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Observations on macrolide resistance and susceptibility testing performance in field isolates collected from clinical bovine respiratory disease cases

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ABSTRACT

The objectives of this study were; first, to describe gamithromycin susceptibility of *Mannheimia haemolytica, Pasteurella multocida*, and *Histophilus somni* isolated from cattle diagnosed with bovine respiratory disease (BRD) and previously treated with either gamithromycin for control of BRD (mass medication = MM) or sham-saline injected (control = CON); second, to describe the macrolide resistance genes present in genetically typed *M. haemolytica* isolates; third, use whole-genome sequencing (WGS) to correlate the phenotypic resistance and genetic determinants for resistance among *M. haemolytica* isolates. *M. haemolytica* (n = 276), *P. multocida* (n = 253), and *H. somni* (n = 78) were isolated from feedlot cattle diagnosed with BRD. Gamithromycin susceptibility was determined by broth microdilution. Whole-genome sequencing was utilized to determine the presence/absence of macrolide resistance genes and to genetically type *M. haemolytica*. Generalized linear mixed models were built for analysis. There was not a significant difference between MM and CON groups in regards to the likelihood of culturing a resistant isolate of *M. haemolytica* or *P. multocida*. The likelihood of culturing a resistant isolate of *M. haemolytica* differed significantly by state of origin in this study. A single *M. haemolytica* genetic subtype was associated with an over whelming majority of the observed resistance. *H. somni* isolation counts were low and statistical models would not converge. Phenotypic resistance was predicted with high sensitivity and specificity by WGS. Additional studies to elucidate the relationships between phenotypic expression of resistance/genetic determinants for resistance and clinical response to antimicrobials are necessary to inform judicious use of antimicrobials in the context of relieving animal disease and suffering.

1. Introduction

Antimicrobial administration has been the mainstay for both the treatment and control of bovine respiratory disease (BRD) for several decades. Considering the ability of bacteria to adapt and survive in changing environmental conditions, it comes as no surprise that the bacterial pathogens associated with BRD are now commonly found to be resistant to these antimicrobials (DeDonder and Apley, 2015).

One of the first reports on macrolide resistance genes found in BRD was an investigation of a strain of *Pasteurella multocida* (strain 36950) isolated from a calf in a Nebraska feedlot in 2005 (Kadlec et al., 2011). This particular strain had shown resistance to most antimicrobials approved for treatment of BRD and prior to their investigation, the mechanism of resistance had not been

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determined. Performing whole genome sequencing (WGS) and a series of cloning experiments they discovered three macrolide resistance genes, (the first of which was described in a nearly simultaneous publication (Desmolaize et al., 2011a)) *erm*(42), *msr*(E), and *mph*(E). The *erm*(42) gene codes for an rRNA methylase gene, the *msr*(E) gene codes for an ABC transporter protein, and the *mph*(E) gene codes for a macrolide phosphotransferase protein (Kadlec et al., 2011; Michael et al., 2012b). The *msr*(E) and *mph*(E) genes are organized in an operon-like structure and separated only by a non-coding spacer sequence of 55 base pairs.

A recent study investigated the effects of these three genes on the minimal inhibitory concentration (MIC) of gamithromycin and tildipirosin (Michael et al., 2012a). In both *P. multocida* and *Mannheimia haemolytica* they reported a pronounced increase in MIC to gamithromycin for those isolates containing *msr*(E)-*mph*(E) and a marked increase in MIC to tildipirosin for *erm*(42) containing isolates. The subset of isolates that harbored all three genes displayed increased MICs to both of the newer macrolides in their study. Similar work found the same to be true; greatly elevated MIC for tildipirosin and tilmicosin in the presence of *erm*(42) and greatly elevated MIC for tilmicosin, tulathromycin, and gamithromycin in the presence of *msr*(E)-*mph*(E) (Rose et al., 2012). These two studies remain the only publications to investigate the molecular effects of the macrolide resistance genes on BRD bacterial pathogen susceptibility to gamithromycin. However, neither investigated the effects of a macrolide antimicrobial administered for control of BRD on subsequent macrolide resistance.

Therefore, the objectives of this work were: first, to describe gamithromycin susceptibility of *M. haemolytica, P. multocida,* and *Histophilus somni* isolated from cattle diagnosed with and treated for BRD, which had previously either received treatment for control of BRD (MM) or sham-saline injection (CON), and second, to characterize the macrolide resistance genes present in the genetically typed *M. haemolytica* isolates. The final objective was to use whole-genome sequencing (WGS) to describe genetic determinants for macrolide resistance and correlate to the phenotypic resistance as determined by broth microdilution among the *M. haemolytica* isolates from this BRD clinical trial.

2. Materials and methods

2.1. Animals and treatment allocation

The randomized clinical trial yielding the isolates described in this manuscript was detailed previously in a pharmacokinetic/pharmacodynamic investigation which utilized the same isolates (DeDonder et al., 2015). One hundred eighty cattle perceived to be at a high risk for developing BRD (165–269 kg) were sourced from three states (Kentucky, Missouri, and Tennessee; 60 head per state) within the United States as part of another study. The cattle were randomly allocated to receive either treatment for control of BRD (mass medication = MM) with gamithromycin (6 mg/kg subcutaneously in the neck) or a sham injection of saline (control = CON) administered upon arrival to a research feedlot near Manhattan, Kansas. The treatments were assigned to cattle within state of source, yielding a total of six pens (30 head/pen) in the study. This study was approved by the Kansas State University Institutional Animal Care and Use Committee.

2.2. Clinical scoring and disease diagnosis

Cattle were observed for symptoms of BRD once daily in their pen for 28 days by a veterinarian masked to treatment allocation. The diagnosis of BRD was determined based on a calf having a rectal temperature of ≥40.0 °C (≥104.0 °F) and a clinical score of ≥1 (general signs of depression and weakness). Cattle diagnosed with BRD based on this clinical scoring system were included in this study and administered gamithromycin at the label dose after the collection of pre-treatment samples. Therefore, cattle enrolled (treated for BRD) in this study had been previously administered either MM (gamithromycin for control of BRD) or CON (sham control injection) on arrival to the feeding facility.

2.3. Sampling allocation and collection procedures

Nasopharyngeal swabs (NPS) were collected, bilaterally and pooled, immediately prior to treatment (0 h) from all cattle diagnosed with BRD. Additionally, cattle were allocated to having bronchoalveolar lavage (BAL) collection at either 0, 12, or 24 h post-treatment with gamithromycin. Cattle sampled by BAL at 12 and 24 h also were simultaneously sampled by NPS. All cattle diagnosed with BRD were sampled by means of both BAL and NPS 120 h after treatment administration.

2.4. Bacterial isolation and MIC determination

After collection, NPS and BAL samples were transported on ice and were plated directly onto trypticase soy + 5% blood, chocolate, and MacConkey agar plates. The plates were incubated in 5% CO_2 at 37° C for 18–24 h. Up to 12 colonies of *M. haemolytica* and up to 6 colonies of both *P. multocida* and *H. somni* displaying growth characteristics typical of each were further isolated in pure culture from each calf. Isolate identity was confirmed with MALDI-TOF (Bruker Daltonics, Billerica, MA, USA) and the selected isolates were frozen for subsequent susceptibility testing.

Gamithromycin susceptibility testing of all isolates was determined by a broth microdilution technique utilizing custom frozen panels (CML1FZAC plates (TREK Diagnostic Systems, Thermo-Fisher Scientific Inc., Waltham, MA, USA)), as per manufacturers' instructions, by personnel masked to treatment group according to CLSI recommended techniques (Clinical and Laboratory Standards Institute, 2013). The frozen panels contained gamithromycin at concentrations ranging from 0.03 to 16 mg/L. Prior to susceptibility testing, isolates were recovered from frozen stock by culturing on Chocolate II agar plates (Becton Dickinson, Co. Sparks, MD) and incubation with increased CO_2 at 37° C for 18–20 h. Suspensions of *M. haemolytica* and *P. multocida,* equivalent to 0.5 McFarland standard, were made in 5 mL of demineralized water. Mueller-Hinton broth tubes (11 mL) were then inoculated with 140 μL of the resulting bacterial suspensions. A 12-channel pipette was used to dispense 50 μL of this suspension into each of 12 wells in the plate, such that the susceptibility of 8 strains could be evaluated per plate. Plates were sealed with seal strips and incubated at 37° C without CO_2 for 18–20 h. *H. somni* isolates were tested as per CLSI guideline VET01-A4, section 12.2 (Clinical and Laboratory Standards Institute, 2013). In brief, *H. somni* cultures were cultivated on Chocolate II agar plates in 5% ± 2% CO_2, at 37 °C, for 24 h. Resulting colonies were picked to 5 mL Mueller-Hinton broth tubes (TREK Diagnostic Systems), to achieve an optical density equivalent to 0.5 McFarland standard. This suspension was used to inoculate 2X Veterinary Fastidious Medium (VFM; TREK Diagnostic Systems) at a ratio of 15 ul per 1 mL, and the resulting bacterial suspension was dispensed 50 ul per well. Plates were sealed prior to incubation in 5% CO_2 at 37 °C for 24 h. For all strains tested, growth was visually inspected and Gamithromycin MIC determined as the lowest concentration preventing visible growth.

Interpretation of susceptible, intermediate or resistant was determined using clinical breakpoints established by CLSI. Gamithromycin breakpoints have been reported for *M. haemolytica, P. multocida,* and *H. somni* at ≤4, 8, ≥16 mg/L for susceptible, intermediate, and resistant, respectively (Clinical and Laboratory