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Molecular characterization of antimicrobial resistance in *Klebsiella pneumoniae* isolated from Brazilian dairy herds

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ABSTRACT

In this observational study, phenotypic and genotypic patterns of antimicrobial resistance (AMR) in *Klebsiella pneumoniae* isolated from intramammary infections, clinical mastitis, fresh feces, rectal swabs, animal hindlimbs, and bulk tank milk samples from Brazilian dairy herds were investigated. In addition, we identified specific genetic variants present among extended-spectrum β-lactamase (ESBL) producers. We obtained 169 isolates of *K. pneumoniae* from 2009 to 2011 on 24 Brazilian dairy farms located in 4 Brazilian states. The AMR profile of all isolates was determined using disk-diffusion assays. The antimicrobial panel included drugs commonly used as mastitis treatment in Brazilian dairy herds (gentamicin, cephalosporins, sulfamethoxazole-trimethoprim, tetracycline) as well as antimicrobials of critical importance for human health (meropenem, ceftazidime, fluoroquinolones). The *K. pneumoniae* isolates resistant to tetracycline, fluoroquinolones, sulfamethoxazole-trimethoprim, or chloramphenicol were screened for presence of drug-specific AMR genes [*tet*, *qnr*, *aac(6′)-Ib*, *floR*, *catA2*, *cm1A*, *dfr*, *sul*] using PCR. In addition, we identified ESBL genes present among ESBL-producers by using whole genome sequencing. Genomes were assembled and annotated, and patterns of AMR genes were investigated. Resistance was commonly detected against tetracycline (22.5% of all isolates), streptomycin (20.7%), and sulfamethoxazole-trimethoprim (9.5%). Antimicrobial resistance rates were higher in *K. pneumoniae* isolated from intramammary infections in comparison with isolates from feces (19.2 and 0% of multidrug resistance in intramammary and fecal isolates, respectively). In contrast, no difference in AMR rates was observed when contrasting hind limbs and isolates from intramammary infections. The genes *tetA*, *sul2*, and *floR* were the most frequently observed AMR genes in *K. pneumoniae* resistant to tetracycline, sulfamethoxazole-trimethoprim, and chloramphenicol, respectively. The *tetA* gene was present exclusively in isolates from milk. The genes $bla_{\text{CTX-M8}}$ and $bla_{\text{SHV-108}}$ were present in 3 ESBL-producing *K. pneumoniae*, including an isolate from bulk tank milk. The 3 isolates were of sequence type 281 and had similar mobile genetic elements and virulence genes. Our study reinforced the epidemiological importance and dissemination of $bla_{\text{CTX-M-8}}$ pST114 plasmid in food-producing animals in Brazil.

Key words: antimicrobial resistance, extended-spectrum β-lactamase, *Klebsiella pneumoniae*, mastitis

INTRODUCTION

Clinical mastitis (**CM**) caused by environmental bacteria such as *Klebsiella pneumoniae* remains a major challenge, threatening the dairy industry worldwide (Schukken et al., 2012). Intramammary infections caused by *K. pneumoniae* tend to have a low spontaneous cure rate (Fuenzalida and Ruegg, 2019a), and recurrence of clinical cases may occur (Oliveira et al., 2013). It is well established that limiting teat-end exposure through increased environmental hygiene is the centerpiece of control plans to curb the spread of *K. pneumoniae* in dairy herds (Zadoks et al., 2011).

There is an ongoing pressure to reduce overall use of antimicrobials in food-producing animals, especially due to the increasing threat of antimicrobial resistance (**AMR**; Van Boeckel et al., 2019). *Klebsiella pneumoniae* are environmental mastitis pathogens that are present in sites of large bacterial communities on dairy

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farms such as the bedding, feces, alleyways, feed, and water (Zadoks et al., 2011), and will occasionally harbor antimicrobial resistance genes (**ARG**; Locatelli et al., 2010). As such, *K. pneumoniae* will play a role in the dissemination of AMR in the farm environment. As management of AMR requires a multifaceted approach that considers the human-animal-environment interface (Sun et al., 2017), there is valuable information to gain by exploring the AMR profile of isolates obtained from different sources in dairy herds, in an attempt to identify potential reservoirs of ARG that may contribute to the within-herd dissemination of AMR.

Mastitis remains one the most frequent reasons to treat cows with antimicrobials in dairy herds (Pol and Ruegg, 2007b; Saini et al., 2012a; Oliveira and Ruegg, 2014). There are many factors that will affect the outcome of antimicrobial treatment of CM, including pathogen-specific characteristics that may affect the efficacy of antimicrobials (Barkema et al., 2006). Studies have suggested that CM caused by *K. pneumoniae* does not respond to antimicrobial treatment as well as CM caused by *Escherichia coli* (Schukken et al., 2011, 2012). Removal and recurrence rates are higher for mastitis caused by *E. coli* than for *Klebsiella* spp. mastitis (Oliveira et al., 2013). In a study from the United States, treated and untreated cows with nonsevere CM caused by drug-susceptible *K. pneumoniae* had comparable posttreatment milk yields, culling rates, and SCC linear scores after resolution of the disease. Yet, cows with CM caused by drug-resistant *K. pneumoniae* had increased risk of culling and voluntary dry-off when compared with cows with CM caused by susceptible *K. pneumoniae* (Fuenzalida and Ruegg, 2019a). This suggests that the susceptibility profile of *K. pneumoniae* that causes CM can be used to inform herd-level strategies aimed to optimize the use of antimicrobials in dairy herds.

Critically important antimicrobials (**CIA**) are routinely used to treat life-threating infections in humans caused by drug-resistant bacteria. Determining whether bacteria isolated from animals are resistant against CIA is important because this information will contribute to the investigation of potential sources of resistant bacteria that cause infections in humans, as previously demonstrated (Paterson and Harris, 2016). Resistance against CIA in *K. pneumoniae* that causes mastitis remains relatively low in dairy herds from the United States, Canada, and Europe (Erskine et al., 2002; Saini et al., 2012b; de Jong et al., 2018). In Brazil, CIA (higher generation cephalosporins, fluoroquinolones, aminoglycosides) are among the most commonly used antimicrobials in dairy herds (Tomazi and Dos Santos, 2020). With increasing concern about antimicrobial usage in livestock leading to resistance against CIA in zoonotic bacteria, it is important to verify whether the high consumption of CIA in Brazilian dairies is associated with high rates of nonsusceptibility in bacteria present on dairy farms.

Extended-spectrum β-lactamases (**ESBL**) are enzymes that inactivate a large number of β-lactam antimicrobials, including ones used to treat infections in humans. They have been increasingly reported from livestock, and some livestock species have been recognized as reservoirs of ESBL-producing bacteria (Carattoli, 2008). Extended-spectrum β-lactamase resistance genes can be disseminated to humans through the food chain or via direct contact with animals (Dahms et al., 2015). It is suggested that, although most community-acquired ESBL-producing coliforms that cause infections in humans are of human origin, part of the intracommunity transmission is sustained by the continuous exposure of humans to coliforms of nonhuman sources (Mughini-Gras et al., 2019). Multidrug-resistant *K. pneumoniae* isolates that cause mastitis, including ESBL-producers, have been isolated from dairy herds (Locatelli et al., 2010; Koovapra et al., 2016). Among ESBL, CTX-M β-lactamases are the most widespread enzymes (D'Andrea et al., 2013). Phylogenetic analysis revealed presence of 5 major groups of CTX-M enzymes: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 (Bonnet, 2004). The mostly frequently reported group in cattle isolated worldwide is CTX-M-1 (Dantas Palmeira and Ferreira, 2020). Thus far, it is unclear whether and which groups of ESBL are circulating in *K. pneumoniae* isolated from Brazilian dairy herds. Such information can be crucial when establishing an antimicrobial stewardship framework to curtail increasing AMR rates in Brazil (Van Boeckel et al., 2019).

Herein, we describe phenotypic and genotypic patterns of AMR in *K. pneumoniae* isolated from IMI, CM, fresh feces, rectal swabs, animal hindlimbs, and bulk tank milk samples from Brazilian dairy herds, and compare the AMR profile of isolates obtained from different sources. In addition, we aimed to identify specific genetic variants present among ESBL-producers.

MATERIALS AND METHODS

Farms and Isolates

We included 169 isolates of *K. pneumoniae* obtained from 2009 to 2011 on 24 Brazilian dairy farms located in 4 Brazilian States (São Paulo, Bahia, Minas Gerais, and Goiás). Isolates belonged to the São Paulo State University bacterial collection (Unesp-Botucatu, São Paulo, Brazil). Briefly, isolates were sampled under 2 protocols. First, a convenience sample of 12 herds were

visited within 4 mo (December 2009 to March 2010) by 2 veterinarians for collection of feces, skin swabs, milk, and bulk tank milk samples. Five herds enrolled in the first sampling protocol housed cows in freestalls, whereas the remaining 7 used an extensive grazing milk production system (Table 1). Test day bulk tank SCC and average herd size ranged from 113 to 826 $\times$ 10^3 cells/mL and from 26 to 970 lactating cows, respectively (Table 1). All herds used blanket dry cow therapy.

Fecal samples were systematically collected from milking parlors (n = 2 per herd), milking parlor holding pens (n = 2), and freestall barns or pasture grazing areas (n = 8). Each location was split in either 2 (milking parlor and holding pens) or 8 (barns or pasture area) equally sized subareas, and fecal samples were collected from each subarea using cotton-tipped wooden swabs, gently introduced in fresh fecal content. In addition, 12 cows per herd were randomly selected during milking for further sampling: 6 cows had swabs collected from hind limbs and 6 from rectum, as previously described (Langoni et al., 2015).

For 3 herds with >1 barn, cows housed at 1 randomly selected barn were selected for sampling. Barns housing replacement heifers or sick animals were not eligible. Accordingly, fecal samples were collected exclusively from selected barns. Swabs were immediately placed in 3 mL of saline solution, stored in cooler boxes containing icepacks, and transported to the laboratory within 10 h. Assuming a low (0.05) intraclass correlation coefficient, and that 80% of our fecal samples would be *K. pneumoniae*-positive, our sampling protocol allowed for estimation of a proportion (e.g., resistance against tetracycline) of 10% in fecal isolates with a 5% margin of error at 90% confidence level.

Milk samples were collected from all lactating cows, except for herds with >1 barn, where we selected cows from sampled barns exclusively. We used the California Mastitis Test (**CMT**), an indicator of milk SCC, to detect cows with moderate or high SCC (Schalm and Noorlander, 1957). From all quarters with a CMT score $\geq$1, we aseptically collected milk samples (~5 mL) following standardized protocols (National Mastitis Council, 1999). In addition, we collected milk samples from all quarters with visible signs of CM (e.g., swelling, heat of mammary gland, changes in normal milk appearance such as watery milk, blood, or clots), providing no antimicrobial treatment was administered up to a week before sampling. Finally, we aseptically collected bulk tank milk samples (~200 mL) following 5 min of agitation using a sterile sampler. Milk samples were stored in cooler boxes containing icepacks and transported to the laboratory within 10 h.

Upon arrival at the laboratory, 1 mL of the saline solution that swabs were stored in was used to

Table 1. Summary of characteristics of the 12 herds enrolled in the first sampling protocol, as well as number of *Klebsiella pneumoniae* isolates according to sample type[1]

									Klebsiella pneumoniae (isolates)[5]						
Herd[2]	Size[3]	Pre-dipping	Pre-washing	Feed after milking	Freestall	Milk production (L/d)	Milkings (no./d)	BTSCC[4]	Milk samples	IMI (no.)	Milking parlor	Holding pens	Hind limbs	Barns or grazing pens	Rectum	Bulk tank
1	74			●		700	2	531	230	√ (1)	√ (2)		√ (4)	√ (7)		√ (1)
2	320	●		●	●	5,800	3	241	1,112	√ (3)		√ (2)	√ (3)	√ (6)	√ (2)	√ (1)
3	970	●	●	●	●	34,000	3	390	1,282	√ (23)			√ (3)	√ (5)	√ (4)	√ (1)
4	113	●		●		2,900	2	315	388		√ (1)		√ (1)		√ (1)	
5	96	●		●	●	2,300	2	NA[6]	290	√ (1)		√ (1)		√ (2)		√ (1)
6	580	●		●	●	19,800	3	113	195			√ (1)	√ (3)	√ (3)	√ (1)	
7	96	●	●	●		2,300	2	640	334					√ (4)	√ (2)	
8	46	●		●		1,050	2	590	177		√ (2)	√ (1)	√ (1)	√ (3)		√ (1)
9	92	●		●		900	2	392	314				√ (1)	√ (2)	√ (1)	
10	26	●		●	●	530	2	143	98		√ (2)	√ (2)	√ (1)		√ (1)	
11	54	●				940	2	735	207		√ (1)	√ (2)		√ (1)	√ (1)	
12	46	●				NA	2	NA	184					√ (3)		

[1]A dot denotes that the management practice or housing type was employed in the herd.
[2]All herds used postdipping.
[3]Lactating cows at sampling day.
[4]Test day bulk tank SCC (BTSCC $\times$ 10^3 cells/mL).
[5]A check mark indicates that *Klebsiella pneumoniae* was isolated from at least 1 sample from that source.
[6]NA = not available.

prepare 10-fold serial dilutions. We streaked 100 μL of each solution onto MacConkey agar plates (Oxoid Ltd.) containing 10 mg/L of ampicillin (Munoz et al., 2006). Following overnight incubation at 37°C, 1 plate per sample containing 30 to 300 cfu was selected for presumptive identification of *K. pneumoniae* colonies based on morphology (Munoz et al., 2006) and oxidase testing. One randomly selected *K. pneumoniae*-like colony per sample was then subcultured onto MacConkey agar for species identification. Plates containing 30 to 300 cfu with no *Klebsiella*-like colony were considered *Klebsiella*-negative.

Ten microliters of quarter-level samples were cultured onto MacConkey agar plates and incubated at 37°C. Plates were observed daily for up to 3 d. Putative *Klebsiella* spp. colonies based on morphology (Munoz et al., 2006) and negative oxidase testing (maximum of 1 colony per plate) were subcultured onto MacConkey agar and incubated overnight at 37°C. *Klebsiella pneumoniae* IMI was defined as a nonclinical milk sample wherein *K. pneumoniae* was detected (CMT score $\geq$1 and detection of at least 1 *K. pneumoniae* colony). For bulk tank milk samples, the same procedures were adopted, but streaking 100 uL onto the entire surface of the MacConkey agar plates and randomly selecting 1 *K. pneumoniae* putative colony for further characterization.

The second sampling scheme consisted of 12 herds participating in a voluntary mastitis control program based on the identification of cows with CM and culture of milk samples, coordinated by a veterinary diagnostic laboratory in Brazil from January 2011 to July 2011. The *K. pneumoniae* isolates (n = 53) were sampled from different cows with CM housed in the 12 herds, with an average of 4.4 *K. pneumoniae* isolates available per herd (ranging from 1–25 isolates/herd; median = 2). No cow contributed >1 isolate. Herd personnel, properly instructed by field veterinarians about aseptic milking procedures, were asked to sample all cases of CM routinely detected based on clinical signs or abnormal milk. Milk samples were aseptically collected from affected quarters before treatment onset. Following teat-end asepsis and discard of first milk strips, 10- to 20-mL samples were collected in 40-mL sterile vials. Milk samples were stored on farms at −20°C and shipped to the veterinary diagnostic laboratory for bacteriological culture following the same procedures described. No information about total number of milk samples collected per farm or any other herd-level data were available. Isolates were kept in 5-mL cryovials at −20°C.

Species identification was carried out in all presumptive *K. pneumoniae*, including ones from second sampling protocol, using a panel of biochemical tests. The *K. pneumoniae* were identified as follows: citrate-positive, hydrogen sulfide–negative, indole-negative, lysine-negative, motility-negative, production of gas in glucose media, tryptophan deaminase–negative, and urease-positive. American Type Culture Collection *E. coli* 25922 and *K. pneumoniae* 13883 were used as positive and negative controls, respectively. All *K. pneumoniae* were stored at −80°C in cryovials containing broth cultures with 20% glycerol.

Antimicrobial Susceptibility Testing

Antimicrobial resistance profile of all isolates was determined using disk-diffusion assays following standardized guidelines (CLSI, 2016). The following antimicrobials and concentrations were tested: cefoxitin (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), meropenem (10 μg), chloramphenicol (30 μg), enrofloxacin (5 μg), ciprofloxacin (5 μg), tetracycline (30 μg), streptomycin (10 μg), gentamicin (10 μg), and the combination of sulfamethoxazole and trimethoprim (25 μg). *Escherichia coli* 25922 was used as the quality control strain in all assays. Antimicrobial resistance was defined as nonsusceptibility to a given compound by grouping resistant and intermediate isolates as a single category. Breakpoints were defined according to Clinical and Laboratory Standards Institute human criteria, as follows: cefoxitin = 18 mm, cefotaxime = 26 mm, ceftazidime = 21 mm, meropenem = 23 mm, chloramphenicol = 18 mm, ciprofloxacin = 21 mm, tetracycline = 15 mm, streptomycin = 15 mm, gentamicin = 15 mm, and the sulfamethoxazole-trimethoprim combination = 16 mm (CLSI, 2016). Breakpoint of enrofloxacin was assumed to be the same as of ciprofloxacin. Isolates with inhibition zones below the breakpoints were deemed as resistant.

All *K. pneumoniae* were screened for production of ESBL using the double disk-diffusion test (Bradford, 2001). In brief, aztreonam (30 ug), ceftriaxone (30 ug), ceftazidime (30 ug), cefotaxime (30 ug), and cefpodoxime (30 ug) disks were placed 20 mm (center to center) to amoxicillin-clavulanic acid (20 ug-10 ug) disks. Presence of an irregular zone of inhibition (also known as ghost zone) was considered as a positive result. *Klebsiella pneumoniae* ESBL-producing ATCC 700603, that was recently reclassified as *Klebsiella quasipneumoniae ssp. similipneumoniae* (Elliott et al., 2016), and *E. coli* ATCC 25922 were used as quality control strains. Multidrug resistance (**MDR**) was defined as nonsusceptibility against 3 or more antimicrobial classes.

The *K. pneumoniae* isolates resistant to tetracycline, fluoroquinolones, sulfamethoxazole-trimethoprim, or

Table 2. Primers and references used

Resistance	Gene	Forward (5′–3′)	Reverse (5′–3′)	Reference
Tetracycline	*tetA*	GCTACATCCTGCTTGCCTTC	CATAGATCGCCGTGAAGAGG	Wedley et al. (2011)
	tetB	CCTTATCATGCCAGTCTTGC	ACTGCCGTTTTTTCGCC	Ryu et al. (2012)
	tetC	ACTTGGAGCCACTATCGAC	CTACAATCCATGCCAACCC	Archambault et al. (2012)
	tetD	ATGATAAGCAGTTTCATACAACG	TCAGTGCATTGCCTCCAATTC	Ryu et al. (2012)
	tetG	GCTGGATGATGCATTGCGCG	ATGGTCTGCGTAGTATTGGC	Archambault et al. (2012)
Fluoroquinolones	*qnrB*	GGMATHGAAATTCGCCACTG	TTTGCYGYYCGCCAGTCGAA	Cattoir et al. (2007)
	qnrS	CGACGTGCTAACTTGCGTGATA	TACCCAGTGCTTCGAGAATCAG	Cavaco et al. (2008)
	aac(6′)-Ib	TTGCGATGCTCTATGAGTGGCTA	CTCGAATGCCTGGCGTGTTT	Kim et al. (2009)
Chloramphenicol	*floR*	TCCTCGCTTCACTGGCGATG	ACCAGCCCCAACGAAACCAG	Chen et al. (2012)
	catA2	GAACAYTTTGCCCTTTATCGTC	TCCTGCTGAAACTTTGCCATCGT	Lu et al. (2011)
	cmlA	GCGGGCTATCTTTGCGTTTC	AAGTAGACTGCCGTGACCGTTCC	Lu et al. (2011)
Sulfamethoxazole-trimethoprim combination	*dfrA5*	ATCGTCGATATATGGAGCGTA	TCCACACATACCCTGGTCCG	Hu et al. (2011)
	dfrA12, dfrA13	CAGGTGAGCAGAAGATTTTT	CCTCGAAGGTTTGATGTACC	Obeng et al. (2012)
	sul1	TTCGGCATTCTGAATCTCAC	ATGATCTAACCCTCGGTCTC	Obeng et al. (2012)
	sul2	CGGCATCGTCAACATAACC	GTGTGCGGATGAAGTCAG	Obeng et al. (2012)
	sul3	GGAAGAAATCAAAAGACTCAA	CCTAAAAAGAAGCCCATACC	Dahmen et al. (2010)

chloramphenicol were screened for presence of drug-specific ARG using PCR (Table 2). In-house positive and negative controls were used in all reactions.

Whole Genome Sequencing and Bioinformatic Analysis

Extended-spectrum β-lactamase producers were whole genome sequenced for screening of virulence and AMR determinants such as ARG. Following DNA extraction using Wizard Genomic DNA Purification kits (Promega), DNA library was prepared using Nextera XT DNA kits (Illumina Inc.). Finally, whole genome sequencing was performed with an Illumina HiSeq2500 (Illumina Inc.) using a paired-end 250-bp reads strategy. The genome assembly was performed using SPAdes version 3.11.1 (Bankevich et al., 2012) and annotation using the National Center for Biotechnology Information (**NCBI**) prokaryotic genome automatic annotation pipeline (https://www.ncbi.nlm.nih.gov/genome/annotation_prok). The assembled data were used to investigate the presence of resistance genes with Antibiotic Resistance Gene-ANNOTation V6 (Gupta et al., 2014) using default parameters, the Comprehensive Antibiotic Resistance Database V3.0.8 (Jia et al., 2017) using high coverage and ≥95% identity (Jia et al., 2017), and ResFinder 3.2 (Zankari et al., 2012), where a threshold for 95% of identity was selected. Thereafter, we used protein sequences of returned hits as queries against the NCBI nonredundant database (https://www.ncbi.nlm.nih.gov/refseq) using the protein basic local alignment search tool (Altschul et al., 1990). Best hits were considered definitive if sharing >80% coverage and percent identity with queries. Multilocus sequence typing (**MLST**) of our samples was determined using MLST 2.0 software (Larsen et al., 2012). PlasmidFinder 2.1 (Carattoli et al., 2014) was used to scan for presence of plasmid replicons types using a threshold of 95% of identity and 60% of minimum coverage. Plasmid MLST was investigated using plasmid MLST 2.0 (Carattoli et al., 2014). Subsequently, basic local alignment search tool was used to compare our samples with plasmid reference sequences in the NCBI database. Plasmid sequences with ≥99% identity and ≥92% query coverage were aligned with our sequences using default parameters in MAUVE 2.1 (Darling et al., 2004). Mauve Contig Mover was used to move the plasmid region of our draft genomes to match the reference plasmid. CGView Beta (Grant et al., 2012) was used to visualize the alignment between our plasmid sequence and the reference plasmid sequence pLV23529-CTX-M-8 (GenBank accession number: KY964068; https://www.ncbi.nlm.nih.gov/nuccore/KY964068).

We used PHASTER (Arndt et al., 2016) to detect intact regions of prophages. The GenBank files generated by SEED (Overbeek et al., 2014) were used to detect virulence factors with virulence factor database using default parameters. The *K. pneumoniae* ssp. *pneumoniae* HS11286 was used as reference (Liu et al., 2019a). Draft genomes of sequenced isolates (ID 48, ID 190, and TQ2) were submitted to NCBI GenBank Database (BioProject PRJNA629588; Biosamples SAMN14783702, SAMN14783703 and SAMN14783704).

Statistical Analyses

Analyses were carried out at the isolate-level. Isolates from fecal (milking parlor, milking parlor holding pens, and barns or pasture grazing areas) and rectal swabs were grouped into a single category. Antimicrobial resistance rates (n of resistant isolates divided by n of tested isolates) were estimated for each antimicrobial and stratified according to sample type. Confidence intervals (95% CI) were estimated using robust standard errors. These were calculated based on the observed variability of resistance phenotypes among herds (clusters) instead of isolates. For comparison of AMR results according to sample type, we first excluded CM isolates, as farm effects would be indistinguishable from effects of sampling types. In addition, bulk tank milk samples were excluded from comparisons, as the relative low number of isolates precluded in-depth assessments. We then used generalized linear mixed models with herd-specific random effects to compare drug-specific resistance phenotypes between sample types collected in same herds (fecal, IMI, hindlimbs). Models were fit using maximum likelihood, with an adaptative Gauss-Hermite quadrature with 50 integration points in R 3.6.1 (https://www.r-project.org/). The lme4 package (Bates et al., 2015) was used for analyses. For uncommon resistance phenotypes (<5 resistant isolates or in presence of 0 cells), exact logistic regression models were used for the same purpose. As there was a lack of independence due to isolates obtained from same herds, herd was specified as a strata variable in exact logistic regression models. We reported *P*-values associated with the conditional probabilities test.

We used a second AMR classification system based on the European Committee on Antimicrobial Susceptibility Testing epidemiological cut-off (**ECOFF**) values (EUCAST, 2020). The goal was to determine whether main findings were dependent on the breakpoints adopted, which are known to be imprecise for animal isolates. First, diameters of inhibition zones were classified according to the *K. pneumoniae* ECOFF value for any given antimicrobial. For classification of tobramycin and enrofloxacin results, the gentamicin and ciprofloxacin ECOFF were used, respectively. Additionally, as the ECOFF of tetracycline is not available for *K. pneumoniae,* the breakpoint of *Salmonella* spp. was used instead. Thereafter, all analyses were repeated, and conclusions were compared. As findings were independent of breakpoints adopted (results not shown), results from the first approach were used for presentation.

Finally, Fisher's Exact test was used to estimate associations between presence of *K. pneumoniae* in swabs and sample type. In addition, we used generalized linear mixed models with random effects to estimate quarter- and animal-level prevalence of *K. pneumoniae.* For these analyses, we reshaped the data accordingly before analyses. Statistical significance was set at 5% level.

RESULTS

Klebsiella pneumoniae–Positive Samples

Overall, 4,811 quarter-level milk samples were collected from 1,244 cows housed in 12 herds, ranging from 26 to 331 cows sampled per herd (average of 103 cows/herd). The remaining 185 quarters were deemed nonfunctional, and thus were not sampled. Out of the 4,811 milk samples, 3,264 were CMT-negative, and 291, 309, and 909 samples yielded CMT scores 1, 2, and 3, respectively. Thirty-eight samples were collected from mastitic quarters. From a total of 1,547 milk samples collected for bacteriological culturing, *K. pneumoniae* was isolated from 28 (Table 1), with an estimated quarter-level prevalence of 0.04 cases per 100 quarters (95% CI: 0.01–0.33). As for cow-level results, *K. pneumoniae* was detected in milk samples from 25 lactating cows (1 or 2 isolates per cow) from 4 herds, with an estimated prevalence of 0.60 cases per 100 cows (95% CI: 0.05–1.82). One herd contributed 23 isolates from IMI (Table 1).

In total, 288 swabs were collected from 12 herds (24 swabs per herd: 18 fecal and 6 hind limbs swabs). We detected *K. pneumoniae* in 83 swabs (29%), with no significant difference between hind limbs and fecal swabs isolation rates ($P = 0.30$). On average, 7 *K. pneumoniae*-positive swabs were collected per herd, ranging from 3 to 13. Additionally, *K. pneumoniae* isolates were recovered from 5 out of 12 bulk tank samples (Table 1).

Antimicrobial Resistance

Resistance was commonly detected against tetracycline (22.5% of all isolates), streptomycin (20.7%), and sulfamethoxazole-trimethoprim (9.5%; Table 3). In contrast, resistance against ciprofloxacin, gentamicin,

Table 3. Absolute frequency (N), estimated proportion (%), and respective 95% CI of drug-specific resistance of 169 *Klebsiella pneumoniae* isolates from Brazilian dairy herds

	Feces (n = 66)		IMI (n = 28)		Hind limbs (n = 17)		Tank (n = 5)		CM[2] (n = 53)		Total (n = 169)	
Antimicrobial[1]	N	% (CI 95%)	N	% (CI 95%)	N	% (CI 95%)	N	% (CI 95%)	N	% (CI 95%)	N	% (CI 95%)
Cefoxitin	0	—	0	—	0	—	0	—	0	—	0	—
Cefotaxime	0	—[a]	2	7.1 (0.1; 90.4)[b]	0	—	1	20.0 (0.8; 88.9)	0	—	3	1.8 (0.2; 13.0)
Ceftazidime	0	—	0	—	0	—	0	—	0	—	0	—
Meropenem	0	—	0	—	0	—	0	—	0	—	0	—
Ciprofloxacin	0	—	0	—	0	—	0	—	1	1.9 (0.5; 6.9)	1	0.6 (0.1; 2.5)
Enrofloxacin	0	—	0	—	0	—	0	—	1	1.9 (0.5; 6.9)	1	0.6 (0.1; 2.5)
Streptomycin	5	7.6 (2.1; 23.9)[a]	8	28.6 (6.6; 69.3)[b]	2	11.8 (3.1; 35.9)	1	20.0 (0.8; 88.9)	19	35.8 (20.9; 54.2)	35	20.7 (13.3; 30.8)
Gentamicin	0	—	0	—	0	—	0	—	2	3.8 (1.0; 13.4)	2	1.2 (0.3; 4.9)
Tetracycline	3	4.5 (1.4; 13.5)[a]	7	25.0 (19.0; 32.1)[b]	5	29.4 (14.2; 51.1)[b]	1	20.0 (0.8; 88.9)	22	41.5 (34.5; 48.8)	38	22.5 (15.2; 32.0)
Chloramphenicol	0	—[a]	5	17.9 (7.7; 36.2)[b]	0	—	1	20.0 (0.8; 88.9)	8	15.1 (5.9; 33.5)	14	8.3 (2.5; 24.3)
SXT[2]	0	—[a]	6	21.4 (15.9; 28.3)[b]	0	—	0	—	10	18.9 (9.1; 35.2)	16	9.5 (3.6; 22.5)
SDR[3]	7	10.6 (3.1; 30.3)[a]	10	35.7 (12.8; 67.8)[b]	5	29.4 (14.2; 51.1)[b]	2	40.0 (3.8; 91.9)	31	58.5 (43.7; 71.9)	55	32.5 (22.2; 44.9)
MDR[4]	0	—[a]	5	17.9 (11.8; 26.0)[b]	0	—	0	—	8	15.1 (4.7; 38.9)	13	7.7 (2.4; 21.8)

[a,b]Proportions followed by different superscripts denote statistically significant differences between sample types at 5% level. Analyses were carried out using generalized linear mixed models or exact logistic regression models. Isolates from bulk tank and clinical mastitis samples were not considered (as described under Material and Methods).

[1]SXT = sulfamethoxazole-trimethoprim combination; SDR = single-drug resistance (resistance to at least 1 antimicrobial); MDR = multidrug resistance (resistance to at least 3 distinct classes of antimicrobials).

[2]CM = clinical mastitis.

cefotaxime, and enrofloxacin was relatively uncommon (<2% for all). Fourteen isolates, or 8.3% of total, were resistant against chloramphenicol. No isolates were resistant against meropenem, cefoxitin, or ceftazidime.

Resistance rates to cefotaxime, streptomycin, tetracycline, chloramphenicol, and sulfamethoxazole-trimethoprim were higher in *K. pneumoniae* isolated from IMI in comparison to fecal isolates (Table 3). Isolates from IMI were 8.2 times more likely (odds ratio = 7.2; 95% CI = 1.5–35.5) to be resistant against streptomycin than fecal isolates. Additionally, the odds of resistance were 35.5, 7.0, 5.3, and 8.9 times higher in *K. pneumoniae* from IMI in comparison to fecal isolates for cefotaxime, tetracycline, chloramphenicol, and sulfamethoxazole- trimethoprim, respectively. Likewise, the odds of single-drug resistance and MDR were 8.6 (95% CI: 1.7–43.7) and 7.4 (95% CI: 1.1–100+) times higher in *K. pneumoniae* isolated from IMI compared with fecal bacteria. No differences were detected between hind limbs and IMI isolates for any tested antimicrobial. In addition, 27.8% of *K. pneumoniae* isolated from hind limbs were resistant against tetracycline; for fecal isolates, the percentage was 4.5% ($P < 0.05$).

Most fecal isolates (89.6%) were susceptible to all antimicrobials (Supplemental Table S1, https://data.mendeley.com/datasets/h74fx6996c/1, Nobrega, 2021). Five IMI (19.2%) and 8 mastitic isolates (15.1%) were considered MDR (Table 3). Most frequently detected resistance patterns included single-drug resistance to tetracycline or streptomycin (Supplemental Table S1). As for MDR isolates, co-resistance against streptomycin, tetracycline, chloramphenicol, and sulfamethoxazole-trimethoprim was the most commonly detected pattern, irrespective of sample type (Supplemental Table S1).

We detected *tetA* exclusively in isolates from milk (IMI and CM), whereas we detected *tetD* in mastitic, fecal, and hind limb isolates (Table 4). No isolate harbored the *tetG* gene. As for resistance determinants against sulfamethoxazole-trimethoprim, 8 isolates from milk harbored the *sul2* gene. Two isolates carrying *sul* resistance determinants also harbored the *dfrA12* gene. The *catA2*, *cm1A* and *floR* genes, associated with chloramphenicol resistance, were detected in isolates from CM exclusively (Table 4). No tetracycline or chloramphenicol resistance genes were detected in *K. pneumoniae* isolated from bulk tank milk.

Extended-Spectrum β-Lactamase and Whole Genome Sequencing

Two *K. pneumoniae* isolates from IMI and 1 from bulk tank milk were ESBL-positive, and thus selected for whole genome sequencing. The remaining 166

isolates were considered ESBL-negative in the double disk-diffusion test. The 3 ESBL-positive isolates were from the same farm (herd 2) and of identical phenotypes (resistance to streptomycin and cefotaxime in addition to ESBL-positive). The 2 isolates from IMI were from distinct cows. Sequencing revealed that all 3 shared the same ARG, virulence factors, and were of same sequence type (ST281).

The ESBL genes $bla_{\text{CTX-M8}}$ and $bla_{\text{SHV-108}}$ were detected in the 3 isolates alongside aminoglycoside phosphotransferases [*aph(6)-ld* and *aph(3″)-lb*]. We also detected ARG related to antimicrobial classes not screened (Table 5).

Further characterization revealed presence of Inc.I1 pST114 plasmids harboring the $bla_{\text{CTX-M8}}$ gene in all isolates. The $bla_{\text{CTX-M8}}$ region was characterized by an insertion sequence element (IS4) in the upstream region. The bulk tank isolate had 2 additional insertion sequence elements (IS26) flanking the $bla_{\text{CTX-M8}}$ region. The local alignment search tool revealed high coverage (>92%) and percent identity (>99%) with plasmid pLV23529-CTX-M8 (accession number: KY964068) recovered in *E. coli* isolates from swine. The pLV23529-CTX-M8 is also an Inc.I1 plasmid, but from a different sequence type (pST113).

The bulk tank isolate contained an Inc.X3 plasmid and a satellite prophage P4 not detected in remaining isolates. In contrast, all isolates shared same plasmids (e.g., Inc.FIB, Inc.FII) and the intact sequence of prophage P88.

DISCUSSION

Herein we reported on the AMR profile of *K. pneumoniae* isolated from dairy herds in Brazil. Isolates from IMI had increased AMR rates in comparison to fecal ones. We have shown that, although *K. pneumoniae* is not routinely detected in CMT-positive quarters, it is present in the environment, corroborating an existing body of literature (Zadoks et al., 2011). We also first reported on 2 ESBL-related genes in dairy herds, and we demonstrated that environmental isolates harbor ARG also encountered in isolates from milk. Finally, resistance to CIA such as meropenem, ciprofloxacin, and ceftazidime was uncommon or absent, regardless of the source of bacteria.

In Brazil, the AMR profile of *Staphylococcus* spp. (de Freitas Guimarães et al., 2013), *Streptococcus* spp. (Duarte et al., 2004), and *E. coli* (Lira et al., 2004) isolated from dairy herds has been well-characterized. In contrast, the literature reporting on *K. pneumoniae* in Brazilian herds is scarce; their role as a potential reservoir of ARG remains unexplored. As such, our study is among a few others reporting on the epidemiology of IMI caused by *K. pneumoniae* and providing estimates of AMR in this group of bacteria.

Fluoroquinolones, aminoglycosides, and sulfonamides are commonly used as mastitis treatment in Brazilian dairy herds (Tomazi and Dos Santos, 2020). Herein, we report on the AMR rates of *K. pneumoniae* that cause CM on Brazilian farms. Although it would be

Table 4. Antimicrobial resistance genes screened according to resistance phenotype and source

Resistance	Gene	Sample type				Total
		CM[1]	Fecal	IMI	Hind limbs	
Tetracycline	*tetA*	1	0	4	0	5
	tetB	5	1	0	1	7
	tetC	0	0	0	1	1
	tetD	6	1	0	2	9
	tetG	0	0	0	0	0
Chloramphenicol	*floR*	2	NT[2]	0	NT	2
	catA2	1	NT	0	NT	1
	cm1A	1	NT	0	NT	1
	qnrB	0	NT	NT	NT	0
Fluoroquinolones	*qnrS*	0	NT	NT	NT	0
	aac(6′)-Ib	0	NT	NT	NT	0
Sulfamethoxazole-trimethoprim combination	*dfrA5*	0	NT	0	NT	0
	dfrA12	1	NT	1	NT	2
	sul1	0	NT	1	NT	1
	sul2	4	NT	4	NT	8
	sul3	0	NT	0	NT	0

[1]CM = clinical mastitis.
[2]NT = not tested due to absence of phenotypic resistance.

Table 5. Comparative results of multilocus sequence typing, presence (+) of phages, plasmids, and resistance genes in 3 extended-spectrum β-lactamase–producing *Klebsiella pneumoniae* isolates sequenced

		Sample type		
		IMI	IMI	Tank
Multilocus sequence typing	Resistance associated	ST281	ST281	ST281
Phages				
P88		+	+	+
P4				+
Plasmids				
Inc.FIB		+	+	+
Inc.FII		+	+	+
Inc.HI1B		+	+	+
Inc.I1-I/pST114		+	+	+
Inc.R		+	+	+
Inc.X3				+
Resistance determinants				
oqxA	Multidrug resistance	+	+	+
$bla_{\text{SHV-108}}$	Carbapenem, cephalosporin, penam	+	+	+
$bla_{CTX\text{-}M8}$	Cephalosporin	+	+	+
KpnE	Multidrug resistance	+	+	+
KpnF	Multidrug resistance	+	+	+
KpnG	Multidrug resistance	+	+	+
aph(6")-Id	Aminoglycoside	+	+	+
aph(3")-Ib	Aminoglycoside	+	+	+
ompK37	Monobactam, carbapenem, cephalosporin, cephamycin	+	+	+
CRP	Macrolide, fluoroquinolone, penam	+	+	+
Mutated *EF-Tu*	Elfamycin	+	+	+
fosA6	Fosfomycin	+	+	+
Mutated UhpT	Fosfomycin	+	+	+
Virulence factors				
entABCDEFS	Enterobactin	+	+	+
fepABCDG		+	+	+
iroE/iroN	Salmochelin	+	+	+
tssA-M	T6SS – locus I and II	+	+	+
ompA	T6SS – locus I	+	+	+
dotU	T6SS – locus II	+	+	+
impF	T6SS – locus II	+	+	+
traBEG	T5SS	+	+	+

tempting to use our estimates to guide selection of antimicrobials to treat CM caused by *K. pneumoniae* in Brazilian herds, we recommend that consideration be given to the selection of cases that are likely to benefit from treatment, as well as to the effect of susceptibility breakpoints on the outcome of antimicrobial therapy. First, not all cases of CM caused by *K. pneumoniae* will require the administration of antimicrobials. In general, treated and untreated quarters or cows have comparable culling, recurrence, and voluntary dry-off rates as well as postinfection SCC levels (Fuenzalida and Ruegg, 2019a), in agreement with results detected when culture-negative cases are enrolled (Fuenzalida and Ruegg, 2019b). However, CM caused by *K. pneumoniae* nonsusceptible to ceftiofur (defined using the *E. coli* susceptibility breakpoint for mastitis) have worse prognosis in comparison to CM caused by susceptible *K. pneumoniae* (Fuenzalida and Ruegg, 2019a). Optimal management of CM caused by drug-resistant *K. pneumoniae* might include, for example, the administration of antimicrobials that are active in vitro against the bacteria. Nevertheless, further research is needed to determine the effect of nonsusceptibility in the course of CM, possibly involving a variety of resistance profiles, as well as to determine optimal strategies to deal with infections caused by *K. pneumoniae* that is resistant to a variety of antimicrobials. Second, we currently lack *K. pneumoniae*-specific breakpoints for antimicrobials used as mastitis therapy. Here, we turned to ECOFF and clinical breakpoints that were based on susceptibility results of bacteria isolated from humans, as done elsewhere (Saini et al., 2012b; de Jong et al., 2018, NORM/NORM-VET, 2019). Antimicrobial resistance defined by using human clinical breakpoints can be imprecise to guide antimicrobial therapy of CM (Hoe and Ruegg, 2005). As such, there is no guarantee that the therapy outcome will be associated with the susceptibility profile of *K. pneumoniae* isolated from CM.

The use of CIA in livestock has come under scrutiny due to their potential effects on human health. Our drug panel included CIA routinely used to treat infections in humans caused by drug-resistant *K. pneumoniae* (e.g., meropenem, ceftazidime, ciprofloxacin). With the exception of streptomycin, resistance to CIA was uncommon, in agreement with previous reports from other countries (Makovec and Ruegg, 2003; Saini et al., 2012b; de Jong et al., 2018). Our results suggested a low contribution of *K. pneumoniae* from dairy herds to resistance against critically important β-lactams (e.g., carbapenems) and fluoroquinolones in bacteria isolated from the human population in Brazil.

In our study, 35.8% of CM isolates were resistant against streptomycin, which is well-above the 17.4% of streptomycin resistance for *K. pneumoniae* isolated from Canadian dairy herds (Saini et al., 2012b). In Brazil, aminoglycosides, which are considered CIA, are among the most commonly used intramammary antimicrobials for treatment of CM (Tomazi and Dos Santos, 2020). The same is not true in Canadian dairy herds, where the use of aminoglycosides is relatively infrequent (Saini et al., 2012a). Although resistance to streptomycin in *K. pneumoniae* can be associated with the administration of drug classes other than aminoglycosides (Saini et al., 2013), it is far more likely that the streptomycin resistance level observed in our study reflected the high consumption of aminoglycosides in Brazilian dairy herds. Indeed, the usage level of antimicrobials commonly administered to treat IMI was associated with drug-specific resistance of bacteria causing mastitis (Pol and Ruegg, 2007a). We recommend that further consideration be given to the contribution of the high consumption of aminoglycosides in Brazilian dairy herds toward recent increases of AMR in zoonotic bacteria in Brazil (Van Boeckel et al., 2019; Rodrigues et al., 2020).

When contrasting AMR rates across studies, there are several factors that should be considered as potential sources of inconsistency between studies, such as the methods used for antimicrobial susceptibility testing, definitions, sampling strategies, and differences in prevalence of AMR (Erskine et al., 2002). Our AMR estimates against tetracycline (41.5%) and sulfa-trimethoprim (18.7%) were higher than those reported from the United States, Europe, and Canada, where resistance rates ranged from 18.3 to 33% for tetracycline, and from 2.7 to 5% for sulfa-trimethoprim (Erskine et al., 2002; Makovec and Ruegg, 2003; Saini et al., 2012b; de Jong et al., 2018). In comparison to a dairy study in China, AMR rates in our Brazilian isolates were increased for tetracycline (41.5% in Brazil vs. 32% in China) and comparable for enrofloxacin (~2% for the 2 countries). Additionally, resistance against carbapenems was infrequent in *K. pneumoniae* from dairy herds of the 2 countries (Cheng et al., 2019). Resistance against chloramphenicol in our Brazilian isolates (15.1%) was higher than the one reported in Canada (1.8%). In contrast, cefoxitin resistance in our *K. pneumoniae* isolates (0%) was lower than the one observed in Canadian herds (6.4%; Saini et al., 2012b). Our gentamicin resistance levels (3.8%) were slightly above those reported from studies in Canada, United States, and China (0–1.8%; Erskine et al., 2002; Saini et al., 2012b; Cheng et al., 2019).

We purposively excluded CM isolates from comparisons because they were sampled from farms that did not contribute to environmental samples. Nonetheless, it is likely that resistance rates of *K. pneumoniae* that cause CM in herds that contributed to environmental samples were comparable to those estimated for IMI isolates from the same farms. For example, Saini et al. (2013) found no differences in resistance levels of *K. pneumoniae* from IMI, subclinical mastitis, and CM isolated from the same farms. In our study, isolates from IMI had increased resistance rates against streptomycin, tetracycline, chloramphenicol, cefotaxime, and sulfamethoxazole-trimethoprim when compared with fecal ones. The increased AMR rates in isolates from milk could be explained by the exposure of quarters to a common extramammary source of resistant bacteria, or a contagious-like spread of udder-adapted resistant clones. Indeed, contagious-like transmission has been reported for outbreaks of CM caused by *K. pneumoniae* (Munoz et al., 2007). It has been suggested that some strains of *K. pneumoniae* that cause IMI will likely have an increased udder adaptability and a contagious nature of transmission due to lower diversity indexes observed in milk isolates when compared with extramammary ones (Cheng et al., 2021). Here, we had 2 almost identical ESBL-positive strains detected in milk samples from different cows with IMI in the same herd. This suggested that the within-herd dissemination of resistant clones partially accounted for the increased rates of AMR observed in isolates from IMI.

Several genes have been linked to drug-specific resistance in bacteria that cause mastitis (Nobrega et al., 2018). Our findings demonstrated that genetic elements such as *sul* and *tet* genes are commonly present in *K. pneumoniae* obtained from Brazilian dairy herds. Not surprisingly, distribution of genes varied according to sample type, as previously observed (Liu et al., 2019b). The *tetA* and *tetB* genes are predominant resistance determinants in *E. coli* isolated from dairy herds (Sawant et al., 2007). The same seems to apply for *K. pneumoniae*, but with *tetD* added to the pool. The most commonly detected genetic profile of tetracycline-resistant *K. pneumoniae* included concomitant presence

of major facilitator superfamily transporter genes *tetB* and *tetD*, irrespective of sample type. Both elements encode tetracycline efflux pumps, and it is unknown whether a synergistic effect exists following the co-presence of the 2 elements in same strains.

Detection of ESBL-producing strains of *K. pneumoniae* in dairy herds is of great concern due to their increasing importance in public health (Seiffert et al., 2013). In our study, 3 *K. pneumoniae* were characterized as ESBL-producers. The ESBL genes $bla_{\text{CTX-M-8}}$ and $bla_{\text{SHV-108}}$ were detected in the 3 sequenced strains. Disturbingly, 1 isolate was from bulk tank milk. In Brazil, 34% of milk production is sold either as part of a state or municipal inspection system, or as part of an informal market under limited surveillance (Spers et al., 2013). It remains uncertain whether increased human exposure to ESBL-encoding genes will result in increased rates of community-acquired nontreatable human infections. Likewise, it is unclear whether these enzymes are linked to reduced efficacy of β-lactams commonly used to treat mastitis. The CTX-M enzymes have been previously described in *K. pneumoniae* isolated from dairy herds worldwide (Locatelli et al., 2010; Timofte et al., 2014; Koovapra et al., 2016). Although there is evidence that $bla_{\text{CTX-M-8}}$ is circulating in Brazilian beef herds (Palmeira et al., 2020), companion animals (Melo et al., 2018), poultry (Norizuki et al., 2017), and hospitals (Bonnet et al., 2000), to the best of our knowledge, the 2 genes have never been reported in isolates from dairy cattle before. The majority of reports in Brazil described a plasmid Inc.I1 harboring the $bla_{\text{CTX-M-8}}$ gene, which is in line with our study. Although a preferential carriage of $bla_{\text{CTX-M-8}}$ Inc.I1 pST113 plasmids have been reported, our study reinforced the epidemiological importance and dissemination of $bla_{\text{CTX-M-8}}$ Inc.I1 pST114 plasmid in food-producing animals in Brazil; the same plasmid is also circulating in Brazilian poultry isolates (Ferreira et al., 2014; Norizuki et al., 2017).

In addition to β-lactamase genes, our isolates also harbored genes encoding aminoglycoside-modifying enzymes and multidrug-resistant efflux pumps, including the *acrAB* operon that, on top of being linked to drug resistance, also plays a role as a virulence factor (Wang-Kan et al., 2017). Moreover, the 3 isolates belonged to same sequence type ST281, previously described in community-acquired infections in humans and companion animals (Garza-Ramos et al., 2018; Marques et al., 2019). In regard to virulence factors, our samples contained the *iroE* and *iroN* genes, associated with salmochelin siderophores, which contribute to cell invasion (Müller et al., 2009). The 2 genes are prevalent in human isolates. *Klebsiella pneumoniae* isolated from cows usually harbors enterobactin, another siderophore type (Yang et al., 2019), also detected in our isolates. Additionally, our samples contained genes that encode proteins of the secretion system type VI locus I and II, which form a needle-like structure similar to a bacteriophage tail responsible for the translocation of effector proteins into host cells, in addition to playing a role in bacterial competition (Storey et al., 2020). Genes from the secretion system type IV were also detected; this system works like a conjugation system transferring molecules intercellularly, increasing diversity among bacterial cells (Christie, 2001).

Mobile genetic elements, such as plasmids and phages, will contribute to bacteria diversification and spread of ARG. Our tank isolate had a phage P4 and plasmid replicon Inc.X3. The plasmid replicons Inc.FII(K), Inc. X3, Inc.R, and Inc.HIIB have been previously reported in resistant *K. pneumoniae* that cause human infections (Holt et al., 2015), which suggests the dissemination of mobile elements associated with AMR in *K. pneumoniae* strains. Phage P88, a P2-like phage detected in our samples, is prevalent in pathogens causing enteric diseases such as enterotoxigenic *E.coli* K88 (Magaziner et al., 2019). The phage P4 detected in our tank isolate is a satellite phage that depends on P2 phages for assembly. Their presence in *K. pneumoniae* has been described (Shen et al., 2020). Prophage regions act as a mode of horizontal gene transfer, playing an important role in bacterial evolution. They carry virulence genes associated with adherence and invasion, affect expression of bacterial genes, and contribute to transfer of ARG.

Our findings need to be considered in face of limitations and caveats. We did not have access to detailed herd-level information for farms enrolled in the second sampling protocol, which precludes estimation of incidence rates of CM caused by *K. pneumoniae* in Brazilian dairy herds. Yet, we must stress that our primary goal was to estimate and compare the AMR rate of *K. pneumoniae* isolated from many sources on dairy farms. Additionally, we did not detect ARG in most resistant isolates, suggesting that elements other than ones screened were responsible for resistant profiles. Indeed, whole genome sequencing of ESBL-positive isolates revealed presence of multidrug resistance elements, aminoglycoside-modifying enzymes, and virulence genes that were not screened using PCR. It is unclear whether similar elements were present in remaining isolates. Finally, the Brazilian dairy industry has experienced a growth in the last 2 decades (IBGE, 2020). Yet, we believe that our findings are still valid for the contemporary Brazilian dairy industry for at least 2 reasons. First, most of the increase in the Brazilian milk production occurred between 1995 and 2010, whereas from 2011 onwards, the relative change in the overall milk production compared with the previous years was much

less pronounced (IBGE, 2020). Second, the antimicrobial formulations available for dairy producers in Brazil have remained relatively constant over the last decade; essentially, the same antimicrobials have been used to treat infectious diseases of dairy cattle over the past 10 yr. As the AMR and drug-usage profile are intertwined (Saini et al., 2013), major changes in AMR rates of mastitis pathogens isolated from Brazilian herds in the last decade are not to be expected.

CONCLUSIONS

Here, we investigated the AMR profile of *K. pneumoniae* isolated from distinct sources in Brazilian dairy herds. Resistance against tetracycline, streptomycin, and combination of sulfamethoxazole and trimethoprim were relatively common. In contrast, no isolates were resistant against meropenem, cefoxitin, or ceftazidime. *Klebsiella pneumoniae* that cause IMI demonstrated increased resistance rates against cefotaxime, streptomycin, tetracycline, chloramphenicol, and sulfamethoxazole-trimethoprim when compared with fecal isolates. We detected *tetA* exclusively in isolates from milk. Resistance against sulfonamides was associated with presence of *sul1* and *sul2*. Whole genome sequencing of ESBL-positive *K. pneumoniae* isolates revealed presence of $bla_{\text{CTX-M8}}$ and $bla_{\text{SHV-108}}$. Isolates were of sequence type ST281 and had similar mobile genetic elements and virulence genes.

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