EXPERIMENTALLY INDUCED DISEASE

Virulence of Two Strains of Mycobacterium bovis in Cattle Following Aerosol Infection

W. R. Waters*, T. C. Thacker*, J. T. Nelson†, D. M. DiCarlo†, M. F. Maggioli*, R. Greenwald†, J. Esfandiari†, K. P. Lyashchenko‡ and M. V. Palmer*

*National Animal Disease Center, Agricultural Research Service, †National Veterinary Services Laboratories, Veterinary Services, Animal and Plant Health Inspection Service, US Department of Agriculture, Ames, IA 50010; ‡Department of Veterinary Pathology, College of Veterinary Medicine, Iowa State University, Ames, IA 50011 and †Chembio Diagnostic Systems, Inc., Medford, NY 11763, USA

Summary
Over the past two decades, highly virulent strains of Mycobacterium tuberculosis have emerged and spread rapidly in man, suggesting a selective advantage based on virulence. A similar scenario has not been described for Mycobacterium bovis infection in cattle (i.e. bovine tuberculosis). An epidemiological investigation of a recent outbreak of bovine tuberculosis in a USA dairy indicated that the causative strain of M. bovis (strain 10-7428) was particularly virulent, with rapid spread within the herd. In the present study, the virulence of this strain (10-7428) was directly compared in the target host with a well-characterized strain (95-1315) of relevance to the USA bovine tuberculosis eradication programme. Aerosol inoculation of 10⁴ colony forming units of M. bovis 95-1315 (n = 8) or 10-7428 (n = 8) resulted in a similar distribution and severity of gross and microscopic lesions of tuberculosis as well as mycobacterial colonization, primarily affecting the lungs and lung-associated lymph nodes. Specific cell-mediated and antibody responses, including kinetics of the response, as well as antigen recognition profiles, were also comparable between the two treatment groups. Present findings demonstrate that M. bovis strains 95-1315 and 10-7428 have similar virulence when administered to cattle via aerosol inoculation. Other factors such as livestock management practices likely affected the severity of the outbreak in the dairy.

Keywords: bovine tuberculosis; immunity; Mycobacterium bovis; virulence

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Introduction
Mycobacterium bovis, a member of the Mycobacterium tuberculosis complex, has a wide host range compared with other species in this disease complex. The organism is infectious to man and is the species most often isolated from tuberculous cattle. In 1917, the United States Department of Agriculture (USDA) initiated a federal bovine tuberculosis eradication programme with significant contributions from state and local authorities as well as producer groups (Palmer and Waters, 2011). Over the past century, the programme has relied primarily on slaughter inspection to iden-
tify bovine tuberculosis-affected herds, as well as test and cull strategies or isolation of infected cattle (i.e. the ‘Bang method’) and movement/border testing policies for the control of bovine tuberculosis. Consequently, disease prevalence in the USA has diminished dramatically from an estimated individual animal prevalence of 5% in 1917 to an approximate herd prevalence of <0.001% in 2011 (Palmer and Waters, 2011; K. Orloski, USDA Animal and Plant Health Inspection Services [APHIS], Veterinary Services, personal communication). Complete eradication of bovine tuberculosis within the USA, however, may be difficult due to ongoing importation of tuberculous cattle from Mexico (McCluskey et al., 2014), spillover from a wildlife reservoir host (i.e.
white-tailed deer, *Odocoileus virginianus*) in Michigan (Schmitt *et al.*, 1997), continued detection of *M. bovis* in captive cervids with transmission to cattle (Waters *et al.*, 2011b) and infrequent interherd spread of the disease within the cattle population in the USA (Miller and Sweeney, 2013).

The prevalence of bovine tuberculosis in England and Wales has been steadily increasing since 1979, despite compulsory testing and an active control campaign (Abernethy *et al.*, 2013). Approximately 10% of English cattle herds were under movement restrictions due to tuberculosis in 2010, with ~25,000 cattle slaughtered at a cost of £91 million (Defra Publications, 2011; Anonymous, 2013). The steady rise in prevalence in Great Britain has been attributed to the failure of the current tuberculin test and slaughter strategy and the presence of a wildlife reservoir (i.e. the Eurasian badger, *Meles meles*) (Defra Publications, 2011; Karolemeas *et al.*, 2011). Bovine tuberculosis also persists, although generally at low levels, within other European Union countries, despite concerted control efforts (Reviriego Gordejo and Vermeersch, 2006). In New Zealand, the presence of another wildlife reservoir (i.e. the brushtail possum, *Trichosurus vulpecula*) seriously hinders bovine tuberculosis control efforts (Ryan *et al.*, 2006). Thus, bovine tuberculosis remains an important animal health problem in developed countries and new tools for control of the disease in wildlife and/or domestic livestock, such as vaccines and improved diagnostic tests, are desperately needed.

An epidemiological investigation was recently conducted by USDA APHIS Veterinary Services on a tuberculosis-affected dairy herd in Colorado, USA, as well as on numerous other dairy herds across the USA determined to be at risk due to animal movement from the affected herd following an epidemiological investigation (i.e. including trace-in and trace-out investigations) (Francisco *et al.*, 2014). Several aspects of this particular outbreak were unique when compared with other tuberculosis-affected herds detected within the past 30 years in the USA. The within-herd prevalence was substantially higher (11.1%) than that typically detected within the USA (average over the past decade is <2%) and ante-mortem test response rates were also particularly high (i.e. 32% for caudal fold test [CFT], 18% for CFT followed by comparative cervical test [CCT] in series and 21% for CFT followed by interferon [IFN]-γ release assay in series) when compared with typical response rates (Francisco *et al.*, 2014). Additionally, numerous calves were infected and at least one cow had supramammary lymph node lesions. One year prior to the outbreak, all adult cattle >2 years of age within this herd were screened for bovine tuberculosis and all were negative. Together, these findings indicate that *M. bovis* infection spread rapidly within the herd. Genotyping indicated that infected animals were colonized with an *M. bovis* strain unique to other currently defined strains within the USA. The USDA APHIS National Veterinary Services Laboratories’ designation for this isolate was *M. bovis* 10-7428.

For cattle infection trials involving experimental challenge to evaluate candidate vaccines and diagnostic strategies, it is critical to select strains of virulence representative of relevant field isolates as well as of geographical and epidemiological significance. Strain virulence significantly influences the outcome of pathogenesis, diagnostic and vaccine efficacy studies with *M. tuberculosis* (Gagneux and Small, 2007; Kato-Maeda *et al.*, 2012; Via *et al.*, 2013); however, few studies have been performed to directly compare the virulence of geographically relevant strains of *M. bovis* in cattle. In one such study, Palmer *et al.* (2002) demonstrated that *M. bovis* 95-1315 has a similar level of virulence in cattle compared with *M. bovis* HC2005T (an isolate obtained from the tracheobronchial lymph node from a dairy cow in Texas, USA). Thus, 95-1315 was selected as a relevant strain for direct comparison with 10-7428 for virulence in cattle. The comparator strain, 95-1315, was isolated from a white-tailed deer in Michigan, USA, in 1995 (Schmitt *et al.*, 1997) and has been used in numerous experimental infection trials (Palmer *et al.*, 2002) and is endemic in Michigan (north east corner of the lower peninsula) white-tailed deer and cattle (i.e. in deer and cattle in the same area). Therefore, the objective of the current study was to compare the recently recognized *M. bovis* 10-7428 (a strain of particular interest to regulatory officials given its perceived high virulence in the field) with a recognized virulent strain (*M. bovis* 95-1315) to specifically address the question of whether a highly virulent strain had emerged within the USA.

**Materials and Methods**

*Mycobacterium bovis* Aerosol Challenge

Two strains of *M. bovis* were used for challenge inoculum: 95-1315 (Schmitt *et al.*, 1997) and 10-7428 (Francisco *et al.*, 2014; Colorado dairy isolate). Low passage (≤3) cultures of both strains were prepared using standard techniques (Larsen *et al.*, 2007) in Middlebrook 7H9 liquid medium (Becton Dickinson, Franklin Lakes, New Jersey, USA) supplemented with 10% oleic acid–albumin–dextrose complex (OADC) plus 0.05% Tween 80. Holstein steers
(n = 23, ~6 months of age) were obtained from a tuberculosis-free herd in Sioux Center, Iowa and housed in a biosafety level-3 (BSL-3) facility at the National Animal Disease Center, Ames, Iowa, USA, according to Institutional Biosafety and Animal Care and Use Committee guidelines (ethical approval via Animal Care and Use protocol #2508). Treatment groups consisted of non-infected steers (n = 7) and animals receiving 10^9 colony forming units (cfu) of *M. bovis* 95-1315 (n = 8) or 10^8 cfu *M. bovis* 10-7428 (n = 8) by aerosol as described by Palmer et al. (2002).

Three months after acquisition, *M. bovis* challenge inoculum was delivered to restrained calves (~9 months of age) by nebulization into a mask via a one-way valve (Trudell Medical International, London, Ontario, Canada) covering the nostrils and mouth. The process continued until the inoculum, a 1 ml phosphate buffered saline (PBS) wash of the inoculum tube and an additional 2 ml PBS were delivered, a process taking ~10 min. Strict biosafety protocols were followed to protect personnel from exposure to *M. bovis* throughout the study, including BSL-3 containment on initiation of *M. bovis* challenge in animal rooms and standard laboratory practices for handling *M. bovis*-infected animals.

**Mycobacterial Isolation and Assessment of Lesions**

All calves were killed ~4 months after challenge by intravenous administration of sodium pentobarbital. Tissues were examined for gross lesions and processed for microbiological analysis and isolation of *M. bovis*. Tissues collected included: lung, liver and mandibular, parotid, medial retropharyngeal, mediastinal, tracheobronchial, hepatic and mesenteric lymph nodes. Lymph nodes were sectioned at 0.5 cm intervals and examined. Each lung lobe was sectioned at 0.5–1.0 cm intervals and examined separately. Lungs and lymph nodes (mediastinal and tracheobronchial) were evaluated using a semiquantitative gross pathology scoring system adapted from Vordermeier et al. (2002). Lung lobes (left cranial, left caudal, right cranial, right caudal, middle and accessory) were assessed individually based on the following scoring system: 0, no visible lesions; 1, no external gross lesions, but lesions seen on slicing; 2, <5 gross lesions of <10 mm in diameter; 3, >5 gross lesions of <10 mm in diameter; 4, >1 distinct gross lesion of >10 mm in diameter; 5, gross coalescing lesions. Cumulative mean scores were then calculated for each entire lung. Scoring of lymph node pathology was based on the following system: 0, no necrosis or visible lesions; 1, small focus (1–2 mm diameter); 2, several small foci; 3, extensive necrosis. Gross pathology data are presented as individual and mean disease scores for lymph nodes (combined scores for tracheobronchial and mediastinal lymph nodes), lung (combined scores including each lung lobe) and a total gross pathology score including both combined pulmonary lymph nodes and lung scores.

Tissues collected for microbiological analysis were fixed in 10% neutral buffered formalin. Fixed tissues were processed routinely and embedded in paraffin wax. Sections (5 µm) were stained with haematoxylin and eosin (HE). Adjacent sections from samples containing caseonecrotic granulomas, suggestive of tuberculosis, were stained by the Ziehl–Neelsen technique for identification of acid-fast bacteria. Microscopical tuberculous lesions were staged (I–IV) based on a scoring system developed by Wangoo et al. (2005). Data are presented as total and mean number of granulomas observed in each histological lesion stage (i.e. I–IV) for lung and mediastinal lymph node sections.

Quantitative assessment of mycobacterial burden was evaluated as described by Waters et al. (2007). Briefly, tracheobronchial lymph nodes were removed, examined for gross lesions, weighed and entire lymph nodes (other than a small ~1 g section for histology) were homogenized in phenol red nutrient broth using a blender (Oster, Shelton, Connecticut, USA). Logarithmic dilutions (10^9–10^–3) of homogenates in PBS were plated in 100 µl aliquots on Middlebrook 7H11 selective agar plates (Becton Dickinson) and incubated for 8 weeks at 37°C. Data are presented as cfu per gram of tissue, based on challenge treatment group. Qualitative assessment of mycobacterial colonization was evaluated using standard mycobacterial culture techniques (Waters et al., 2010) using Middlebrook 7H11 selective agar plates (Becton Dickinson) incubated for 8 weeks at 37°C as well as IS6110 real time polymerase chain reaction (PCR) for confirmation of colonies as described by Thacker et al. (2011).

**Measurement of Delayed Type Hypersensitivity Responses**

Ten days prior to necropsy examination, the calves received 0.1 ml (100 µg) of *M. bovis* purified protein derivative (PPD) and 0.1 ml (40 µg) of *M. avium* PPD intradermally at separate clipped sites in the mid-cervical region according to guidelines described in USDA APHIS circular 91-45-01 (USDA APHIS, 2007) for the CCT. Skin thickness was measured with calipers prior to PPD administration and 72 h after injection. Balanced PPDs were obtained from the *Brucella* and Mycobacterial Reagents section of the National Veterinary Services Laboratories (NVSL),
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Ames, Iowa, USA. Data are presented as individual and treatment group mean responses (i.e. change in skin thickness 72 h after injection) to *M. bovis* PPD and to *M. avium* PPD. Additionally, a scattergram for interpretation of CCT was used to characterize responses as negative, suspect or reactor as prescribed by USDA APHIS Veterinary Services (USDA APHIS, 2007).

**Antibody Detection**

The IDEXX *M. bovis* antibody enzyme-linked immunosorbent assay (ELISA; IDEXX, Portland Maine, USA) was performed at NVSL as described by Waters *et al.* (2011a) and according to the manufacturer’s directions. Briefly, immulon I 96-well microtitre plates (Thermo Scientific, Hudson, New Jersey, USA) were coated with a proprietary blend of mycobacterial antigens (MPB83 and MPB70). Diluted serum samples (100µl/well; 1 in 50 dilution with kit diluent) plus kit controls (positive and negative) were added to the plates, incubated at room temperature (18–26°C) for 60 min and then wells were washed four times before adding 100 µl of horseradish peroxidase-conjugated monoclonal anti-bovine IgG to each well for 30 min. After a further four washes, 100 µl of tetramethyl benzidine (TMB) substrate was added to each well and incubated for 15 min at room temperature. Colour development was stopped by addition of an acidified stop solution. Plates were read at 450 nm (Vmax, Molecular Devices, Sunnyvale, California, USA) and results were presented as sample-to-positive ratios (S/P) derived by subtracting the mean kit negative control optical density (OD) from each sample and dividing this value by the corrected positive control value (mean positive control OD minus mean negative control OD). Samples with S/P ratios greater than or equal to 0.30 were considered positive for *M. bovis* antibodies.

The multi-antigen print immunoassay (MAPIA, Lyashchenko *et al.*, 2000) was performed as described for use with samples from cattle (Waters *et al.*, 2006). Briefly, twelve *M. tuberculosis* complex antigens (*M. bovis* culture filtrate [MBCF], *M. bovis* PPD, ESAT-6 [Rv3875], CFP-10 [Rv3874], MPB59 [Rv1886c], MPB64 [Rv1980c], MPB70 [Rv2875], MPB83 [Rv2873], Acr1 [Rv3391], 38 kDa protein [Rv0934], ESAT-6/CFP10 [polyepitope fusion of CFP10 and ESAT-6] and F10 [polyepitope fusion of Mtb8, Rv0379; CFP10 and 38 kDa protein]) were immobilized on nitrocellulose membrane strips, blocked for 1 h with 1% non-fat skimmed milk in PBS with 0.05% Tween 20 and then incubated for 1 h with serum samples diluted 1 in 40 in blocking solution. After washing, strips were incubated for 1 h with peroxidase-conjugated protein G (Sigma, St Louis, Missouri, USA) diluted 1 in 1,000, washed and developed with 3, 3′, 5, 5′-TMB (Kirkegaard and Perry Laboratories Inc., Gaithersburg, Maryland, USA).

Dual-path platform (DPP) technology developed by Chembio (Lyashchenko *et al.*, 2013) was used to design a DPP assay measuring bovine IgG and IgM antibody responses independently to two polypeptide fusion proteins, MPB70/MPB83 and CFP10/ESAT-6. As a detector system, goat anti-bovine IgG and anti-bovine IgM antibodies (Kirkegaard and Perry Laboratories) were conjugated to colloidal gold nanoparticles using Chembio standard procedure. Serum samples were tested at a dilution of 1 in 40 in assay running buffer and results were recorded at 20 min using an optical reader to measure test band reflectance in relative light units (RLU), as previously described (Lyashchenko *et al.*, 2013).

**Statistical Analysis**

Data were analyzed by analysis of variance followed by Tukey’s multiple comparisons test or Student’s *t* test using a commercially available statistics programme (Prism 6.0c, GraphPAD Software, La Jolla, California, USA).

**Results**

**Assessment of Mycobacterial Colonization and Gross and Microscopical Lesions**

Aerosol inoculation of *M. bovis* 95-1315 or 10-7428 resulted in similar (*P* >0.05) levels of colonization in tracheobronchial lymph nodes between the two treatment groups (Fig. 1). Additionally, *M. bovis* was isolated from all calves within the two infection groups and *M. bovis* was not isolated from any of the animals within the non-inoculated group. The distribution of tuberculous lesions was similar between the two *M. bovis* infection groups with gross and microscopical lesions detected within lungs and lung-associated lymph nodes from all but one of the *M. bovis*-inoculated animals. One animal in the *M. bovis* 95-1315 group did not have any detectable lesions within its lung-associated lymph nodes, despite having a lung lesion. Microscopical lesions were detected in the parotid lymph node of one animal in the *M. bovis* 10-7428 group and in the liver of a different animal in the same group. Otherwise, lesions were not detected in additional tissues examined. Weights (mean ± SEM) of lung-associated lymph nodes (tracheobronchial and mediastinal) tended (*P* = 0.1) to be greater for *M. bovis*-infected (i.e. 95-1315- and 10-7428-infected) groups combined,
50.95 ± 14.26 g) versus non-infected calves (16.42 ± 2.78 g). Lung-associated lymph node weights (tracheobronchial, mediastinal or combined) did not differ (P > 0.1) between M. bovis 95-1315- and 10-7428-infected groups. Using a semi-quantitative gross pathology scoring system, lesion severity did not differ (P > 0.05) between M. bovis 95-1315- and 10-7428 infection groups (Fig. 2). Representative images of gross lung lesions are depicted in Fig. 3. Similarly, thorough assessment of microscopical lesions via histological staging did not reveal differences (P > 0.05) in lesion severity between M. bovis 95-1315- and 10-7428 infection groups (Tables 1 and 2).

Cell-mediated Immune Responses

Both M. bovis strains elicited robust cell-mediated immune responses. IFN-γ responses were detectable beginning at 2–3 weeks after aerosol inoculation; however, these responses did not differ between strains (Bass et al., 2013). Significant delayed type hypersensitivity (DTH) responses to PPDs were also elicited by both strains (Fig. 4). As with IFN-γ responses, DTH responses as measured by CCT did not differ (P > 0.05) between strains; however, responses (PPDb and PPDb) from both 95-1315 and 10-7428 treatment groups each exceeded (P < 0.05) the respective responses by non-infected animals.

Fig. 1. Quantitative assessment of M. bovis colonization. To eliminate bias based on organ sampling site, entire tracheobronchial lymph nodes (except a 1 g section obtained for histology) were homogenized and cultured for M. bovis. Data are presented as individual and mean (horizontal lines) cfu per gram of tissue according to treatment group (non-infected, n = 7; M. bovis strain 95-1315-infected, n = 8; M. bovis strain 10-7428-infected, n = 8). Colonization did not differ (P > 0.05) between 95-1315 and 10-7428 infection groups. Mycobacterium bovis was not detected within tissues from the non-infected group.

Fig. 2. Quantitative assessment of gross pathology. At necropsy examination (~4 months after M. bovis challenge), tracheobronchial and mediastinal lymph nodes, as well as lungs dissected into individual lobes, were evaluated for lesions based on a scoring system adapted from Vordermeier et al. (2002). Data are presented as individual (closed circles and squares) and mean (horizontal lines) disease scores for: (A) lymph nodes (combined scores for tracheobronchial and mediastinal lymph nodes), (B) lung (combined scores including each lung lobe) and (C) total gross pathology score, which includes both combined pulmonary lymph nodes and lung lobes scores. Treatment groups included non-infected (n = 7) and M. bovis strain 95-1315- and 10-7428 infected groups (n = 8/group), as shown in the lower margin. Lesion severity did not differ (P > 0.05) between challenge strains; however, lesion severity for each of the infected groups exceeded (P < 0.05) that of the non-infected group. Tuberculous lesions were not detected within tissues from the non-infected (n = 7) group (data not shown).
Serum Antibody Responses

Approximately 4 months after challenge (immediately prior to necropsy examination), all calves within the two *M. bovis* infection groups had serum antibody responses considered positive (>0.3 S/P) by the *M. bovis* IDEXX ELISA. Two animals (calf 221 and 222) in the 95-1315-infected group had responses >0.3 S/P at 8 and 12 weeks after challenge; however, responses did exceed (*P* <0.05) respective responses by non-infected animals at 16 weeks after challenge (Fig. 5). Consistent with prior studies (Waters *et al.*, 2011a), injection of PPDs for CCT increased (*P* <0.05) the antibody response with the 10-7428 group (from 0.05 ± 0.06 pre-CCT to 3.52 ± 1.06 post-CCT). The response also tended (*P* = 0.1) to increase on injection of PPDs for CCT with the 95-1315 group (from 1.39 ± 1.18 pre-CCT to 4.62 ± 1.66 post-CCT).

MAPIA was used to determine antigen recognition profiles of serum antibodies elicited by infection with *M. bovis* strains 95-1315 and 10-7428 (Table 3). By MAPIA, specific antibody responses were detected as early as 2 weeks after challenge with strain 10-7428 (to ESAT-6/CFP10 in one animal and MPB59 in another animal). By 4 weeks after challenge, 6/8 and 7/8 animals in the 10-7428 and 95-1315 groups, respectively, had serum antibody responses detectable by MAPIA (Table 3). Specific serum antibody responses were not detected with serum samples from non-infected calves. As with the IDEXX ELISA, antibody responses as measured by MAPIA did not differ noticeably in onset, kinetics or antigen recognition profiles between the two animal groups based on infection strain. These findings were consistent with detection of serum IgG or IgM by the DPP assay, which did not reveal any significant difference between the two *M. bovis* challenge groups (Table 4).

**Table 1**

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<th>107</th>
<th>206</th>
<th>227</th>
<th>205</th>
<th>208</th>
<th>209</th>
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<th>225</th>
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<th>221</th>
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<td>20</td>
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Histological staging procedures are defined in Materials and Methods. Samples for microscopical evaluation were obtained from cattle killed 4 months after aerosol infection with 10⁴ cfu *M. bovis* strain 10-7428 or strain 95-1315. Data are presented as the mean number of granulomas per visual field for individual animals with a tally of the mean ± SEM for each histological stage for the two groups. Lesion severity, as measured by microscopical assessment, did not differ (*P* >0.05) between infection groups. Bold was used to set apart the animal numbers and mean (SEM) results to better represent the data.
IgM responses to MPB70/MPB83 were first detected at week 3 or 4 post-challenge, usually preceding IgG seroconversion observed at week 4 in most cases. In some of the infected cattle, however, IgM and/or IgG responses were not detected until week 15 post inoculation of M. bovis or 2 weeks after PPD administration for the intradermal tuberculin test.

**Table 2**

Summary of histological staging of granulomas in the lungs of M. bovis-infected cattle

<table>
<thead>
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<th>Animal number</th>
<th>M. bovis strain 10-7428</th>
<th>M. bovis strain 95-1315</th>
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<td>Stage I</td>
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<td>178 222 225 214 221 216 181 220</td>
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<tr>
<td>Stage II</td>
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<td>0 2 2 1 0 2 1 1 0</td>
<td>1.0 (0.3)</td>
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<tr>
<td>Stage IV</td>
<td>0 1 0 0 2 1 0 1 1</td>
<td>0.6 (0.3)</td>
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</table>

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**Discussion**

Typical M. tuberculosis complex pathogens (including M. bovis) are slow growing, >99.95% homologous (genetically), rarely undergo horizontal genetic exchange and have a strictly clonal population structure (Smith et al., 2006). Despite these attributes, which limit rapid changes in population structure, highly virulent strains of M. tuberculosis have emerged recently and spread rapidly amongst human non-infected animals. Fig. 5. Comparison of serum antibody responses as measured by IDEXX M. bovis ELISA. Data are presented as sample-to-positive ratios (S/P, mean ± SEM) derived by subtracting the mean kit negative control optical density (OD) from each sample and dividing this value by the corrected positive control value (mean positive control OD minus mean negative control OD). Samples with S/P ratios greater than or equal to 0.30 (noted with a dotted line on the graph) were considered positive for M. bovis-specific (i.e. to MPB83/70) antibodies. Responses did not differ (P > 0.05) between M. bovis strain 95-1315 and 10-7428 treatment groups; however, responses (PPDb and PPDb) from both 95-1315 and 10-7428 treatment groups each exceeded (P < 0.05) the respective responses by non-infected animals.
populations (de Jong et al., 2008; Djelouadji et al., 2011; Brites and Gagneux, 2012). In contrast, to the authors’ knowledge, no reports describe highly virulent strains of M. bovis in cattle. The reason for this is unclear, but variation in human strains may result from differential selective pressures such as wide-scale use of BCG vaccination in many developing countries, use of antimycobacterial compounds for treatment of tuberculosis and the impact of human immunodeficiency virus co-infection. Similar selective pressures do not exist in the case of bovine tuberculosis. By contrast, few studies compare the virulence of M. bovis strains in cattle, despite reports suggesting that highly virulent strains exist, as determined by inoculation of mice (Aguilar León et al., 2009; Nishibe et al., 2013). Thus, lack of evidence for the emergence of highly virulent strains of M. bovis may simply be due to a lack of investigation via comparative virulence studies in cattle. In the present study, a strain of M. bovis (10-7428) with epidemiological evidence suggesting high virulence was determined to be of similar virulence (including analysis of pathology as well as cellular and humoral immune responses) to another well-characterized clinical isolate. Thus, parameters other than strain virulence likely contributed to the rapid spread of M. bovis 10-7428 within the affected dairy herd, possibly relating to management practices (e.g. feeding non-pasteurized milk to calves), transmission potential of the organism, herd immune status (i.e. recent or concurrent infection with immunosuppressive agents such as bovine viral diarrhoea virus or bovine immunodeficiency virus) or undetermined host genetic factors. These findings support the notion that hypervirulence is not a significant selective pressure for M. bovis infection in cattle.

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Conflict of Interest Statement

The authors declare that they have no conflict of interests with respect to their authorship or to the publication of this article.

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