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Short communication

Comparison of *Mannheimia haemolytica* isolates from an outbreak of bovine respiratory disease

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ABSTRACT

The objective of this study was to determine the clonal relatedness of *Mannheimia haemolytica* isolates responsible for an outbreak of bovine respiratory disease in a commercial feedlot. The isolates were obtained from the lungs of 21 calves with fatal pneumonia that were part of a group of 206 total calves. All isolates were serotyped and analyzed by pulsed-field gel electrophoresis (PFGE) and for antibiotic sensitivity patterns. ELISA and immunoblotting assays were performed to compare serum antibody levels to *M. haemolytica* antigens in calves with fatal pneumonia to those calves that survived the outbreak. Isolates were categorized into 14 different PFGE groups based on 90% similarity. Two Group D isolates (1 and 6), and 3 Group H isolates (14, 15, and 16) were characterized as 100% similar. Antimicrobial susceptibility profiles defined 8 groups based on differences in patterns of resistance between isolates. The two 100% similar isolates from PFGE Group D were both in susceptibility Group 1. All but isolate 14 from PFGE Group H (3, 15, 16, and 19) were in susceptibility Group 4a. Serum antibody levels to *M. haemolytica* antigens in the dead calves were not different than the antibody levels in the 185 calves that survived the outbreak. Immunoblots of selected isolates from each of the PFGE groups demonstrated only minimal differences in antigenic profiles between strains when reacted with serum from calves that either died from or survived the outbreak. Based on the characteristics of these isolates, multiple strains of *M. haemolytica* were responsible for fatal pneumonia during this outbreak.

1. Introduction

Bovine respiratory disease (BRD) is a multifactorial condition that causes substantial economic loss to the feedlot industry. Of the bacterial entities most consistently associated with BRD, *Mannheimia haemolytica* is considered the most common and important cause of severe or fatal bronchopneumonia (Rice et al., 2007). Under normal conditions *M. haemolytica* is a commensal of cattle and sheep that is present at low concentrations and remains localized within the upper respiratory tract (Zecchinon et al., 2005; Singh et al., 2011). However, a complex interaction of physical and physiological stressors, bacteria, and viruses can change the upper respiratory microenvironment to favor increased bacterial growth and/or a shift to a more virulent phenotype of the *M. haemolytica* (Duff and Galyean, 2007; Frank, 1986; Taylor et al., 2010). Increased numbers, increased virulence, or both allow the organism to extend into the lower respiratory tract to cause pneumonia. In outbreaks of BRD it is often unclear whether severe pneumonia and fatalities are due to pulmonary proliferation of non-contagious commensal strains of *M. haemolytica*, or a single contagious strain that is transmitted between calves during the outbreak. The purpose of this study was to compare DNA typing and antimicrobial susceptibility profiles of *M. haemolytica* isolates obtained from the lungs of feedlot calves that died in an outbreak of BRD. Host responses to these isolates were compared by ELISA and immunoblotting using serum from both survivors and calves that died during the outbreak.

2. Materials and methods

2.1. Calves and Mannheimia haemolytica isolates

Calves were purchased at auction market and shipped to a private feedlot in South-Central Kansas. From a group of 206—six months old (~500 lb.) calves, 21 died of pneumonia. *M. haemolytica* were isolated from the affected lung of each dead calf and stored at −80 °C. A sample from each isolate was sent to Dr. Robert Briggs, National Animal Disease Center, Ames, Iowa for serotyping.

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2.2. Sera

Blood samples were collected from the jugular vein of all calves ($n = 206$) upon arrival at the feedlot. Serum was collected from each sample, divided into three aliquots and stored at −80 °C.

2.3. Pulsed field gel electrophoresis (PFGE)

Genomic DNA was prepared according to the CDC Pulsenet protocol (http://www.cdc.gov/pulsenet/PDF/ecoli-shigella-salmonella-pfge-protocol-508c.pdf) with some modifications. Colonies from 16 h blood agar plate cultures of *M. haemolytica* from the 21 isolates plus two *M. haemolytica* control strains were suspended in cell suspension buffer (1 M Tris, 0.5 M EDTA, pH 8.0). Chromosomal DNA plugs of the suspensions were prepared in 1% SeaKem Gold agarose (Lonza, Rockland, ME, USA) containing 1% sodium dodecylsulfate (SDS). Bacterial cells within the plugs were lysed (1 M Tris, 0.5 M EDTA, 10% sarcosyl), and extensively washed (sterile water and TE buffer [10 mM Tris, 0.5 M EDTA, pH 8.0]) prior to incubation in 10X enzyme buffer (1:10) (Promega, Madison, Wis.). Two restriction enzymes, *Sal*I and *Sma*I (Promega, Madison, Wis.) were used to cleave DNA of *M. haemolytica* and *Xba*I (Promega, Madison, Wis.) was used to cleave DNA of CDC PFGE marker-Branderup isolate H9812. Plugs were digested with 10 U/μl of restriction enzyme (Promega, Madison, Wis.) and DNA fragments were resolved by electrophoresis in 1% Seakem Gold agarose gel with 0.5X TBE buffer (Thermo Fisher Scientific, MA, USA). Digested fragments were separated using a CHEF-DRII drive apparatus (Bio-Rad Laboratories, Richmond, CA) in 3000 ml of 0.5 X TBE buffer plus 1 ml of 1 M thiourea for 20 h at 14 °C. After staining with ethidium bromide (1 μg/ml), macrorestriction profiles were visualized with the Bio-Rad gel documentation system (Gel Doc 2000) and TDS Quantity One software. The PFGE profiles were analyzed and compared using the BioNumerics Software package (Applied Maths, Belgium) and clustered using an identity cut off of 90%.

2.4. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed on all *M. haemolytica* isolates using guidelines developed by the Clinical and Laboratory Standards Institute (CLSI 2004) for microwell dilution testing. Briefly, bacterial colonies were suspended in sterile water, adjusted to a 0.5 McFarland standard, and transferred (10 μL) into cation-adjusted Mueller–Hinton broth. Fifty-microliters (50 μL) of the final suspension was placed into each well of a commercially available, antimicrobial susceptibility panel[1] that contained a fluorescent compound conjugated to a bacterial surface enzyme substrate (final inoculum 5×10^4 CFU/well). Inoculated panels were placed in an automated reader instrument[2] for 18–24 h and MICs were determined by comparing the fluorescent signal (the amount of bacterial growth) in the test and control wells (*Enterococcus faecalis* ATCC® 29212, *Escherichia coli* ATCC® 25922, *Pseudomonas aeruginosa* ATCC® 27853 and *Staphylococcus aureus* ATCC® 25923). *M. haemolytica* isolates were considered different when the MIC values for any antimicrobial agent were greater than 3 dilutions (8-fold) apart.

2.5. Enzyme-linked immunosorbent assays

An enzyme-linked immunosorbent assay (ELISA) was used to determine serum antibody levels to whole-cell antigens of a *M. haemoltycia* strain isolated from a fatal case of pneumonia in a feedlot calf. Briefly, bacteria were grown in BHI broth for 16 h at 37 C, washed, resuspended in phosphate-buffered saline (PBS) to an OD (600 nm) of 1.035, and mixed with a 1:20 dilution in coating buffer to make the whole-cell antigen. Whole-cell antigen (100 μl) was added to each well of ninety-six well polystyrene microtiter plates (Pierce, Rockford, IL), incubated overnight, washed with wash buffer (0.05% Tween 20 in PBS [PBS-T]), then incubated with duplicate 1:100 dilutions of serum samples, or positive (hyperimmune serum) and negative (fetal bovine serum) controls (100 μl/well) for 1 h. Peroxidase-labeled affinity purified anti-bovine IgG antibody (Pierce, Rockford, IL) was added at a dilution of 1:100 to each well (100 μl/well) and incubated for 1 h. After washing with wash buffer, *o*-phenylenediamine dihydrochloride peroxidase substrate (Thermo Scientific, Wilmington, DE) was added and incubated for 20 min. The OD for each well was measured at 490 nm as an estimate of antibody concentration.

2.6. Immunoblotting

Immunoblotting analysis was performed using 28 randomly selected sera (14 surviving calves and 14 dead calves), serum from a calf that had been hyperimmunized with *M. haemolytica* (positive *M. haemolytica* control), and anti-leukotoxin (LKT) monoclonal antibody (MM601; LKT positive control) (Gentry and Srikumaran, 1991). The monoclonal antibody was kindly provided by Dr. Subramaniam Srikumaran, Washington State University. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the *M. haemolytica* isolate used for the ELISA study (112.5 μg protein) was carried out using 12% Tris–HCL ready made gels (Bio-Rad Laboratories, Hercules, CA), followed by electrophoretic transfer to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was cut into individual strips which were blocked in PBS-T with 3% (wt/vol) bovine serum albumin (BSA), incubated with selected serum samples or controls diluted 1:500 in PBS-T with 0.1% (wt/vol) BSA, followed by washing and incubation with horseradish peroxidase (HRP)-labelled goat anti-bovine IgG (Thermo Scientific, Rockland, IL). For the LKT positive control incubation was carried out with goat anti-mouse IgG labeled with HRP (Kirkegaard and Perry Laboratories [KPL], Gaithersburg, Md.) at a dilution of 10^{-3}. Protein bands were visualized by reaction of each strip with Opti-4CN substrate (BioRad, Hercules, CA). Density of the leukotoxin band was quantitated by densitometry using NIH Image J software and standardized based on the density of the LKT band from the *M. haemolytica* control (Schneider et al., 2012).

2.7. Statistical analysis

Descriptive and statistical analyses were conducted using a commercial statistical program (JMP, SAS Institute, Cary, NC). The distribution of ELISA adjusted values was right skewed and the immunoblot relative densities were bimodal and right skewed. Both distributions were tested for normality using the Shapiro–Wilk goodness-of-fit test and rejected ($P < 0.001$); therefore Kruskal–Wallis non-parametric tests were used to evaluate potential differences among those calves that lived or died. The level of significance was $\alpha = 0.05$.

3. Results

All 21 *M. haemolytica* isolates belonged to serotype 1. The enzyme *Sma*I produced several fragments of small molecular weight that were very poorly separated (data not shown), therefore, *Sal*I was chosen for PFGE cluster analysis. A dendrogram combining data for all 21 *M. haemolytica* isolates was constructed

[1] Sensititre Bovine/Porcine with Tulathromycin MIC format plate, Trek Diagnostic Systems, Oakwood Village, OH.
[2] ARIS 2X, Trek Diagnostic Systems, Oakwood Village, OH.