± 7.4). Additional comparisons between the groups showed calves challenged with the UK strain of *P. multocida* had heavier lymph nodes than those challenged with the USA strain and this was approaching significance (individual confidence level = 97.99%, Tukey's 95% simultaneous confidence intervals).

Suppurative lymphadenitis consistent with a bacterial infection was present in the lymph nodes of many, although not all, of the calves challenged with *P. multocida* (Table 3) and those challenged with the UK

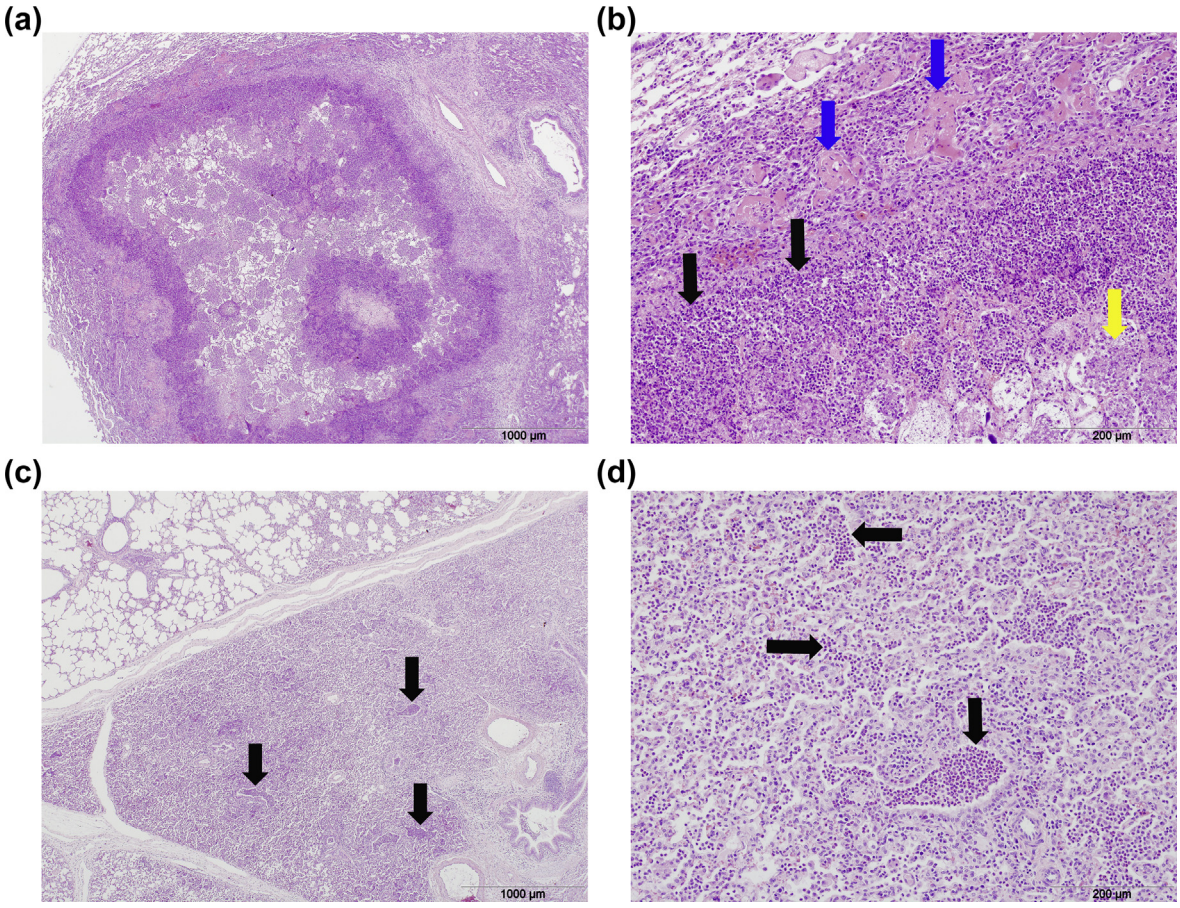

Fig. 2. Histology of lung lesions present in calves challenged with *Pasteurella multocida* A. (a) UK strain (group 2). Note sequestra/early abscess formation with central necrotic tissue. (b) UK strain (group 2). Higher magnification of (a). Note large numbers of leucocytes, primarily neutrophils with fewer macrophages (black arrows), fibrin deposits (blue arrows) and necrotic tissue (yellow arrow). (c) USA strain (group 3). Note presence of cellular infiltrate within bronchioles (black arrows) and lack of abscess formation. (d) Higher magnification of (c). Note cells infiltrating the lumina of bronchioles and alveoli are predominantly neutrophils with fewer macrophages (arrows). HE.

strain of *P. multocida* had significantly ($P = 0.02$) more severe lymph node pathology than those infected with the USA strain.

No clinical signs of disease were observed in group 1 (negative control) calves, rectal temperatures remained at 38.7 ± 0.47°C (mean ± SD) and all organs and tissues appeared normal on gross and histological examination.

Bacteriology

Recovery of *P. multocida* after experimental challenge from two ante-mortem and one post-mortem BALF samples showed mean values (cfu/ml) of 8.0×10^4, 3.1×10^3 and 2.0×10^4 (group 2) and 4.5×10^4, 6.7×10^3 and 1.5×10^2 (group 3) on 1 and 4 dpc and on the day of post-mortem examination,

Table 2
Frequency and severity of histological pneumonic lesions in each lung lobe within different calf groups challenged with *Pasteurella multocida* A

Calf group	*Lung lobe — number affected (total lesion severity score)*						*Total severity score*[*]
	Left cranial	*Right cranial*	*Left caudal*	*Right caudal*	*Right middle*	*Accessory*	
Negative control	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0
UK strain	6/8 (29)	6/8 (18)	1/8 (5)	2/8 (12)	8/8 (28)	8/8 (38)	130[†]
USA strain	2/8 (7)	5/8 (11)	2/8 (6)	1/8 (5)	8/8 (17)	1/8 (3)	49[†]

[*]A highly significant difference ($P = 6.2 \times 10^{-5}$, Fisher's exact test for count data) was present between the three groups.
[†]Significantly more severe lesions were present in the group challenged with the UK strain compared with the group challenged with the USA strain ($P = 0.006$, Fisher's exact test for count data).

Table 3
Frequency and severity of suppurative lymphadenitis in each lymph node within different calf groups challenged with *Pasteurella multocida* A

Calf group	*Lymph node – number calves affected (N), mean weight [g] (W), total lesion severity score (S)*												*Total severity score*
	Left bronchial			*Cranial bronchial*			*Mid-mediastinal*			*Caudal mediastinal*			
	N	*W*	*S*	*N*	*W*	*S*	*N*	*W*	*S*	*N*	*W*	*S*	
Negative control	0/3	3.0	0	0/3	1.2	0	0/3	1.3	0	0/3	5.3	0	0
UK strain	4/8	9.2	11	5/8	2.6	12	6/8	4.6	17	6/8	13.1	15	55*
USA strain	1/8	7.1	3	3/8	2.3	8	3/8	2.4	9	1/8	10.5	2	22*

*Calves challenged with the UK strain had significantly ($P = 0.02$, Fisher's exact test for count data) more severe lesions than those challenged with the USA strain.

respectively. Recovery from gross pulmonary lesions or grossly normal lung tissue taken post-mortem was 7.2×10^6 and 1.4×10^4 (group 2) and 4.9×10^5 and 4.3×10^3 (group 3), respectively. The mean bacterial counts in lung tissue and lavage fluids were not significantly different between groups 2 and 3. No *P. multocida* was recovered from any group 1 BALF or lung tissue.

Pulsed-Field Gel Electrophoresis Analysis of P. multocida *Isolates*

Analysis, in triplicate, of UK and USA strains of *P. multocida* from frozen stocks by PFGE demonstrated different banding patterns with some common and other different bands (Fig. 3). For both groups 2 and 3, PFGE analysis of isolates recovered from

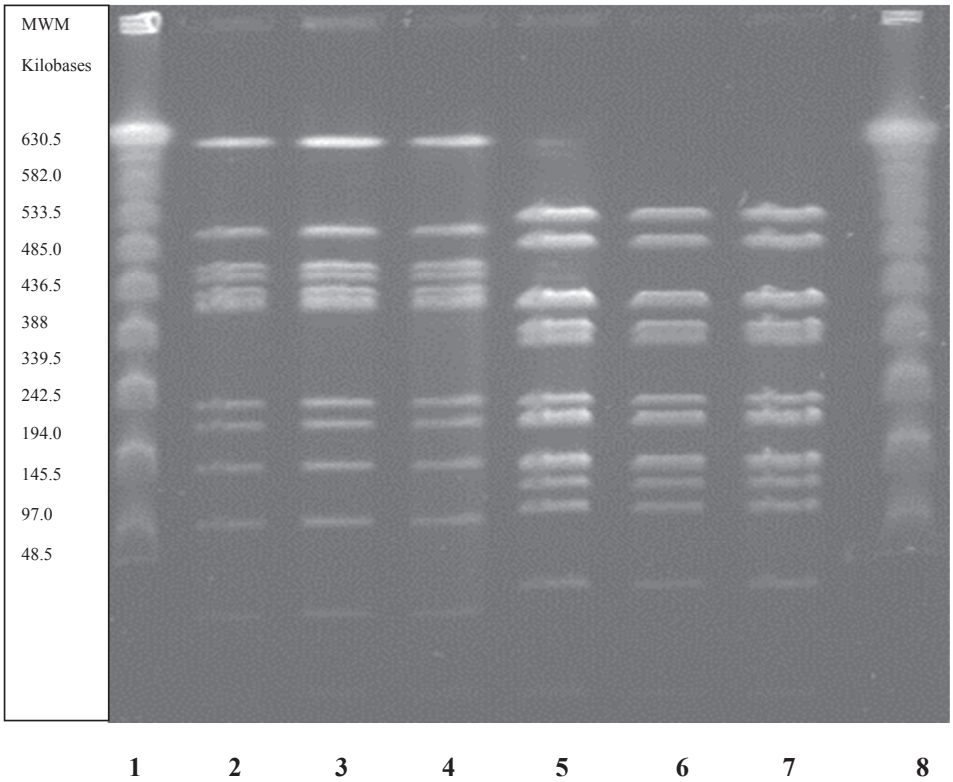

Fig. 3. Pulsed-field gel electrophoresis of stock suspensions, in triplicate, of *Pasteurella multocida* A strains used as challenge inocula originally isolated from clinical cases of bovine pasteurellosis in the UK (lanes 2, 3 and 4) and USA (lanes 5, 6 and 7). Note UK and USA isolates demonstrate distinct banding patterns with minimal conformity, indicating distinct genetic differences between isolates. Lanes 1 and 8 Lambda ladder PFG marker (mwm kilobases: New England Biolabs Inc., Ipswich, Massachusetts, USA).

lung and tonsil samples indicated that *P. multocida* from both locations had the same banding pattern as their respective challenge strain derived from frozen stocks (data not shown).

Discussion

This is the first report comparing the clinical disease, pathological lesions and bacterial load in cattle challenged experimentally with different strains of *P. multocida* capsule type A. Both strains were isolated originally from field cases of clinical bovine pneumonic pasteurellosis and this shows that significant strain-related variations in clinical disease and pathological severity were recognized. Results overall indicated a much more severe clinical disease associated with the UK strain of *P. multocida* compared with the USA strain, and this correlated with the extensive and severe pathological lesions associated with this strain. The animals challenged with the UK *P. multocida* strain reproduced accurately the disease model in terms of the clinical and pathological responses observed in previous work (Dowling *et al.*, 2002, 2004; Dagleish *et al.*, 2010). The milder clinical disease and lesser extent and severity of pulmonary lesions as a result of challenge with the USA strain of *P. multocida* were not due to differences in *P. multocida* cell numbers present within the pulmonary tissues, as both strains of bacteria were able to replicate to a similar degree. Therefore, the differences must lie in either their direct effects on bovine tissues or host responses. It has been suggested that excessive or inappropriate host response(s) to *P. multocida* in bovine pneumonic pasteurellosis may be a significant factor with respect to tissue damage and, consequently, the severity of the pathology and clinical disease (Dowling *et al.*, 2004; Dagleish *et al.*, 2010).

Subjecting the two strains of *P. multocida* to analysis by PFGE generated banding patterns that indicated, despite some similarities, a different overall genetic composition. This would be expected as although both strains had the same capsular type (A), which denotes major host species susceptibility and disease presentation (Ewers *et al.*, 2006), there was variation in the serotype with the UK strain being serotype 3 and the USA strain serotype 3/4. Although the virulence attributes of *P. multocida* are not well established, it is tempting to speculate that these genetic differences resulted in the production of different virulence-associated bacterial proteins responsible for the observed significant variations in disease severity *in vivo* of these two isolates in our calf model of pneumonic pasteurellosis. Outer membrane proteins and capsule types make significant contributions to virulence (Ewers *et al.*, 2006) and two key core structures for *P. multocida* lipopolysaccharide that may be linked to differences in virulence have been identified (Harper *et al.*, 2007). However, in avian isolates of *P. multocida*, inactivation of the *hgbA* or *hgbB* genes, involved in iron acquisition, did not affect the virulence of fowl cholera-associated *P. multocida* (Bosch *et al.*, 2002; Cox *et al.*, 2003). The clear and significant variation in experimentally-induced clinical disease under highly controlled conditions shown in the current work between two strains of *P. multocida* A recovered from geographically separate clinical cases of bovine pneumonia indicate the importance of establishing whether this outcome was linked to the bacterial strain or due to host response differences. This would require full genome sequencing and transcriptome analysis to determine if any of the genetic differences between *P. multocida* strains were associated with any known expressed virulence-associated factors and was outwith the scope of this study.

A better understanding of the diversity between bovine clinical pneumonic disease-associated *P. multocida* A isolates from different areas of the world may allow prudent selection of common antigenic proteins, resulting in the eventual production of a more universal, effective and safe vaccine. An effective vaccine to protect against *P. multocida* bovine pneumonic pasteurellosis is still required as extensive pulmonary pathology occurs prior to clinical signs developing (Dagleish *et al.*, 2010), giving no opportunity to instigate treatment to prevent lung damage. However, great care is needed, as a simple bacterin preparation aimed at inducing pulmonary mucosal immunity has been shown to exacerbate disease rather than protect against it (Dowling *et al.*, 2004). If differences in virulence exist between *P. multocida* isolates from animals in the same geographical area, it may be possible to screen and differentiate between true commensal strains versus facultative and obligate pathogenic ones in order to identify diagnostic markers or potential new vaccine candidates. Differentiation of commensal from pathogenic strains may also be required due to the emergence of antibiotic resistance among *P. multocida* serotype A strains in Europe (Kehrenberg *et al.*, 2001). Unpublished observations from our own laboratory have shown great difficulty in eliminating *P. multocida* from the upper respiratory tract (URT) in clinically healthy calves using a range of antibiotic preparations, which makes effective choice of antibiotic a key issue in limiting the spread of this ever increasing problem (Kehrenberg *et al.*, 2001; Dabo *et al.*, 2008).

Antibiotic resistance may arise by several different mechanisms including survival of *P. multocida* bacteria

within macrophages (J. C. Hodgson personal observation) or within other pharmacologically or immunologically privileged sites (e.g. tonsilar crypt) thereby avoiding administered medication. Previous work suggested that planktonic and biofilm formation by both *P. multocida* and *Mannheimia haemolytica* had no effect on antibiotic sensitivity (Olson *et al*., 2002). However, more recent studies on *Histophilus somni*, a related organism, has indicated that biofilms formed by this organism may be inhabited by *P. multocida* also (Sandal *et al*., 2009) and the consequences of this mixed population are unknown. These factors may require development of novel control methods to target calves carrying potentially dangerous strain(s) of *P. multocida*, thereby avoiding metaphylaxis with its inherent dangers of encouraging development of antibiotic resistance.

In summary, the calves infected with the UK strain of *P. multocida* had significantly more extensive and severe lesions present in the lung lobes and pulmonary lymph nodes than those infected with the USA strain, which resulted in a more severe clinical presentation. Further investigation of the genetic differences between different strains may lead to the determination of bacterial-associated virulence factors and antigens, which should aid in the understanding of these differences and in the development of a safe, effective vaccine. With careful screening it may be possible to find low- or even non-pathogenic strains of *P. multocida* A as a starting point for developing a live vaccine, which, in the light of failed bacterin preparations (Dowling *et al*., 2004), may be more successful.

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