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Genetic characterization of susceptible and multi-drug resistant *Mannheimia haemolytica* isolated from high-risk stocker calves prior to and after antimicrobial metaphylaxis

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ABSTRACT

Bovine Respiratory Disease (BRD) is a major threat to animal health and welfare in the cattle industry. Strains of *Mannheimia haemolytica* (*Mh*) that are resistant to multiple classes of antimicrobials are becoming a major concern in the beef industry, as the frequency of isolation of these strains has been increasing. Mobile genetic elements, such as integrative conjugative elements (ICE), are frequently implicated in this rapid increase in multi-drug resistance. The objectives of the current study were to determine the genetic relationship between the isolates collected at arrival before metaphylaxis and at revaccination after metaphylaxis, to identify which resistance genes might be present in these isolates, and to determine if they were carried on an ICE. Twenty calves culture positive for *Mh* at arrival and revaccination were identified, and a total of 48 isolates with unique susceptibility profiles (26 from arrival, and 22 from revaccination) were submitted for whole-genome sequencing (WGS). A phylogenetic tree was constructed, showing the arrival isolates falling into four clades, and all revaccination isolates within one clade. All revaccination isolates, and one arrival isolate, were positive for the presence of an ICE. Three different ICEs with resistance gene modules were identified. The resistance genes *aphA1*, *strA*, *strB*, *sul2*, *floR*, *erm42*, *tetH/R*, *aadB*, *aadA25*, *blaOXA-2*, *msrE*, *mphE* were all located within an ICE. The gene *bla-ROB1* was also present in the isolates, but was not located within an ICE.

1. Introduction

Bovine respiratory disease (BRD) is the most common cause of morbidity and mortality in North American beef cattle. While BRD is a multifactorial disease syndrome, clinical signs of disease are often associated with colonization of the lower airway with specific bacterial pathogens. *Mannheimia haemolytica* (*Mh*) is one of the most important bacterial pathogens associated with the development of BRD, and is frequently isolated from the airway of feedlot and stocker cattle with disease (Fulton et al., 2009; Timsit et al., 2017). Recently, there have been increasing concerns regarding the presence of extensively drug resistant (XDR) strains of *Mh*, as many diagnostic labs have observed a decrease in the susceptibility of *Mh* to a growing number of antimicrobials (DeDonder and Apley, 2015; Sweeney et al., 2018). Isolates that are XDR are resistant to at least one drug in all but one or two classes of antimicrobials (Sweeney et al., 2018). This observation has been verified by the discovery of *Mh* isolates collected in the field, from both live and dead animals, that exhibit similar extensive resistance patterns (Crosby et al., 2018; Klima et al., 2014; Snyder et al., 2017; Woolums et al., 2018). The presence of *Mh* strains resistant to many of the drugs available for the management of BRD is concerning, as it limits the ability of producers and veterinarians to effectively treat and prevent disease in at-risk populations.

The ability of XDR to rapidly develop within a population of microbes with limited antimicrobial exposure suggests something more than simple selection pressure for singly resistant mutants. One such way that XDR can arise rapidly is via horizontal gene transfer (HGT), that is, the transfer of genes between microbes laterally, rather than inherited from the parent or arising spontaneously (Ochman et al., 2000). Bacteria are known to be able to acquire genes horizontally a number of ways, but conjugation is thought to be the mechanism most often responsible for the transfer of multiple resistance genes and virulence factors (Ochman et al., 2000). One type of mobile genetic element, the Integrative Conjugative Element (ICE), has been identified

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in many different microbes, including *Pasteurellaceae*, that can transfer a large number of closely linked resistance genes to another microbe in one transfer event (Beker et al., 2018; Bhatt et al., 2018; Eidam et al., 2015; Johnson and Grossman, 2015; Michael et al., 2012a). These ICEs are modular mobile genetic elements that can integrate into the host genome, but in contrast to plasmids, they also encode the machinery in their genes to initiate excision and transfer on their own, independent of their host (Johnson and Grossman, 2015). This makes them particularly troublesome, as those microbes that have an ICE carrying multiple resistance genes can be selected for with the use of only one antimicrobial, and can also go on to share that ICE with neighboring microbes.

Recent work from our lab has documented a rapid and dramatic increase in the isolation of XDR *Mh* isolates from several groups of stocker cattle after metaphylactic treatment with antimicrobials (Snyder et al., 2017). This sudden shift in susceptibility within the *Mh* population between arrival and revaccination 10–14 days later raised a number of questions. First, for calves *Mh* culture positive at both arrival and revaccination, was the *Mh* the same, or were the *Mh* populations genetically distinct between those two time points? Second, was the dramatic increase in resistance between the two collection time points due to a shift in the *Mh* population from susceptible to resistant strains, or had the calf's own strains suddenly acquired resistant genes, such as in the form of an ICE? Thus, the objectives of this study were to determine the genetic relationship between *Mh* isolates collected at arrival and those collected at revaccination, to determine which antimicrobial resistance genes were present in our samples, and to determine if resistance bearing ICEs were present in our isolates.

2. Materials and methods

2.1. Animals and sampling procedures

The isolates in this study were collected as part of a previous investigation into the prevalence of multi-drug antimicrobial resistant *Mh* in high risk stocker cattle (Snyder et al., 2017). A detailed description of animal selection and sampling is described in Snyder et al. Briefly, 169 bull and steer calves were enrolled in that study. These calves were castrated if necessary, and processed at arrival following an established protocol. Each calf also received a weight appropriate dose of the macrolide antimicrobial tulathromycin at this time (Draxxin, Zoetis Inc., Florham Park, NJ). A deep nasopharyngeal swab (NPS) was collected from each calf at arrival, and a second swab was collected 10 to 14 days later at revaccination, or at the time of treatment if a calf was treated for BRD prior to revaccination. All calves arriving in a given week were placed together in one pasture at the end of the week; calves arriving during different weeks did not have nose to nose contact. From these calves, a total of 365 *Mh* isolates were collected and stored as frozen stabilates. For this study, only *Mh* isolates collected from calves ($n = 20$) that were culture positive at both arrival and revaccination sampling or *Mh* isolates from these same calves that had unique antimicrobial susceptibility profiles were selected for analysis; that is, a calf could have 1 to 3 isolates from a given time point included if each isolate had a unique susceptibility profile. This yielded a total of 48 isolates; 26 isolates from arrival and 22 isolates from revaccination.

2.2. DNA extraction, sequencing, and assembly

DNA was extracted from 24-h cultures of the selected isolates grown on blood agar plates at 37 °C, using a commercially available kit (Ultraclean Micobial DNA Isolation Kit, Qiagen, Germantown, MD), and assessed for purity and concentration with a NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA); all samples had A260/A280 values > 1.80. Extracted DNA was submitted to the Georgia Genomics and Bioinformatics Core for sequencing. Sequence libraries were synthesized with a Kapa Hyper Prep Kit (Kapa Biosystems, Wilmington, MA) and sequencing was performed on an Illumina NextSeq 500 instrument (Illumina Inc., San Diego, CA). Sequence data was quality trimmed, assembled and annotated by the UGA Georgia Genomics and Bioinformatics Core. FastQC, version 0.11.5, was used to assess quality metrics for both raw and trimmed reads (Andrews, 2010). Quality trimming and filtering of reads was performed using Trimmomatic, version 0.36, with only paired-end reads ≥ 50 bases maintained for downstream assembly (Bolger et al., 2014). The average number of reads per sample for the raw and trimmed reads was 7.3 million and 6.5 million, respectively. The total number of trimmed, paired reads ranged from a low of 3.9 million (isolate UGA-R5-126-1) to a high of 9.3 million (isolate UGA-A6-146-1), with most reads ≥ 150 bp in length. Coverage ranged from 144x to 369x; average coverage was 253x.

De novo assembly of paired-end reads was performed using the SPAdes assembler, version 3.11.1, and assembly quality was assessed with QUAST, version 4.5 (Table S1) (Bankevich et al., 2012; Gurevich et al., 2013). Draft assemblies were queried using the NCBI BLAST+ algorithm (BLASTn) against GenBank bacterial accessions in order to identify those *Mh* isolates having the greatest homology (Altschul et al., 1990). Cohorts of assemblies with homology to specific *Mh* accessions were then aligned individually against those reference sequences using MAUVE, version 2.4.0, for the purpose of ordering the scaffolds into a more biologically relevant orientation prior to annotation (Darling et al., 2004). The three *Mh* strains that the assemblies aligned most closely to were D171 (CP006573.1), 89010807N (CP011098.1), and USDA-ARS-USMARC-185 (CP004753.2). Annotation of the ordered assemblies was performed using RASTtk version 1.3.0 (Brettin et al., 2015). All sequences from our study can be found under the BioProject PRJNA517942.

2.3. Construction of phylogenetic tree

Isolate assemblies were uploaded to the CSI Phylogeny web interface to produce an alignment, along with a number of *Mh* strains from NCBI for comparison (CP006573.1, CP011098.1, CP004753.2 CP005972.1); the *Mh* strain M42548 (CP005383.1) was selected as the reference sequence and included in the tree (Kaas et al., 2014). For assembled genomes, the CSI Phylogeny algorithm uses NUCmer to produce an alignment, and only uses those regions of the genome shared by all submitted sequences to build it; thus, mobile genetic elements and insertions not shared by all sequences are excluded (Delcher et al., 2002). The resulting alignment file was used to create a maximum likelihood tree using RAxML version 8.2.11 in Geneious v11.0.4, using a GTR GAMMA nucleotide model and running 1000 bootstrap replicates (Kearse et al., 2012; Stamatakis, 2014). The resulting tree was uploaded into FigTree version 1.4.3 for further refinement (Rambaut, 2016).

2.4. In silico identification of resistance genes

A list of resistance genes and their sequences was obtained from the Comprehensive Antibiotic Resistance Database (CARD, accessed July, 2017) and from the Microbial Ecology Group Antibiotic Resistance Database (MEGARes, accessed July 2017) (Jia et al., 2017; Lakin et al., 2017). Additional resistance associated genes not present in the databases at the time that the initial search was done were identified and downloaded from GenBank. These sequences included the multi-copper oxidase gene *mco* from *Pasteurella multocida (Pm)* 3361 (CP026861.1) and the macrolide efflux gene *msrE* from the same organism. All of these genes were queried against all assemblies using BLASTn (Altschul et al., 1990). Resistance gene hits identified in the assemblies were assessed for length, the presence of a start and stop codon, and errors due to misidentification in the databases to eliminate any false positives. Each identified resistance gene was additionally queried with BLASTn against a known susceptible reference gene in order to identify any point mutations that might result in gene inactivation or premature termination (Table S2). The genes *gyrA* and *parC* from each assembly were additionally queried against these respective genes from the *Mh* isolate, M42548 (CP005383.1), which is known to be susceptible to fluoroquinolones, to identify any point mutations in these genes known to be associated with fluoroquinolone resistance. Specifically, each isolate was searched for the mutations S83F, S83Y, A84P, D87G, or D87N in *gyrA*, and S80I, S80L, E84K in *parC* (Katsuda et al., 2009; Kong et al., 2014; Ozawa et al., 2009).

2.5. In silico identification of integrative and conjugative elements (ICEs)

The ICE associated genes *int1*, *int2*, *parB* and *ICErel1* from *Pm* 3358 (CP029712.1) and *ICErel2* from *Mh* M42548 (CP005383.1) were queried using BLASTn against every assembly. Positive identification of any ICE associated genes was only considered if the gene had ≥95% identity and the full length of the gene was present (Altschul et al., 1990). Positive identification of *parB* was only considered relevant if it was in close proximity to other ICE associated genes or resistance genes within each assembly. Those assemblies with similar resistance gene and ICE associated gene patterns were grouped together for ICE assembly, as it was thought that they were likely to have more similar ICEs. To construct the first ICE, a full assembly from one isolate was mapped to the reference *ICEPMu1* from *Pm* 36950 (CP003022.1) using Geneious v11.0.4; all those contigs that mapped to this reference ICE were identified, along with any additional contigs containing ICE associated genes and resistance genes previously identified by BLASTn (Kearse et al., 2012). These contigs were extracted, and a *de novo* assembly of the ICE was made using the Geneious Assembler. These newly generated larger contigs were ordered with reference to other known ICEs, specifically those of ICE*Pmu1* and ICE*Mh1* (from *Mh* M42548), and the sequences concatenated into one complete ICE sequence. This sequence was subsequently used to identify other likely ICE containing contigs in all isolates within each assembly group, and a *de novo* assembly was done to construct a consensus ICE for each group. Each fully assembled ICE was annotated using a custom database of genomes known to contain ICEs, also in Geneious.

3. Results

3.1. Phylogeny

The result of the phylogenetic analysis of the isolates is presented in Fig. 1. With strain M42548 (2.7 Mbp) as the reference for the dendrogram, 75.5% of the reference genome was shared by all isolates; individually, isolates shared 84–100% of positions compared to the reference genome. The isolates fell into a total of 4 clades, with all isolates from revaccination falling into one clade, and arrival isolates falling into four clades. One arrival isolate was grouped with the revaccination isolates; all other arrival isolates were in separate clades distinct from the revaccination isolates. Boostrap support (Figure S1) was 100% for all major branches to each of the clades; within the largest arrival clade, bootstrap support for smaller branches ranged from 47 to 100% (Fig. S1, blue clade), whereas in the predominately revaccination clade, bootstrap support ranged from 3 to 100% (Fig. S1, red clade). A Single Nucelotide Polymorphism (SNP) matrix also produced by CSI Phylogeny showed that the large clade of arrival isolates had no more than 351 SNPs of difference between any two study isolates, with most falling below 170 SNPs of difference (Fig. S2). The revaccination clade (excluding the arrival isolate UGA-A5-128-1) had no more than 15 SNPs of difference between any two study isolates. Comparing all arrival isolates to revaccination isolates, SNPs of difference between any two isolates ranged from a low of 147 (UGA-A5-128-1) to a high of 6834. Bootstrap support is an indicator of confidence in the position of a particular branch or clade within a tree, so when low, it can indicate more uncertainty for that branch being in that location. When isolates are very closely related, as in this instance, there can be more uncertainty in the location of that branch relative to another closely related branch because there are not enough differences between the isolates to separate them into distinct groups. In this instance, the low bootstrap support is an additional indicator of clonality within the revaccination clade.

3.2. Identification of resistance genes

There were a total of 13 resistance gene/gene complexes identified in the isolates (Table 1). These include the *tetH/tetR* complex associated with tetracycline resistance, as well as genes associated with resistance to the phenicols, aminoglycosides, macrolides, sulfonamides, and β-lactams. In addition, it was found that many of these genes, specifically *aphA1*, *strB*, *strA*, *sul2*, *floR*, and *erm42* were commonly located in one contig, and often times very close together. This was also observed with the genes *aadB*, *aadA25*, *bla-OXA2*, *msrE*, and *mphE*. There were also point mutations identified in genes associated with fluoroquinolone resistance (Tables 1 and 2); specifically S83F in *gyrA*, D87N in *gyrA*, and E84K in *parC*. The S83F mutation has been previously identified in fluoroquinolone resistant strains of *Mh*, whereas the D87N and E84K mutations have been found in *Pasteurella multocida* (*Pm)* (Table 2) (Katsuda et al., 2009; Kong et al., 2014; Ozawa et al., 2009). No mobile quinolone resistance genes were detected in any isolates. Of the arrival isolates, only two were positive for any resistance genes; one was positive for *bla-ROB1*, and one was positive for *tetH/tetR*. All 22 revaccination isolates were positive for all identified tetracycline, sulfonamide, aminoglycoside, and macrolide resistance genes, as well as fluoroquinolone point mutations. All 22 were also positive for the β-lactam resistance gene *bla-OXA2*, and 18 were positive for *bla-ROB1*. Eight of the revaccination isolates were also positive for the phenicol resistance gene *floR*.

3.3. Identification of putative ICEs

ICE associated genes present in the isolates are presented in Table 3. One arrival isolate (UGA-A5-128-1) was positive for the ICE-associated genes *ICErel1*, *int1*, *int2*, and *parB*; no other arrival isolates were positive for any ICE-associated genes. All revaccination isolates were positive for *ICErel1*, *ICErel2*, *parB*, and two copies of *int2*. Gene *int1* was not present in any revaccination isolates. From all of those isolates positive for ICE-associated genes, a total of 3 putative consensus ICEs were assembled; ICE*Mh*-UGA1, ICE*Mh*-UGA2, and ICE*Mh*-UGA3 (Fig. 2). A total of eight revaccination isolates were positive for ICE*Mh*-UGA1, while 14 were positive for ICE*Mh*-UGA2. One arrival isolate was positive for ICE*Mh*-UGA3.

ICE*Mh*-UGA1 was approximately 105,588 bp in length, while ICE*Mh*-UGA2 was 101,244 bp, due to the absence of a 4.3 kbp region containing *floR*. These ICEs otherwise shared a high degree of homology. Within both of these ICEs, there were two separate regions containing resistance genes. The first region contained the genes *aphA1*, *strB*, *strA*, *sul2*, *erm42*, and when present, *floR*. The second resistance region contained the genes *aadB*, *aadA25*, *bla-OXA2*, *msrE*, *mphE*, *tetR* and *tetH*. These two resistance gene clusters were separated by a number of genes involved in type 4 secretion systems (T4SS), specifically the genes *traC*, *traD*, *traG*, *topB* and *pilL*, as well as a number of other uncharacterized genes. The multicopper oxidase gene *mco* was also identified within this region. The ICE*Mh*-UGA3 identified in one arrival isolate was substantially smaller (59,848 bp) than those identified in the revaccination isolates, and only carried the resistance genes *tetR* and *tetH*, as well as *mco*.

3.4. Bla-ROB1 and putative plasmid

The β-lactam resistance gene *bla-ROB1* was not located within the ICE of any isolates positive for the presence of that gene. The gene was

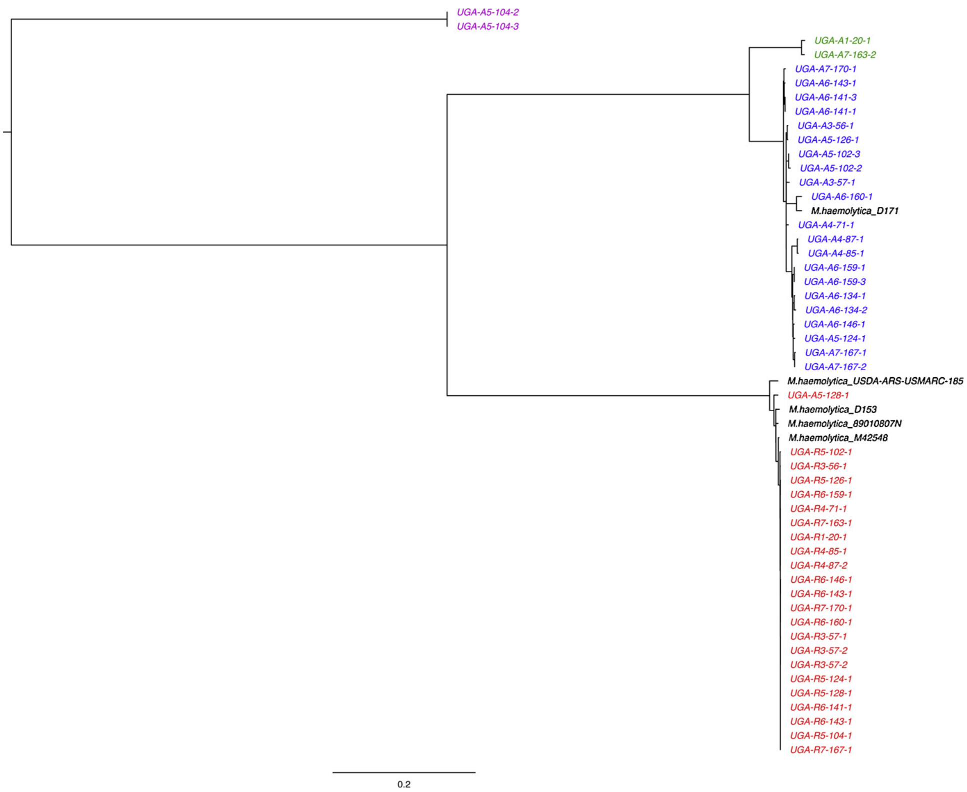

Fig. 1. Phylogenetic tree of matched isolates collected from stocker cattle at arrival and revaccination. Isolate M42548 was used as the reference for construction of the tree. Clades are grouped by color. Other isolates in black are included to illustrate genetic relationships to other *Mh* sequences found on NCBI.

Table 1
Genes identified by BLAST of whole genome sequences of 48 *Mh* isolates against antimicrobial resistance gene databases from CARD and MEGARes, with total number of isolates at each time point positive for a given resistance gene.

		Time Point (No. isolates)	
Antimicrobial Class	Gene	Arrival (26)	Revaccination (22)
Phenicols	*floR*	0	8
Tetracyclines	*tetH*	1	22
	tetR	1	22
Sulfonamides	*sul2*	0	22
Aminoglycosides	*strB (aph6)*	0	22
	strA (aph3")	0	22
	aphA1 (aph3')	0	22
	aadA25 (ant3")	0	22
	aadB (ant2")	0	22
Beta - Lactams	*bla OXA-2*	0	22
	bla ROB-1	1	18
Macrolides	*erm42*	0	22
	msrE	0	22
	mphE	0	22
Fluoroquinolones	*gyrA* S83F†	0	22
	gyrA D87N†	0	22
	parC S80I/L†	0	0
	parC E84K†	0	22
Metal Tolerance	*mco*	1	22

† Amino acid abbreviations: S = Serine, F = Phenylalanine, D = Aspartic Acid, N = Asparagine, E = Glutamic Acid, K = Lysine.

found to be located within a 5377 bp contig in all revaccination isolates positive for the gene. Examination of the annotations of all contigs carrying this gene revealed the presence of the genes *mobA*, *mobB*, and *mobC* (Francia et al., 2004). These genes are known to be associated with HGT in plasmids. Furthermore, these contigs bear a high degree of similarity to the *Mh* strain 48 plasmid pKKM48 (MH316128.1). However, the putative plasmid identified here additionally carries an alpha/beta hydrolase gene that is not present in plasmid pKKM48 (MH316128.1). There was additionally one ICE negative arrival isolate positive for the *bla-ROB1* gene on a smaller, 2386 bp contig, however it was negative for all other plasmid associated genes present in the larger 5377 bp putative plasmid.

4. Discussion

The current cattle marketing system puts cattle through a series of events that increase the risk of developing BRD. To address this risk and to initiate therapy in animals that may not yet be showing signs of disease, the practices of metaphylaxis and of administering antimicrobials in feed have been approved and implemented for use in these high risk animals. Metaphylaxis has been shown to result in reductions in calf morbidity and mortality, but concerns regarding its contributions to antimicrobial resistance are leading some to question its use (Nickell and White, 2010; Snyder et al., 2017; Timsit et al., 2017). Indeed, based on diagnostic lab reports, increases in antimicrobial resistance in pathogens involved in BRD, specifically *Mh*, are becoming more commonplace; and multi-drug resistance appears to be on the rise as well (DeDonder and Apley, 2015; Sweeney et al., 2018).

How might resistance to multiple antimicrobials occur suddenly after exposure to only one drug? One such way this may occur is via horizontal gene transfer (HGT). This can occur any number of ways, such as the acquisition of genes in the environment via transformation, the transfer of genetic material by a phage via transduction, or via the acquisition of a mobile genetic element in the form of a plasmid or ICE (Johnson and Grossman, 2015; Ochman et al., 2000). However, the following discussion will focus on the acquisition of resistance genes due to ICEs, since this seems to be the primary method that *Pm* and *Mh* use to acquire a large number of resistance genes in one event (Beker et al., 2018).

As previously mentioned, ICEs are mobile genetic elements that integrate into the host chromosome, and can be propagated and passed

Table 2
Point mutations present in gyrA and parC of 48 susceptible or resistant strains of *Mh*.

Gene	Susceptible	Resistant	
gyrA	S83[†]	S83F[†]	Associated with resistance in *Mh* (Katsuda et al., 2009; Ozawa et al., 2009)
	D87[†]	D87N[†]	Associated with resistance in *Pm* (Kong et al., 2014)
parC	S80[†]	S80[†]	
	E84[†]	E84K[†]	Associated with resistance in *Pm* (Kong et al., 2014)

[†] Amino acid abbreviations: S = Serine, F = Phenylalanine, D = Aspartic Acid, N = Asparagine, E = Glutamic Acid, K = Lysine.

Table 3
ICE associated genes identified by BLAST of whole genome sequences of 48 *Mh* isolates with total number of isolates at each time point positive for a given ICE associated gene.

		Time Point (No. isolates)	
Gene Class	Gene	Arrival (26)	Revaccination (22)
Relaxases	*ICErel1*	1	22
	ICErel2	0	22
Integrases	*int1*	1	0
	int2	1	22
Partitioning Protein[†]	*parB*	1	22

[†] In proximity to ICE associated contigs/resistance gene clusters.

on to progeny, but can also excise themselves, replicate as a circular intermediate, and transfer to other neighboring cells via a T4SS (Johnson and Grossman, 2015). Frequently, other genes are carried along within the ICE that are not essential to ICE function (Hall et al., 2017). If these genes happen to code for antimicrobial resistance, a large number of resistance genes can be transferred all at once to new cells, and not necessarily cells of the same species (Klima et al., 2014; Michael et al., 2012b; Ochman et al., 2000).

To date, a number of different ICEs have been documented in *Mh* and other *Pasteurellaceae* associated with BRD. In 2011, Michael et al. were the first to document the presence of an ICE in *Pasteurella multocida* (ICE*PMu1*) (Michael et al., 2012a). The isolate was resistant to tetracyclines, florfenicol, sulfonamides, spectinomycin, enrofloxacin, tilmicosin and tulathromycin. Whole genome sequencing (WGS) of this isolate revealed an 82 kbp ICE with two regions containing resistance genes. The first region contained the resistance genes *aphA1, strB, strA, sul2, floR* and *erm42*. The second region contained the resistance genes *tetH, aadA25, blaOXA-2, msrE* and *mphE*, and the regulator *tetR*. In *Mh*, the first documented ICE was ICE*Mh*1, discovered in *Mh* strain 42,548 in 2007 (Eidam et al., 2015). Although it was longer in length than the ICE*PMu*1, being about 92 kbp, it had fewer resistance genes present in its genome, lacking *floR*, *erm42*, *aadB*, *aadA15*, *blaOXA-2*, *msrE* and *mphE* (Eidam et al., 2015). Both ICEs insert at a tRNA-Leu, but have differences in their flanking repeats (Eidam et al., 2013; Michael et al., 2012b).

The ICEs identified in the isolates collected at revaccination in the current study bore the greatest resemblance to the ICE*Pmu*1 for about the last ¾ of the sequence, while the front ¼ was most similar to the ICE*Mh*1. The two resistance gene regions identified in ICE*Mh*-UGA1 shared all of the same resistance genes as ICE*Pmu*1, while ICE*Mh*-UGA2 lacked *floR*. The lengths of the regions were different, however, with the first being about 13.5 kbp for ICE*Mh*-UGA1, and ICE*Mh*-UGA2 being about 4.3 kbp shorter, due to the lack of the *floR* region. The second resistance gene region was longer in our sequences, being about 12.3 kbp compared to the 9.8 kbp region of ICE*Pmu*1. These differences in the resistance gene regions, along with those in the inter-resistance-module regions, could indicate recombination, but may just indicate more diversity within this ICE family. Additionally, the left flanking sequence in both ICE*Mh*-UGA1 and ICE*Mh*-UGA2 are the same as that in the same flanking sequence of ICE*Mh*1, but are in reverse. The consequence of this is not clear, but could suggest transposition of this insertion site. The summary of the total differences between all of these ICEs only adds to the known diversity of this type of mobile genetic element within this family of bacteria.

4.1. Other genes of interest

The annotation of the ICEs additionally identified a number of other genes that may play important roles in the lifecycle of the element. All three of the ICEs we identified were positive for the multi-copper

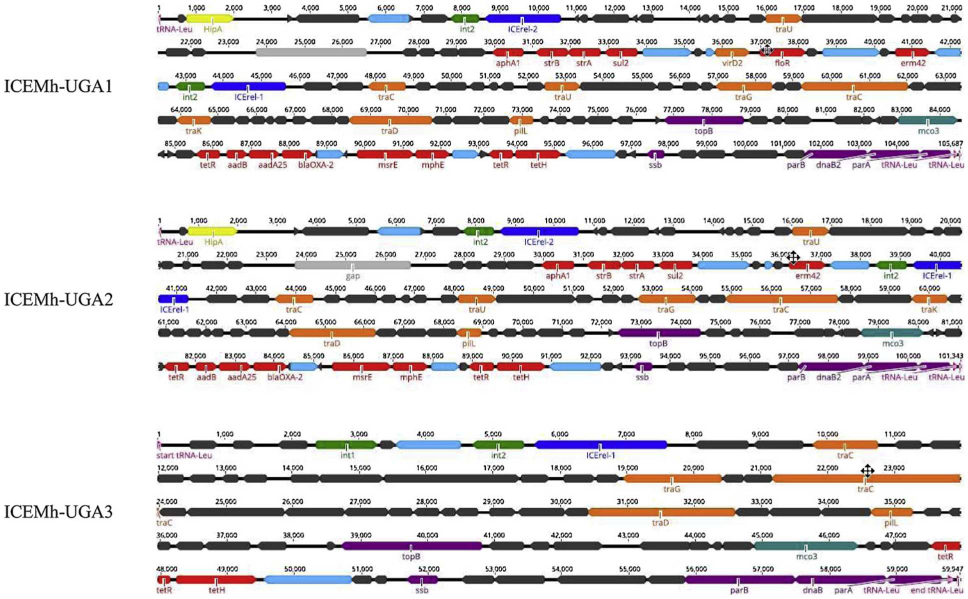

Fig. 2. Schematic representation of ICEs. Arrows represent open reading frames (ORFs). Genes are color coded as follows; tRNA-Leu (pink), toxin-antitoxin system (yellow), transposase (light blue), integrase (green), relaxase (blue), T4SS (orange), antimicrobial resistance (red), metal-resistance (teal), replication/repair (purple), other genes (dark grey), gaps (light grey). There is a disrupted tRNA-Leu at the end of each ICE which is the site of insertion into the bacterial chromosome. The disrupted tRNA-Leu is replaced by a complete tRNA-Leu at the end of the ICE. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

oxidase gene *mco* type 3. This gene, or forms of it, have been identified in numerous other *Pasteurellaceae*, including *Mh*, *Pm*, and *Histophilus somni* (*Hs*), and is responsible for copper detoxification (Bhatt et al., 2018; Eidam et al., 2015; Klima et al., 2016; Michael et al., 2012b). Feeding levels of copper above the recommended National Research Council (NRC) values is a common practice in feedlot and stocker operations, and, because incoming mineral status is unknown, cattle are frequently injected with multi-mineral supplements at arrival, which occurred with the cattle in this study (Cameron and McAllister, 2016; Snyder et al., 2017; Vasconcelos and Galyean, 2007). It is known that elevated levels of copper can have an antimicrobial effect, which is why they may be fed at levels greater than physiologically required (Cameron and McAllister, 2016). However, bacteria can develop metal tolerance by acquiring genes that code for metal tolerance, such as *mco*, that can help them to ameliorate the toxic effects of these metals (Cameron and McAllister, 2016). Frequently in *Pasteurellace*, these metal tolerance genes are carried on ICEs that also harbor antimicrobial resistance genes (Bhatt et al., 2018; Eidam et al., 2015; Klima et al., 2016; Michael et al., 2012b). As a result, increasing levels of copper that animals and their microbes are exposed to can co-select for antimicrobial resistance, and may be an additional method that bacteria use to maintain ICEs within the bacterial population.

The presence of a *hipA*-like module within ICE*Mh*-UGA1 AND ICE*Mh*-UGA2 may also help explain how these elements are conserved in the population when antimicrobial selection pressure is absent. This gene module codes for a toxin-antitoxin system that has been shown to promote maintenance of ICEs within the bacterial population (Carraro et al., 2015). The mechanism of action of HipA for promotion of ICE maintenance is not fully known, but in other ICE associated toxin-antitoxin systems, such as that coded by the module *mosAT*, the secreted toxin is able to inhibit the growth of neighboring microbes not carrying an antitoxin, while those cells with an antitoxin gene within their ICE are spared (Wozniak and Waldor, 2009). Additionally, the *hipA* module has been shown to play a major role in persister cell formation, allowing those with this module to evade antimicrobial killing by entering a dormant state and by forming biofilms (Schumacher et al., 2009). Thus, it is possible that the ICEs identified in this study may carry genes which exert a self-selective pressure in their offspring that additionally offer another mechanism of antimicrobial resistance and environmental persistence.

4.2. Phylogeny

The phylogenetic analysis of the isolates collected in this study revealed a number of unexpected and surprising results. The change in diversity between isolates collected at arrival and revaccination was dramatic, as the arrival isolates were distributed between 4 different clades, and the revaccination isolates were all grouped together in one. It is not clear what caused this change in *Mh* diversity, but two possible explanations come to mind. One explanation might be that this change could be something that is a natural occurrence in calves after arrival to a stocker facility, perhaps due to changes in calf physiology or management that happen around the time of revaccination. Another explanation might be that the use of metaphylactic drugs might eliminate all isolates except those that carry resistance genes, leaving only multidrug resistant strains to become the predominant ones present in the nasopharynx. Nevertheless, whether the change in *Mh* isolate diversity in the present study is a normal occurrence or a sequelae to metaphylaxis cannot be definitively determined, as there were no isolates from calves that had not received antimicrobials.

The data in the tree does present evidence of another surprising result; the contagious spread of *Mh* between calves. At revaccination, all of the isolates were so genetically similar that there were no more than 15 SNPs of difference between any two isolates. It is also important to remember that mobile genetic elements not shared by all isolates were excluded in the production of the alignment, so any transposons, ICEs or plasmids would not have any influence on the organization of the tree because not all isolate genomes contained these sequences. What this means is that the genetic similarity of the sequences at revaccination was not biased by the presence of the ICEs or plasmid, and that the high genetic similarity of the sequences was not artificially influenced by the sudden acquisition of the same mobile genetic element in an unrelated strain.

It has long been presumed that cattle developing BRD tend to become sick from their own native *Mh* strains (Purdy et al., 1993; Timsit et al., 2013). Previous work by others has demonstrated that the diversity of *Mh*, as evaluated by PFGE, within a pen of cattle remained high during BRD outbreaks, and that there was a high level of agreement between isolates collected from a single calf's NPS and transtracheal aspirate, which would indicate that calves are becoming ill from their own resident *Mh*, rather than acquiring a virulent strain contagiously from their penmates (Timsit et al., 2013).

Still, there is evidence for the contagious spread of BRD, and even the contagious spread of *Mh*. Work investigating animal-to-animal and community contact patterns within the first 28 days of feedlot arrival demonstrated that increased contact with cattle shedding BRD pathogens increased the risk of developing BRD (Shane et al., 2018). Furthermore, in the previously referenced 2013 study by Timsit, some of the bulls from different pens within the study acquired isolates with identical PFGE types after only having had contact with one another at arrival, suggesting that they were able to exchange *Mh* strains within a relatively short period of time (Timsit et al., 2013).

Another issue to consider is the discriminatory ability of PFGE to accurately classify isolates. While long considered the gold standard for characterizing an outbreak prior to the establishment of WGS, more recently questions have been raised regarding the accuracy of PFGE as WGS has become more widely implemented. In fact, in hospital outbreaks of *Acinetobacter baumanii, Clostridium difficile,* and vancomycin resistant *Enterococci*, WGS has been shown to have superior discernment and more accurately classify strains when compared to PFGE (Dominguez et al., 2016; Fitzpatrick et al., 2016; Lytsy et al., 2017). Considering the superiority of WGS in characterizing outbreaks in all of these species, relationships inferred in *Mh* based on PFGE may require re-evaluation using WGS as well.

Whether or not *Mh* is spread contagiously may depend on the management decisions that are made when cattle arrive at the stocker or feedlot facility, and the history of those animals prior to entry. Factors such as antimicrobial treatment and stress that decrease respiratory tract microbial diversity could predispose cattle to more easily acquire foreign bacteria, as could the frequency of contact between animals and potential environmental sources serving as fomites (Shane et al., 2018; Stroebel et al., 2018). Furthermore, the selective advantage of strains carrying resistance genes in the face of antimicrobial usage may allow those strains to dominate the nasopharynx and spread more easily into other niches depleted of microbial competition.

Our study suggests a potential environmental exposure acting as the point of infection for some of these calves, as calves arriving on different weeks and placed in separate pens without fenceline contact were nevertheless positive for the same *Mh* clone at revaccination. This may indicate that the point of exposure could be the holding pen that all cattle were placed in following processing before being sent to their home pen at the end of the week. Evidence for environmental survival of *Mh* does exist; a study published in 1997 demonstrated that *Mh* can survive in water at room temperature for up to 3 days, and up to 7 days at 4 °C (Burriel, 1997). It could be that sources such as water tanks, feed bunks, or congregation areas within the pen are facilitating the spread of the single *Mh* clone that was observed in our study. Additional research is needed to determine the impact of the environment's role in the spread of *Mh* between cattle in the feedlot and stocker setting.

5. Conclusion

The role of ICEs in multi-drug antimicrobial resistance is an emerging issue in the beef cattle industry. These elements may prove to be one of the greater challenges the industry faces, as they code not only for resistance to numerous drugs, but metal resistance, and may carry genes that allow them to self-select and better persist in the environment. Additionally, this paper presents evidence for the contagious spread of *Mh*, even between groups of cattle without contact. This means that we as an industry may need to readdress how we handle cattle at receiving and sick cattle that have been treated, as well as investigating the role of the environment in the spread of *Mh*. More research is needed to investigate the role that ICEs may have in the persistence of multi-drug resistant *Mh* in cattle and the environment.

Authors' contribution

Sample culture and DNA extraction was performed by ES. Dendrogram construction was performed by ES, with the assistance of SA. ICE assembly, annotation, and illustration was performed by ES. BC assisted with data analysis and manuscript creation

Declaration of Competing Interest

The authors do not have any conflicts of interest that might influence the current work.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.06.012.

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