Characterization of *Klebsiella* isolates obtained from clinical mastitis cases in dairy cattle

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ABSTRACT

Klebsiella spp. are important opportunistic pathogens commonly defined as environmental clinical mastitis agents. Despite *Klebsiella* mastitis being clinically impairing in cows and costly to the industry, only a few studies describe *Klebsiella* isolated from mastitis cases. The aim of this work was to characterize species of *Klebsiella* involved in clinical mastitis cases in Canada. *Klebsiella* isolated from clinical mastitis cases ($n = 53$) were identified to the species level using a biochemical test panel and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The *rpoB* gene sequence was used as the gold standard method and identified *Klebsiella pneumoniae* ($n = 40$), *Klebsiella oxytoca* ($n = 9$), *Raoultella ornithinolytica* ($n = 2$), and *Raoultella planticola* ($n = 2$). *Raoultella*, a genus closely related to *Klebsiella*, was also accurately identified using mass spectrometry but not via biochemical testing. Using the disc diffusion technique, 31 (58%) isolates were found to be susceptible to all antimicrobials tested ($n = 18$). The remaining 22 (42%) isolates were resistant to 1 or more of the following antimicrobials: kanamycin (2%), streptomycin (38%), spectinomycin (13%), sulfisoxazole (13%), and tetracycline (19%). The following antimicrobial resistance genes were identified: *tetA*, *tetB*, *sul1*, *strA/strB*, and *aadA*. Random amplified polymorphic DNA revealed the majority of our isolates as unrelated and having different patterns, indicating environmental contamination as the primary source of infection. All isolates were shown to be biofilm producers. In conclusion, although antimicrobial resistance was low for both *Klebsiella* and *Raoultella* species, genetically related *Klebsiella* spp. isolates appeared to be more resistant.

Key words: cattle, clinical mastitis, *Klebsiella*, *Raoultella*

INTRODUCTION

Klebsiella and *Raoultella* belong to the *Enterobacteriaceae* family. They are gram-negative bacteria found in the rumen, in bovine fecal material, and in the dairy environment (Munoz et al., 2007; Zadoks et al., 2011a). *Klebsiella* is an opportunistic pathogen causing environmental clinical mastitis (Schukken et al., 2012). It is isolated in 2 to 9% of milk samples from clinical mastitis cases (Olde Riekerink et al., 2008; Oliveira et al., 2013; Levison et al., 2016). Infections of the mammary gland by *Klebsiella* spp. are characterized by severe clinical signs and poor bacteriological cure rates following administration of antimicrobials (Schukken et al., 2012; Oliveira et al., 2013; Fuenzalida and Ruegg, 2019). *Klebsiella* infection is also associated with a major decrease in milk production (Gröhn et al., 2004) and thus leads to important economic losses. Affected animals are frequently removed from the herd (Oliveira et al., 2013). The reported pathogenicity in the udder is not well understood, but some virulence determinants have been shown to play a role: K1, K2, *magA* (mucoviscosity-associated gene, specific to K1 capsule serotype), *uge* (uridine diphosphate galactose 4 epimerase encoding gene, responsible for capsule and smooth lipopolysaccharide synthesis), *kfu* (iron uptake system gene), and the *rmpA* gene (regulator of the mucoid phenotype; Osman et al., 2014). Because *Raoultella* is a new genus that was previously classified as *Klebsiella* (Drancourt et al., 2001), no data is presently available on this bacterium for mastitis in dairy cattle.

In diagnostic laboratories, biochemical tests are frequently used for identification of *Klebsiella*, and identification beyond the genus level is not often attempted. Molecular techniques such as *rpoB* sequencing (β subunit of RNA polymerase; Diancourt et al., 2005) would allow for species identification and are commonly

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used in research (Brisse and Duijkeren, 2005; Munoz et al., 2007; Zadoks et al., 2011a). These techniques, however, are time-consuming, expensive, and difficult to implement in a routine diagnostic laboratory. However, MALDI-TOF mass spectrometry offers rapid, precise, and economical routine identification to the species level and is gaining in popularity in veterinary diagnostic laboratories. Moreover, guidelines for this technique are now available from the Clinical and Laboratory Standards Institute (CLSI, 2017). The most important *Klebsiella* species isolated from the milk of cows with clinical mastitis is *Klebsiella pneumoniae*, followed by *Klebsiella oxytoca* (Munoz et al., 2007; Zadoks et al., 2011a,b). One case of *Klebsiella variicola* has also been reported (Podder et al., 2014).

Klebsiella pneumoniae can also cause many nosocomial infections in humans. It belongs to a group of bacteria frequently associated with antimicrobial resistance (**AMR**) known as ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.; Rice, 2010). One of the proposed mechanisms for the persistence of *K. pneumoniae* in human infections is the formation of biofilms, which consist of bacteria embedded in a self-produced polysaccharide matrix (Chung, 2016). The ability to form biofilms has been correlated with AMR (Vuotto et al., 2014). In a study by Schönborn et al. (2017), 84% of *Klebsiella* spp. isolates were shown to produce visible quantities of biofilm in a tube method. To our knowledge, this is the only study describing biofilm formation for this pathogen in isolates recovered from bovine mastitis cases. In *Klebsiella* isolated from bovine milk, AMR is reported as uncommon (Saini et al., 2012b; de Jong et al., 2018), and, when it was present, the genes responsible for the observed resistances were not described. Molecular analysis, as by random amplified polymorphic DNA (**RAPD**), can assess relatedness of isolates and thereby identify sources and transmission routes for *Klebsiella*. This analysis has been used to help distinguish between contagious transmission and opportunistic infection from the environment (Munoz et al., 2007; Paulin-Curlee et al., 2008; Podder et al., 2014). The aim of this study was to characterize *Klebsiella* in clinical mastitis in Canada in terms of microbiological identification and discrimination, AMR, biofilm production, and genetic diversity.

MATERIALS AND METHODS

Culture Collection

Isolates were obtained from the Mastitis Pathogen Culture Collection (**MPCC**) of the Canadian Bovine

Mastitis and Milk Quality Research Network. This collection was assembled during a 2-year cohort study conducted on 91 Canadian dairies. Herd selection, sampling, and bacterial culturing were previously described by Reyher et al. (2011), and the MPCC itself is described by Dufour et al. (2019). Briefly, during that study quarter-milk sample series were collected from apparently normal milking cows and at diagnosis of clinical mastitis. Routine bacteriological milk culture was performed, and *Klebsiella* spp. were identified based on the following criteria: gram-negative rods, lactose fermentation on MacConkey agar, negative oxidase testing, non-motility, production of urease, utilization of citrate, and usually indole negativity. All *Klebsiella* spp. isolates recovered in concentration >1 cfu/0.01 mL and from a plate that was not considered contaminated (i.e., ≥ 3 phenotypically different types of colonies on the plate) were sent for conservation in the MPCC to the Faculté de médecine vétérinaire of the Université de Montréal. The MPCC contained 194 presumed *Klebsiella* spp. isolates identified via traditional biochemical methods, and, of these, 139 had some AMR data (Saini et al., 2012b). We selected isolates retrieved from clinical mastitis cases only, and only 1 isolate was included per quarter for each cow presenting clinical mastitis, yielding a total of 53 presumed *Klebsiella* spp. Verification in our database revealed that isolates came from 21 farms. Frozen isolates were thawed at room temperature, cultured on Columbia agar with 5% sheep blood (Oxoid, Nepean, Canada), and incubated overnight at 35°C. A single colony was subcultured on fresh Columbia agar with 5% sheep blood and incubated overnight at 35°C.

Species Identification

Biochemical identification for each isolate was performed using API-20E test strips (Biomérieux, Marcy L'Etoile, France) according to the manufacturer's recommendations. Isolates were also identified via MALDI-TOF using a Microflex LT instrument (Bruker Daltonics, Bremen, Germany). Each isolate was applied on a polished steel MSP 96 target (Bruker Daltonics), extracted with 1 μ L of 70% formic acid, and overlaid with 1 μ L of HCCA matrix before automatic spectra acquisition was launched, using default settings. The instrument was calibrated with a bacterial test standard, and identification to the species level was deemed valid when a score ≥ 2.0 was obtained. Spectra analyses were conducted using the manufacturer reference database, containing, at the time, 6,903 different spectra. Sequencing of the *rpoB* gene (β -subunit of RNA polymerase B) was also performed for species-level identification, using primers VIC2 and VIC3, as

previously described (Diancourt et al., 2005). Following PCR amplification, products migrated in a 1.5% agarose gel for 25 min at 150 V. Gels were stained with bromide ethidium and visualized with UV transillumination. A single band at approximately 1,000 bp was present for each isolate and purified by QIAquick PCR purification kit (Qiagen, Hilden, Germany). Purified DNA products were sequenced via pyrosequencing at the Plateforme d'analyse génomique (Université Laval, Québec, Canada). Sequences were manually proofread using SnapGene viewer software (GSL Biotech, Chicago, IL) and were compared with BLAST public database for species identification. *Klebsiella pneumoniae* ATCC 13883 was used as a control. When identification could not be obtained with the *rpoB* sequence, 16S sequencing was performed at the Animal Health Laboratory (AHL, University of Guelph, Guelph, Canada).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing for 18 antimicrobials was performed on all isolates of this study, using the disc diffusion method on Mueller Hinton agar, according to CLSI (2018a). The following commercially available discs (Becton Dickinson, Franklin Lakes, NJ) were tested (disc concentrations, breakpoints for susceptibility): amikacin (30 µg, ≥17 mm), amoxicillin/clavulanic acid (20/10 µg, ≥18 mm), cefoxitin (30 µg, ≥18 mm), ceftiofur (30 µg, ≥21 mm), ceftriaxone (30 µg, ≥23 mm), cephalotin (30 µg, ≥18 mm), chloramphenicol (30 µg, ≥18 mm), ciprofloxacin (5 µg, ≥21 mm), florfenicol (30 µg, ≥19 mm), gentamicin (10 µg, ≥15 mm), kanamycin (30 µg, ≥18 mm), nalidixic acid, (30 µg, ≥19 mm), neomycin (30 µg, ≥17 mm), spectinomycin (100 µg, ≥14 mm), streptomycin (10 µg, ≥15 mm), sulfamethoxazole/trimethoprim (23.75/1.25 µg, ≥16 mm), sulfisoxazole (250 µg, ≥17 mm), and tetracycline (30 µg, ≥15 mm). Isolates were defined as susceptible, intermediate, or resistant according to CLSI VET08 (CLSI, 2018a) for ceftiofur (breakpoint of *Escherichia coli* in mastitis), florfenicol, and spectino-

mycin (for both, breakpoints of respiratory pathogens in cattle: *Mannheimia haemolytica*, *Histophilus somni*, and *Pasteurella multocida*). For all other antimicrobials, except neomycin and cephalotin, the interpretation was based on human-derived zone diameters for *Enterobacteriaceae* from CLSI M100 (CLSI, 2018b). Interpretation for cephalotin came from a previous version of CLSI M100 (CLSI, 2012). No zone diameter is available for neomycin; thus, that of amikacin was used (<17 mm indicates resistance). For the current study, intermediate and resistant isolates were grouped together in a “resistant” category.

Genotyping

Bacteria from a fresh overnight culture was suspended in 100 µL of 10% Chelex (Bio-Rad, Hercules, CA) and heated at 95°C for 20 min. After centrifugation at 13,000 × *g* for 2 min, the supernatant was used as bacterial DNA. All PCR assays were performed in a total volume of 25 µL mixture containing the following reagents: 10× PCR Buffer (2.5 µL; New England Biolabs, Ipswich, MA), dNTP (0.4 mM), bacterial DNA (2.5 µL), MgCl₂ (1 mM), Taq polymerase (1 U), and the corresponding primers for AMR genes (Table 1). After migration on 1.5% agarose gel for 25 min at 150 V with bromide ethidium, all PCR products were visualized using UV transillumination.

To determine relatedness between isolates, RAPD PCR was conducted, using a method previously described by Munoz et al. (2007) and Vogel et al. (1999), with slight modifications. Briefly, ERIC-2/ERIC-1026 primers were used, and thermal conditions were as follows: 2 cycles (94°C for 5 min, 35°C for 5 min, and 72°C for 5 min), followed by 31 cycles (94°C for 1 min, 62°C for 1 min, and 72°C for 2 min) and a final extension at 72°C for 8 min (Vogel et al., 1999). After migration on 1.5% agarose gel with bromide ethidium for 90 min at 90 V, products were visualized using UV transillumination. The gels were read visually (2 independent readers) to compare banding patterns for each isolate.

Table 1. PCR conditions, primers, and controls for detection of antimicrobial resistance (AMR) genes

PCR	Resistance phenotype	Gene	Primers and PCR conditions	Final primer concentration (µM)	Product size (bp)	Annealing temperature (°C)	Control strain
1	Tetracycline	<i>tetA</i>	Bokaeian et al., 2014	0.2	888	55	<i>Escherichia coli</i> SAS1393
2	Tetracycline	<i>tetB</i>	Fluit et al., 2005	1	305	56	<i>E. coli</i> CT4afooB
3	Tetracycline	<i>tetD</i>	Fluit et al., 2005	1	477	61	<i>E. coli</i> D7–5
4	Sulfonamide	<i>sul1</i>	Frank et al., 2007	1	433	61	<i>E. coli</i> pACYC184
5	Streptomycin	<i>strA/strB</i>	Kozak et al., 2009	0.4	893	63	AMR 075
5	Streptomycin/spectinomycin	<i>aadA</i>	Kozak et al., 2009	0.1	525	63	AMR 075

Identical patterns (no band differences) were classified as a single RAPD pattern. *Klebsiella pneumoniae* ATCC 13883 was used in all reactions as a control.

Biofilm Formation

Biofilm production and quantification were conducted as described by de Campos et al. (2016), with some modifications. Briefly, 1 colony from an overnight culture was added to 5 mL of brain heart infusion (BHI) broth and incubated for 24 h at 35°C with rotation at 180 rpm. This broth was diluted 1:125 in a fresh 5 mL of BHI broth, then 200 µL was added in triplicate to a 96-well flat-bottom polystyrene plate (Costar 3595, Corning Life Sciences, Kennebunk, ME). After static incubation for 24 h at 35°C, plates were washed twice with deionized water, and 200 µL of crystal violet 0.1% was added and incubated for 15 min at room temperature. Plates were washed twice with deionized water and allowed to dry for 30 min at 35°C; then 200 µL of ethanol 70% was added for 15 min for elution. Absorbance was read at 570 nm with a spectrophotometer plate reader. Three independent technical replicates were performed on 3 different days. The negative control was BHI broth without bacteria, and optical density for this control was subtracted to all samples. Two controls (low and high biofilm-forming isolate) were added according to Naparstek et al., (2014). *Klebsiella pneumoniae* ATCC 13883 and internal control *K. pneumoniae* 12 were used as controls in each assay. Wells with optical densities greater than 2 times the average of the triplicate were considered contaminated.

Statistical Analysis

Descriptive statistics and statistical analyses were computed using SPSS Statistics version 25 (IBM Corp.,

Armonk, NY). Comparison of species as functions of different factors (biofilm production, mastitis severity score, DIM, lactation number, and AMR) was performed using the Kruskal-Wallis test for independent samples. Fisher's exact test was used to compare numbers of AMR between 2 groups, according to RAPD type: the first group constituted isolates with similar RAPD patterns (thus, related isolates); the second represented isolates with independent RAPD patterns (thus, the remaining unrelated isolates). An α of 0.05 was considered for inferring statistical significance.

RESULTS

Bacterial Identification

Table 2 presents the comparison between biochemical tests, MALDI-TOF, and *rpoB* sequencing for bacterial identification. The *rpoB* sequencing could not give an accurate identification for 1 isolate, identified as either *K. pneumoniae* or *K. variicola* (506/506 bp for both). Complete 16S RNA sequencing revealed this isolate to be a *K. pneumoniae* (1,166/1,167 bp) with a close association to *K. variicola* (1,164/1,167 bp). Except for this isolate of *K. pneumoniae* mistakenly identified as *K. variicola* by MALDI-TOF, we found no disparities between the MALDI-TOF results and the *rpoB* sequencing data for *Klebsiella* and *Raoultella* spp. On the other hand, biochemical tests could not identify *Raoultella planticola* and *Raoultella ornithinolytica* misidentified as *K. pneumoniae* and *K. oxytoca*, respectively.

AMR Phenotypes

Among the 53 isolates tested, 31 (58%) were susceptible to all antimicrobials tested. The remaining 22 (42%) isolates were resistant to 1 or more of the fol-

Table 2. Comparison between biochemical tests, *rpoB* sequencing, and MALDI-TOF identification for *Klebsiella* and *Raoultella* isolates (n = 53) and their specific characteristics¹

Species	Identification technique			Average $\pm$ SD				
	<i>rpoB</i>	Biochemical tests	MALDI-TOF	Biofilm formation (OD _{570nm})	DIM	Mastitis severity scores ²	Lactation number	AMR ³
<i>Klebsiella pneumoniae</i> ⁴	40	43	39	0.77 $\pm$ 0.57	157 $\pm$ 127	1.8 $\pm$ 0.7	2.8 $\pm$ 2.0	0.9 $\pm$ 1.2
<i>Klebsiella oxytoca</i>	9	10	9	0.80 $\pm$ 0.85	83 $\pm$ 65	1.8 $\pm$ 0.8	3.0 $\pm$ 1.3	0.7 $\pm$ 1
<i>Raoultella planticola</i>	2	0	2	0.68 $\pm$ 0.26	189 $\pm$ 189	1.5 $\pm$ 0.7	2.0 $\pm$ 0.7	1.5 $\pm$ 2.1
<i>Raoultella ornithinolytica</i>	2	0	2	0.10 $\pm$ 0.06	149 $\pm$ 49	1.5 $\pm$ 0.7	3.5 $\pm$ 1.4	0.5 $\pm$ 0.7
<i>Klebsiella variicola</i>	0	0	1	—	—	—	—	—

¹AMR = antimicrobial resistance; OD = optical density.

²Mastitis severity scores were attributed by the dairy producer at clinical mastitis diagnosis according to the scale proposed by Sears and McCarthy (2003): 1 = only the milk is abnormal, 2 = milk and quarter appearance are abnormal, and 3 = the animal is sick.

³Tested for 18 antimicrobials via disc diffusion technique.

⁴RNA 16S identified as *K. pneumoniae* (1,166/1,167 bp), also closely related to *K. variicola* (1,164/1,167 bp).

lowing antimicrobials: kanamycin (2%: 1 *K. oxytoca*), streptomycin (38%: 3 *K. oxytoca*, 16 *K. pneumoniae*, and 1 *R. planticola*), spectinomycin (13%: 1 *K. oxytoca*, 5 *K. pneumoniae*, and 1 *R. planticola*), sulfisoxazole (13%: 1 *K. oxytoca*, 5 *K. pneumoniae*, and 1 *R. planticola*), and tetracycline (19%: 9 *K. pneumoniae*, and 1 *R. ornithinolytica*; Table 3). Only 1 (2%) *K. pneumoniae* isolate was resistant to 3 antimicrobial classes (aminoglycosides, tetracyclines, and sulfonamides) and thus considered multi-drug resistant (Table 3). Isolates resistant to sulfisoxazole ($n = 7$) were also resistant to spectinomycin and streptomycin, and vice versa. No resistance to the remaining 13 antimicrobials was observed.

AMR Genotypes

Table 3 shows the distribution of tested AMR genes *tetA*, *tetB*, *sul1*, *strA/strB*, and *aadA* found in resistant isolates of *Klebsiella* and *Raoultella*. Briefly, all *K. pneumoniae* and *R. ornithinolytica* that were resistant to tetracycline ($n = 10$) harbored *tetB* and *tetA* genes, respectively. All sulfisoxazole-resistant strains ($n = 7$) carried the *sul1* gene. Of 7 isolates resistant to streptomycin and spectinomycin, 6 harbored the gene *aadA*. The remaining isolates resistant to streptomycin ($n = 13$) harbored the gene *strA/strB*. The *tetD* gene was

not present in any of the resistant isolates of *Klebsiella* or *Raoultella*.

RAPD Typing

Overall, 40 RAPD patterns were observed among isolates tested ($n = 53$; Figure 1). From those, 36 unique patterns originated from 20 different farms, and the remaining 4 patterns (A, B, C, and D) contained 8, 3, 2, and 4 isolates and originated from 2, 2, 1, and 1 farm or farms, respectively (Table 3). Those related isolates showed greater AMR (13 out of 17 were resistant to at least 1 antimicrobial) compared with the 36 unrelated isolates with individual RAPD patterns (9 out of 36 were resistant to at least 1 antimicrobial; $P < 0.001$).

Biofilm Formation

Klebsiella pneumoniae ATCC 13883 and internal control *K. pneumoniae* 12 were used as relatively low and high biofilm-forming isolates, respectively, with average optical density values of 570 nm and standard deviations of 0.035 ± 0.038 and 1.575 ± 0.583 , respectively. All *Klebsiella* and *Raoultella* in this study were shown to produce biofilms. Figure 2 shows the variable quantities of biofilm observed for each species, and Table 2 presents average biofilm production for each species.

Table 3. Antimicrobial resistance (AMR) phenotypes and genotypes observed in the random amplified polymorphic DNA (RAPD) patterns of this study

RAPD patterns (no. of isolates)	Farms of origin (no. of isolates)	AMR phenotype ¹	AMR genotype ²	Species ³
A (8)	Farm 101 (5)	5: STR-TET	<i>strA/strB-tetB</i>	5 <i>Klebsiella pneumoniae</i>
B (3)	Farm 101 (2), farm 103 (1)	3: None	Not tested	3 <i>K. pneumoniae</i>
	Farm 415 (2)	2: STR	<i>strA/strB</i>	2 <i>K. pneumoniae</i>
C (2)	Farm 103 (1)	1: None	Not tested	1 <i>K. pneumoniae</i>
	Farm 104 (1)	1: STR-SPE-TET-FIS ⁴	<i>strA/strB-aadA-tetB-sul1</i>	1 <i>K. pneumoniae</i>
	Farm 104 (1)	1: STR-TET	<i>strA/strB-tetB</i>	1 <i>K. pneumoniae</i>
D (4)	Farm 110 (4)	4: STR-SPE-FIS	<i>aadA-sul1</i>	4 <i>K. pneumoniae</i>
Other patterns (36)	15 different farms (27)	27: None	Not tested	5 <i>Klebsiella oxytoca</i> , 20 <i>K. pneumoniae</i> , 1 <i>Raoultella ornithinolytica</i> , 1 <i>Raoultella planticola</i>
	Farm 104 (1), farm 110 (1)	2: STR-TET	<i>strA/strB-tetB</i>	2 <i>K. pneumoniae</i>
	Farm 202 (1)	1: STR-SPE-FIS	<i>aadA-sul1</i>	1 <i>K. oxytoca</i>
	Farm 407 (1)	1: STR-SPE-FIS	Not found- <i>sul1</i>	1 <i>R. planticola</i>
	Farm 408 (2), farm 310 (1)	3: STR	<i>strA/strB</i>	2 <i>K. oxytoca</i> , 1 <i>K. pneumoniae</i>
	Farm 416 (1)	1: KAN	Not tested	1 <i>K. oxytoca</i>
	Farm 101 (1)	1: TET	<i>tetA</i>	1 <i>R. ornithinolytica</i>

¹STR = streptomycin; TET = tetracycline; FIS = sulfisoxazole; SPE = spectinomycin; KAN = kanamycin.

²Antimicrobial resistance genes tested using simplex PCR for *tetA*, *tetB*, *tetD*, and *sul1*, and multiplex PCR for *strA/strB* and *aadA*.

³Obtained via *rpoB* or 16S RNA sequencing.

⁴Multidrug resistant patterns.

Figure 1. Random amplified polymorphic DNA (RAPD) banding patterns for *Klebsiella* and *Raoultella* spp. obtained from dairy cattle clinical mastitis cases. (A) Results for *Raoultella ornithinolytica* (samples 1 and 37) and *Raoultella planticola* (samples 35 and 47). (B) Results for *Klebsiella oxytoca*. (C) Results for *Klebsiella pneumoniae*; letters above the gel represent isolates with the same RAPD banding pattern. One isolate (number 25) is missing due to technical difficulties and is not presented here; however, it was not correlated with any RAPD patterns. M = molecular weight marker; – = negative control (water); + = positive control (*K. pneumoniae* ATCC 13883); Ko = positive control (*K. oxytoca* ATCC 13182).

Five out of 561 wells were considered contaminated and were excluded from the results.

DISCUSSION

Mastitis contributes to major economic losses in the dairy industry (Aghamohammadi et al., 2018). The prevalence of mastitis varies between geographical locations due to differences in legislation, veterinary and laboratory services, and management practices (Osman et al., 2014). Among environmental agents causing mastitis, *Klebsiella* spp. are frequently identified by biochemical tests. As previously reported (Munoz et al., 2007; Zadoks et al., 2011a; Oikonomou et al., 2012), these tests do not yield accurate *Klebsiella* and *Raoultella* discrimination. As indicated by the name, *R. ornithinolytica* is the only species of the genera *Klebsiella* or *Raoultella* positive for ornithine decarboxylase (Drancourt et al., 2001). However, even if the reference strains of *R. ornithinolytica* are positive, most clinical isolates described to date are negative for this test (Walckenaer et al., 2008; Ponce-Alonso et al., 2016), as noted for the 2 *R. ornithinolytica* in this study identified via API-20E. The *rpoB* gene sequencing was described and developed as a valid identification technique for

Klebsiella (Diancourt et al., 2005). It has been used for *Klebsiella* and *Raoultella* identification in dairy farms (Munoz et al., 2007; Zadoks et al., 2011a; Podder et al., 2014). In our study, we had only 1 identification

Figure 2. Whisker plot of biofilm formation analyzed by a static technique in 96 wells per plate for *Klebsiella* and *Raoultella* spp. obtained from dairy cattle clinical mastitis cases. The midline represents median observation. Lower and upper boxes represent first and third quartile, respectively. Whiskers are minimum and maximum observed values. Empty circles represent outlier observations. OD 570 nm = optical density at 570 nm.

problem, regarding an isolate whose *rpoB* sequence was identical for both *K. variicola* and *K. pneumoniae*. The final identification had to be confirmed using 16S RNA, which was more discriminant for identification of this isolate. In contrast, a past study reported adequate identification for *K. pneumoniae* and *K. variicola* by *rpoB* even if MALDI-TOF could not yield a definitive identification (Munoz et al., 2007). All other identifications in our study were the same between *rpoB* and MALDI-TOF, as reported in another study (Ponce-Alonso et al., 2016), indicating that MALDI-TOF is a suitable alternative to sequencing for identification.

After molecular identification, 1 study reported *Raoultella* in 4.6% (11 out of 240) of isolates first identified as *Klebsiella* (Ponce-Alonso et al., 2016). This concurs with our results of 7.5% (4/53). The *Raoultella* genus was created in 2001, after phylogenetic analysis. Before that, it had been classified as *Klebsiella* (Drancourt et al., 2001). This explains the close relationship between the 2 genera and the difficulty for their accurate differentiation. *Raoultella* was previously reported on farm environments and in the rumen and manure samples of cows, but not in mastitis cases (Zadoks et al., 2011a; Oikonomou et al., 2012). To our knowledge, this is the first study to publish on *Raoultella* as a clinical mastitis agent.

In an antimicrobial susceptibility surveillance program conducted in Europe (VetPath; de Jong et al., 2018), low percentages of AMR for *Klebsiella* spp. isolated from acute mastitis were observed, with the greatest levels of resistance being toward tetracyclines (19%). The same tetracycline resistance percentage is reported in this study; however, the most prevalent resistance we observed was toward streptomycin, which was not reported in the European program (de Jong et al., 2018). As previously reported (de Jong et al., 2018), we also did not observe resistance to first-, second-, or third-generation cephalosporins and fluoroquinolones. The most commonly used antimicrobials in 2008 in Canada for clinical mastitis were β -lactams (Saini et al., 2012a). However, acquired resistance to β -lactams was not observed in our study. Interestingly, *Raoultella* was previously described as naturally resistant to ampicillin through a chromosomally encoded class A β -lactamase (Ponce-Alonso et al., 2016) similar to *Klebsiella*. In general, the AMR phenotypes observed were well explained by the AMR genes tested, with the exception of 1 isolate that did not carry the *aadA* gene. The correlation observed between sulfonamide and spectinomycin resistance suggests a common mobile genetic element such as an integron for both *sul1* and *aadA*, as previously reported (Fluit and Schmitz, 2004). Susceptible isolates were not tested for the presence of

resistance genes, because they did not express AMR on disc diffusion. In a study by Tyson and colleagues (2015), sensitivity and specificity were higher than 97% between phenotypes (minimum inhibitory concentration) and genotypes (whole-genome sequencing) in *E. coli* from cattle. The only exception was for streptomycin, for which half of susceptible *E. coli* carried a resistance gene (Tyson et al., 2015).

Biofilms have been associated with tolerance to antimicrobials (Tremblay et al., 2014). Biofilm production was previously reported for 84% of *Klebsiella* spp. isolated from mastitis cases (Schönborn et al., 2017). It is difficult to compare our results with those of Schönborn and colleagues, because the methodologies used in both studies were different. Also, no significant associations were observed between bacterial genus, species, AMR, biofilm production, and cow variable such as mastitis severity score, DIM, and lactation number. In this study, biofilm production has been tested only in isolates from clinical cases and not in isolates from the environment for a comparison.

Interestingly, some isolates with the same RAPD pattern did not have similar AMR genotypes and phenotypes. This suggests that various AMR profiles can be obtained within a unique RAPD pattern, indicating that this technique is not sufficiently discriminant to group DNA fragments associated with AMR. A unique RAPD pattern or closely related RAPD patterns associated with a single farm may indicate contagiousness or a common point-source environmental origin, whereas unrelated patterns are commonly associated with environmental contamination. Previous studies have observed both routes of transmission for *Klebsiella* on dairy farms (Munoz et al., 2007; Paulin-Curlee et al., 2008; Podder et al., 2014). In our case, the majority of our isolates were unrelated based on RAPD patterns, indicating environmental contamination. Only a small number of isolates showed similar RAPD patterns. Interestingly, isolates with an identical RAPD pattern carried more AMR phenotypes, indicating a possible link between AMR and contagiousness, but this hypothesis warrants further investigation.

CONCLUSIONS

Characterization of *Klebsiella* species involved in clinical mastitis cases in Canada revealed that accurate identification was provided by MALDI-TOF. Antimicrobial resistance was generally low, with AMR genes correlating well with AMR phenotypes. All isolates were shown to produce biofilms. This study also determined that environmental contamination was likely the primary source of *Klebsiella* infection in cows.

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