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Molecular Subtyping of Mastitis-Associated *Klebsiella pneumoniae* Isolates Shows High Levels of Diversity Within and Between Dairy Herds

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ABSTRACT

Despite advances in controlling mastitis (inflammation of the mammary gland), udder infections caused by *Klebsiella pneumoniae* continue to affect dairy cattle. Mastitis caused by *K. pneumoniae* responds poorly to antibiotic treatment, and as a consequence, infections tend to be severe and long lasting. We sought to determine whether a nonrandom distribution of specific genotypes of *K. pneumoniae* was associated with mastitis from 6 dairy herds located in 4 different states. A total of 635 isolates were obtained and fingerprinted by repetitive DNA sequence PCR. Significant genetic diversity was observed in 4 of the 6 dairy herds analyzed, and a total of 49 genotypic variants were identified. Within a herd, Simpson's diversity indices were 91.0, 94.1, 91.7, 88.6, 53.3, and 64.3% for dairies A, B, C, D, E, and F, respectively. The association between matrices of genetic similarity and matrices of temporal distance was negative in all the dairies analyzed. Four dairies had a high incidence of *K. pneumoniae* mastitis during the winter. The majority of genotypes were unique to herds of origin, and only 5 genotypes were detected in more than 2 dairies. Genotype 1 (arbitrary designation) occurred most frequently across dairies and was found in 25.2% of all mastitis cases and among 22.8% of reinfected and culled cows in dairy A. Specific genotypes also tended to be associated with a specific bedding type and dairy location. Analysis of molecular variance showed that 18% of the genetic diversity was due to variation among herds within states, and 82% of the genetic diversity was accounted for by variation of genotypes within herds. The data support the idea that mastitis is caused by a diverse group of *K. pneumoniae* genotypes and thus has major implications for the diagnosis, prevention, and treatment of udder infections in dairy cows.

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INTRODUCTION

Coliform bacteria are a common cause of mastitis and mastitis-related death in dairy cows (Roberson et al., 2004). Mastitis caused by *Klebsiella pneumoniae* can be particularly severe because of its poor response to conventionally applied antibiotic therapy and rapid progression to toxic shock and death (Sampimon et al., 2006). Thus, mastitis caused by *Klebsiella* spp. results in higher losses to the dairy producer compared with *Escherichia coli* (Grohn et al., 2004). Wenz et al. (2001) isolated *K. pneumoniae* from 42% of the coliform mastitis cases classified as severe, and Cebra et al. (1996) found that up to 32% of cows with coliform mastitis developed bacteremia. Other studies have reported that coliform bacteria were responsible for a large proportion (40% to greater than 50%) of acute mastitis episodes (Erskine et al., 1991), with up to 39.4% of mastitis cases caused by gram-negative bacteria attributable to *Klebsiella* spp. (Todhunter et al., 1991).

Currently, the most effective control measures for coliform mastitis are prevention by implementing appropriate management strategies and immunization by using the core antigen bacterin of *E. coli* strain J5 or *Salmonella* Typhimurium Re17 (Hogan et al., 1995; Wilson and Gonzalez, 2003). Vaccination is beneficial in reducing the clinical severity of coliform mastitis; however, it is not efficacious in preventing new infections (Wilson and Gonzalez, 2003). Thus, mastitis caused by *Klebsiella* spp. continues to affect the dairy industry. A better understanding of how *Klebsiella* spp. is involved in mastitis and its epidemiology is needed. Molecular characterization of the *K. pneumoniae* responsible for causing bovine mastitis is lacking (Kikuchi et al., 1995). Understanding the molecular diversity would assist in the development of appropriate strategies for the prevention and control of udder infections in dairy cows. The economic losses, ineffectiveness

of conventional control methods, and unsuccessful treatment rates of *Klebsiella* mastitis make this study timely and relevant. In our recent studies with a variety of *K. pneumoniae* isolates from clinical cases of mastitis in a single dairy herd, we identified a high degree of genetic diversity in *K. pneumoniae* (Paulin-Curlee et al., 2007). To determine whether a similar level of genetic diversity is present across dairy herds, we analyzed mastitis-associated *K. pneumoniae* isolates by repetitive DNA sequence PCR (**rep-PCR**) from 6 dairy herds from 4 different states.

MATERIALS AND METHODS

K. pneumoniae Isolates

Klebsiella pneumoniae isolated from animals with clinical mastitis (n = 598) or subclinical mastitis (n = 18) were collected from 1 mo up to 1.5 years. Samples were obtained from 6 dairy herds in 4 states (Indiana, Minnesota, Wisconsin, and Pennsylvania). Isolates (n = 12) from one bulk tank milk sample and from a bedding sample (n = 10) from recycled manure solids were also obtained from one dairy located in Wisconsin. Each dairy was identified by alphabetic designation, and the state locations and bedding types, respectively, are as follows: dairy A (WI, recycled manure solids); dairy B (IN, sand); dairy C (WI, sawdust and wood shavings); dairy D (MN, sawdust); dairy E (PA, sawdust and wood shavings); and dairy F (WI, wood shavings). A total of 635 isolates were analyzed, 67.4% originating from dairy A (n = 428) and 32.6% from dairy B (n = 93), dairy C (n = 27), dairy D (n = 54), dairy E (n = 15), and dairy F (n = 18), respectively. Milk, bulk tank milk, and bedding samples were sent to the Laboratory for Udder Health (University of Minnesota, St. Paul), where they were processed using routine culture methods to isolate and identify *K. pneumoniae* by culturing the gram-negative and lactose-fermenting bacteria on MacConkey agar plates. The samples selected for use in this study were those in which *K. pneumoniae* was the only organism isolated. Following incubation at 37°C for 18 h, the identity of the isolates was verified by using the API 20E (bioMérieux Vitek Inc., Hazelwood, MO). Three separate colonies from each clinical mastitis sample and 10 to 12 colonies from bedding and bulk tank milk were restreaked and incubated as above. Each colony originating from the same milk sample was identified with the cow number followed by an alphabetic designation (A, B, or C) to indicate different isolates originating from the same sample. All isolates identified as *K. pneumoniae* were preserved in a glycerol-blood solution and frozen at –80°C. Three *K. pneumoniae* isolates from hospitalized human patients at Fairview-University Medical Center (Minneapolis, MN) were included for comparison. The human isolates were derived from blood, sputum, and urine and were provided by the Clinical Microbiology Laboratory (Fairview-University Medical Center, Minneapolis, MN).

rep-PCR

Bacterial genomic DNA was extracted by PrepMan Ultra Reagent (Applied Biosystems, Foster City, CA). Briefly, frozen cultures were streaked onto MacConkey agar plates and incubated. Two individual colonies were harvested and suspended in 300 μL of PBS and pelleted by centrifugation for 3 min at 7,500 × *g*. The supernatant was discarded, 200 μL of PrepMan was added, and the tubes were placed in a heat block for 10 min at 100°C. After incubation, the solution was pelleted for 3 min at 7,500 × *g*, and the supernatant containing DNA was diluted 1:1 with sterile DNase-free water. Repetitive DNA sequence PCR fingerprints were obtained by using a boxA1R primer (5′ CTACGGCAAGGCGACGCTGACG 3′; Goldberg et al., 2006). The 25-μL PCR mixture contained 25 m*M* $MgCl_2$, 10× Buffer II (Applied Biosystems), 25 pmol of boxA1R primer, 100 m*M* deoxynucleotide 5′-triphosphate mix and AmpliTaq Gold DNA polymerase (Applied Biosystems), and 2 μL of DNA (150 ng) template. Polymerase chain reaction conditions were 95°C for 7 min, followed by 30 cycles consisting of 94°C for 1 min, 66°C for 8 min, 71°C for 1 min, and a final extension of 71°C for 15 min, followed by storage at 4°C (Goldberg et al., 2006). The DNA bands were separated by 2% agarose gel electrophoresis at 4°C for 5 to 6 h at 62 V (6 V/cm). Gels were stained in ethidium bromide-1× Tris-acetate-EDTA buffer, and the images were obtained with Labworks 4.0 Image Acquisition and Analysis Software (UVP Inc., Upland, CA) and saved as tagged image files. Reproducibility of PCR was verified as described previously (Paulin-Curlee et al., 2007) by repeating rep-PCR 4 times with 16 separate isolates and freshly extracted DNA each time. Analysis for reproducibility showed an average of 93.6% fingerprint similarity for the same isolate based on 4 iterations with 16 distinct isolates and freshly extracted DNA each time, and was used to establish the 90% similarity cutoff for genotype definition used in this study.

rep-PCR Fingerprint Analysis

Gel images were uploaded and analyzed by using BioNumerics software, version 4.0 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). The positions of the bands in each gel were normalized by using a 1-kb DNA ladder (Hi-Lo DNA Marker, Minnesota Molecular Inc., Minneapolis, MN). Band matching and similarity

of the fingerprints was accomplished by using the band-based Dice coefficient of similarity at 1.5% tolerance and 1.0% optimization.

Statistical Analysis

The MacAnova statistical analysis program (School of Statistics, University of Minnesota, Minneapolis, MN) was used to calculate the correlation between fingerprint similarity matrices and matrices of temporal distances (sample interval). The Spearman rank coefficient was used to calculate the temporal correlation to determine whether isolates collected at shorter sampling intervals were more likely to have similar fingerprints. MacAnova was also used to analyze the association between genotypes and herds, genotypes and the type of bedding used by each dairy herd, and genotypes and the dairy location. A P-value of ≤ 0.05 was considered significant for the temporal correlation and association analysis. Simpson's index of diversity was used for the measurement of genetic diversity and discriminatory power of the typing techniques (Hunter and Gaston, 1988). Simpson's index of diversity was calculated by using BioNumerics software, version 4.0 (Applied Maths BVBA), and it was based on the total number of isolates, the total number of genotypes described, and the number of isolates belonging to each genotype. Dendrograms were generated by using the unweighted pair group method with arithmetic means. Because identical DNA fingerprints obtained from the same clinical mastitis case could bias our analysis (Johnson et al., 2004), all of the duplicate DNA fingerprints originating from the same mastitis case were eliminated in the final analysis. Thus, isolates originating from the same sample having exactly the same DNA fingerprint were represented singly in the analysis.

In addition to the descriptive analysis, analysis of molecular variance (**AMOVA**) was used to partition the variation among isolates within herds, among herds within states, and among states. A matrix was constructed based on the presence or absence of rep-PCR bands for each isolate to compute the genetic distance for each pair of isolates. The analysis was performed by using Genalex 6 (Peakall and Smouse, 2006).

RESULTS

Genetic Diversity of K. pneumoniae Within Herds

Unique genotype patterns were defined by rep-PCR as DNA fingerprints from *K. pneumoniae* whose similarity coefficients were less than 90%. Similarities of 90% or greater by Rep-PCR were assigned to the same genotype and were considered as a cluster. The percentage for similarity cutoff was established at 90% on the basis of reproducibility of the technique, as described previously (Paulin-Curlee et al., 2007).

Dairy A (WI, Recycled Manure Solids). A total of 428 *K. pneumoniae* isolates originating from 141 clinical mastitis cases were collected over 18 mo (June 2003 to December 2004) and the genetic diversity was determined by rep-PCR fingerprinting. The 406 isolates originated from up to 3 separate colonies per mastitis sample. After eliminating replicate isolates with identical DNA fingerprints, a total of 167 isolates were used for the cluster analysis. Simpson's index of diversity was 91.0% for the 30 distinct banding patterns identified, indicating wide genetic diversity and a high degree of discrimination by rep-PCR. The dendogram was arbitrarily divided into 2 major groups, I and II. All the genotypes comprising ≥10 isolates belonged to group I. Group II consisted of all the genotypes comprising ≥1 to ≤ 9 isolates. Genotypes detected a single time were termed unique. Six genotype patterns contained between 11 and 37 isolates each, which accounted for 64.7% of the isolates (group I; Table 1) and were detected throughout the study. The remaining 24 genotypes (35.3%) contained 1 to 7 isolates in each cluster (group II), and 7 unique genotypes were present (R3, R11, R14, R15, R21, R26, and R27). More than one genotype per mastitis case was present 12.1% of the time.

Some cows (17.6%, 16/91) were diagnosed with clinical mastitis 2 to 5 times throughout the study. The majority of these animals (81.3%, 13/16) were infected with a different genotype of *K. pneumoniae* at each infection (Table 1). Nineteen distinct genotypes were detected in 24 cases of reinfection among 16 cows. In 25% (6/24) of the cows that became reinfected, more than one genotype per mastitis case was detected. Genotypes R5, R6, R7 (group I), and R8 (group II) were the most frequently (53.2%, 25/47) isolated genotypes in reinfected cows. Reinfections occurred throughout the study and were the most frequent during winter (45.8%, 11/24) and summer (29.2%, 7/24). Some cows (11%, 10/91) were culled because of persistent *K. pneumoniae* mastitis. Genotype R5 was isolated the most frequently (30%, 3/10) among the 8 distinct genotypes from the 10 culled cows. Some of the genotypes isolated from reinfected cows were also detected in the culled cows (R2, R5, R7, R16, R21, R23, and R24). Genotype R5 was the most frequently isolated genotype in reinfected and in culled cows (22.8%, 13/57), whereas the other genotypes were isolated from reinfected and culled cows 1.8% (1/57) to 10.5% (6/57) of the time.

Analysis of isolates collected on the same date as the bedding and the bulk tank milk samples identified 6 genotypes among 12 isolates (Figure 1). A common genotype was detected among isolates from mastitis, bed-

Table 1. Repetitive DNA sequence PCR (rep-PCR) genotypes of 167 *Klebsiella pneumoniae* isolates from dairy A, divided into groups I and II[1]

Rep-PCR genotypes	Times detected in mastitic cows, n	Times detected in reinfected cows, n	Times detected in culled cows, n
R1	11	2	—
R2	12	3	1
R5[2]	37	10	3
R6	14	5	—
R7	22	5	1
R12	12	4	—
R3	1	1	—
R4	3	—	—
R8	7	5	1
R9	2	—	—
R10	4	1	—
R11	1	—	—
R13	2	1	—
R14	1	—	—
R15	1	—	—
R16	4	1	1
R17	3	—	—
R18	3	1	—
R19	2	2	—
R20	4	—	—
R21	1	—	1
R22	2	1	—
R23	3	—	1
R24	3	1	1
R25	2	1	—
R26	1	—	—
R27	1	1	—
R28	2	1	—
R29	3	1	—
R30	3	—	—

[1]Data analysis of the number of times each genotype was isolated from clinical cases of mastitis, from reinfected cows (including the first infection), and from cows that were culled.

[2]Most common genotype in dairy A.

ding, and bulk tank milk. The percentage of *K. pneumoniae* mastitis was similar during winter (36.2%, 51/141) and summer (32.6%, 46/141). The spring and fall seasons had a lower percentage (15.6%, 22/141) of *K. pneumoniae* mastitis cases. There was a small negative association between the fingerprint similarities matrix and the temporal distances matrix (R = –0.083, P = 0.005), indicating that isolates that were collected closer in time had higher similarity than those collected over a greater interval of time (Table 2).

Dairy B (IN, Sand Bedding). Thirty-one clinical mastitis cases occurring during a 1-yr period (September 2003 to 2004) yielded 93 *K. pneumoniae* isolates for further analysis. Thirty-four isolates were included in the cluster analysis, with 15 distinct genotypes identified. Each genotype comprised 1 to 5 isolates having substantial diversity (Simpson's index, 94.1%). Seventy-one percent (22/31) of *K. pneumoniae* mastitis cases occurred during fall and winter, with summer (19.4%, 6/31) and spring (9.7%, 3/31) accounting for the remaining cases. More than one genotype was isolated from a single mastitis case 9.7% of the time. The remaining 90.3% of the mastitis cases had the same genotype for the 3 separate colonies. When analyzing only the clinical cases for which a single genotype was isolated, 14 genotypes were scattered on the dendrogram and, again, substantial genetic diversity was observed (Simpson's index, 94.7%). As observed in dairy A, there was a negative association between the fingerprint similarities matrix and the temporal distances matrix (R = –0.063, P = 0.165; Table 2).

Dairy C (WI, Sawdust and Wood Shavings). Nine mastitis cases occurred over 6 mo (June through December 2004). Simpson's index of diversity was 91.7% for the 6 distinct genotypes identified among the 27 isolates evaluated. The 3 separate isolates originating from the same clinical mastitis case showed the same genotype. As identified for other dairies, summer (44.4%, 4/9) and winter (33.3%, 3/9) had the highest number of cases of *K. pneumoniae* mastitis, with the remaining 22.2% (2/9) of mastitis cases occurring during fall. A negative association between the fingerprint similarities matrix and the temporal distances matrix was observed (R = –0.162, P = 0.147; Table 2).

Dairy D (MN, Sawdust). Eighteen mastitis cases occurring during November 2004 yielded 54 isolates. Twenty-one isolates were included in the analysis and 18 distinct genotypes were identified. Among the isolates that originated from the same clinical case, a unique genotypic pattern was observed 83.3% of the time. Simpson's index of diversity was 88.6%, indicating the higher degree of genetic diversity of *K. pneumoniae* occurring within this herd. Despite the fact that the majority of mastitis samples (60.0%) were collected at a single time point, a considerably high degree of genetic diversity was observed. There was a negative association between matrices of fingerprint similarities and temporal distances (R = –0.30, P = 0.003; Table 2).

Dairy E (PA, Sawdust and Wood Shavings). Fifteen isolates were obtained over 30 d (September to October 2004), 6 of which were included in the cluster analysis. Simpson's index of diversity was 53.3%, indicating relatively lower genetic diversity than those observed for other herds. The fact that the mastitis cases were identified during a 1-mo period may have limited the identification of genetic diversity. Only 2 unique genotypes were identified among 6 isolates and, as previously observed, a single genotype per mastitis case was detected most of the time (80%).

Dairy F (WI, Wood Shavings). Six subclinical *K. pneumoniae* mastitis cases were identified in cows during the dry period (July and August 2004). Five unique genotypes were identified among 8 isolates; Simpson's index of diversity was 64.3%, and a single genotype

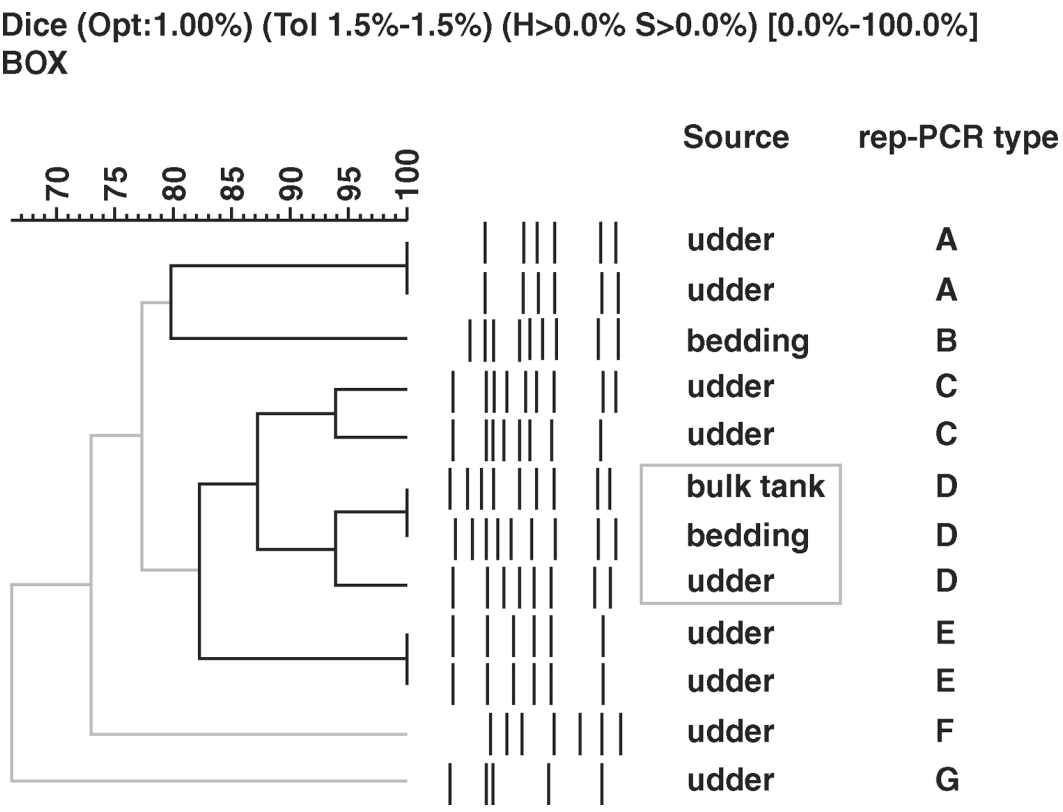

Figure 1. Repetitive DNA sequence PCR (rep-PCR) clustering (BOX primers) of 12 *Klebsiella pneumoniae* isolates originating from clinical mastitis cases from 9 different cows, bedding, and bulk tank milk collected on the same date. Similarities of 90% or greater for rep-PCR were assigned to the same genotype and were considered a cluster. The common genotype (94.1% similarity) detected in bedding, bulk tank milk, and 9 cows with mastitis is highlighted.

pattern was isolated from the majority (66.7%) of the mastitis cases. Sixty percent of the genotypes detected among the subclinical isolates were also detected among the clinical isolates. The remaining genotypes (40.0%) with arbitrarily assigned alleles, 20, 25, and 32 (Table 3), were unique to the subclinical mastitis cases. Genetic diversity was lower when compared with other dairies. As in dairy E, it is possible that the short period of time in which samples were collected (1 mo) may have limited identification of the true diversity in the population. To confirm this hypothesis, we analyzed the genetic diversity of other dairies for the period of approximately 1 mo and, whenever possible, at the same point in time as in dairies E and F. A decrease from 28.0% (dairy D) to 8.4% (dairy C) in Simpson's index was observed when other dairies were analyzed for isolates obtained in a 1-mo period.

Genetic Diversity of K. pneumoniae Among Herds

Forty-nine distinct genotypes were identified when mastitis isolates from the 6 dairy herds [A (n = 167),

Table 2. Number of mastitis cases and isolates examined for each dairy herd and their respective location, herd size, type of bedding, Simpson index of diversity, and temporal correlation values

Dairy	Mastitis cases, n (isolates, n)	Location	Herd size	Type of bedding	Simpson's index of diversity, %	Temporal correlation
A	141 (406)	WI	1,500	Recycled manure solids	91.0	R = –0.083*
B	31 (93)	IN	1,000	Sand	94.1	R = –0.063
C	9 (27)	WI	1,000	Sawdust and wood shavings	91.7	R = –0.162
D	18 (54)	MN	1,100	Sawdust	88.6	R = –0.30*
E	5 (15)	PA	600	Sawdust and wood shavings	53.3	—
F	6 (18)	WI	400	Wood shavings	64.3	—

*Statistically significant at $P \leq 0.05$.

Table 3. Repetitive DNA sequence PCR (rep-PCR) genotypes among 245 *Klebsiella pneumoniae* mastitis cases isolated from 6 dairy herds,[1] the dairies in which each genotype was detected followed by the respective bedding type, and state location

Rep-PCR type	Dairy
8,[2] 10, 11,[3] 15, 16,[2] 17,[2] 27,[2] 28, 34, 35,[2] 40,[3] 42, 44,[2] 45,[2] 46, 48,[2] 49[2,3]	A
31, 38, 41	B
36, 37	C
26, 47	D
20, 25, 32	F
2,[2] 4, 13,[2] 14,[2,3] 24,[3] 39,[2] 43	A, B
7, 21,[2] 22	A, C
5,[2] 6[2,3] 30, 33	A, D
9	A, E
19[5,6]	A, F
12	B, C
3,[2] 29	A, B, D
23[5,6]	A, E, F
18[5]	A, B, F
1[2,3,4]	A, B, C, D, E

[1]Dairy A (n = 167), dairy B (n = 34), dairy C (n = 9), dairy D (n = 21), dairy E (n = 6), and dairy F (n = 8).

[2]Genotypes also detected in reinfected cows from dairy A.

[3]Genotypes also detected in culled cows from dairy A.

[4]Genotype comprising the largest number of isolates (n = 62), and also the most common genotype across dairies.

[5]Matching genotypes in clinical and subclinical cases of mastitis.

[6]Matching genotypes in reinfected cows and subclinical cases of mastitis.

B (n = 34), C (n = 9), D (n = 21), E (n = 6), and F (n = 8)] were combined for comparative fingerprint analysis (n = 245; Table 3). The 49 genotypes defined among all dairies included the genotypes originating from mastitis cases that were also found on dairy A (Table 1 and Figure 1). Genotypes from dairy A were redefined to the cluster analysis including isolates from all dairy herds. Genotype 1 comprised 25.2% (n = 62) of all isolates, whereas the remaining genotypes comprised no more than 0.4% (n = 1) to 6.9% (n = 17) of the isolates. Genotype 1 was also identified in a dairy analyzed previously (Paulin-Curlee et al., 2007). Association analysis was performed to identify any relationship between genotypes and herds, genotypes and the type of bedding used by each dairy herd, and genotypes and dairy location (state). Of the 49 genotypes identified among the 6 dairy herds, 55.1% were unique to the dairy of origin ($P = 0.015$), suggesting that specific genotypes occurred in a particular dairy herd (Table 3). Among the genotypes detected in more than one herd, 34.7, 8.2, and 2.0% occurred in 2, 3, and 5 dairy herds, respectively. Genotype 1 was the most common across all dairies in the study.

An association was also observed between genotypes and the types of bedding used by each dairy. Sawdust and wood shavings were combined into a single bedding category, termed wood by-products. A total of 55.1% of the genotypes were unique to each bedding type used ($P = 0.011$), indicating an association between genotypes and particular types of bedding material. Among the genotypes associated with a particular bedding type, 34.7% occurred with the use of recycled manure solids, 14.3% occurred with the use of wood by-products, and 6.1% occurred with sand. A total of 44.9% of the genotypes were associated with more than one bedding type. Most of those (18.4%) occurred with the use of recycled manure solids and wood by-products, followed by recycled manure and sand (14.3%), recycled manure, wood by-products, and sand (10.2%), and wood by-products and sand (2.0%).

The 6 dairies were located in 4 different states (Wisconsin, Minnesota, Indiana, and Pennsylvania), and an association between genotypes and dairy location was also observed. More than 63% of the genotypes were unique to the state of origin ($P = 0.017$), whereas 36.7% occurred in more than one state. Among the genotypes detected in more than one state, 18.4% occurred in Wisconsin and Indiana, 8.2% occurred in Wisconsin and Minnesota, and 4.1% occurred in Wisconsin and Pennsylvania. Two genotypes (4.1%) were detected in Wisconsin, Indiana, and Minnesota, and only one (2.0%) was detected in all 4 states. The results of AMOVA showed that none of the genetic diversity was due to variation among the states. Eighty-two percent ($P = 0.001$) of the genetic diversity was accounted for by variation among genotypes within herds. Herds within the same state accounted for 18% ($P = 0.001$) of the variation.

The genotypes isolated from reinfected and culled cows in dairy A were also detected in other dairies. Twenty-three genotypes matched those of reinfected or culled cows in dairy A and 52.2% were isolated from infected cows on other dairies. Genotype 1 was the most common among dairies A, B, C, D, and E and was among the genotypes isolated from reinfected and culled cows; it was identical to genotype R5, which was predominant in dairy A (Table 1). Two genotypes from subclinical cases of mastitis (19 and 23) were also found in cases of reinfection.

Overall, the incidence of *K. pneumoniae* mastitis was highest during winter for all of the dairies in this study except one (Figure 2). One dairy had a higher incidence of *K. pneumoniae* during summer. Dairy A had the highest incidence of *K. pneumoniae* mastitis overall.

DISCUSSION

Genetic diversity among *K. pneumoniae* isolates from mastitis and environmental sources is expected to provide critical missing information on the sources and spread of this mastitis pathogen. We studied 6 dairies

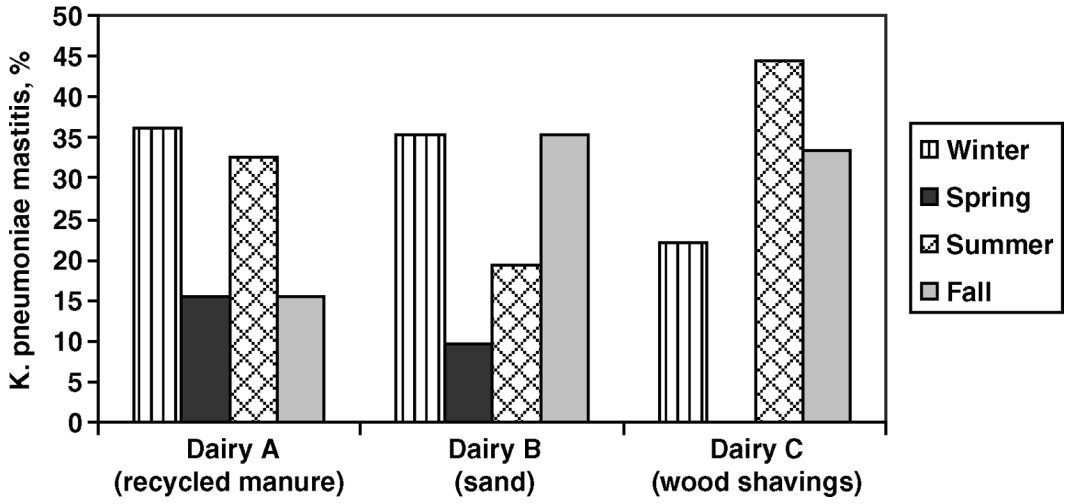

Figure 2. Percentage of *Klebsiella pneumoniae* mastitis during summer, fall, winter, and spring on dairies A (recycled manure solids), B (recycled sand), and C (wood by-products).

in 4 states from a period of 1 to 18 mo to evaluate the diversity of *K. pneumoniae* isolated from these herds. A significant degree of genetic diversity of *K. pneumoniae* mastitis occurred on most of the dairies studied. The 2 dairies in which a lower genetic diversity was detected (E and F) were analyzed for a shorter period of time, which may have partially affected the genetic diversity results. Dairies E and F also had smaller herd sizes (400 to 600 cows) in comparison with the other dairies (1,000 to 1,500 cows). Whether this lower degree of diversity is a function of time or herd size will require further analysis in a long-term longitudinal study.

Differences among dairies could have been due to variations in the genotyping technique used in this study. Repetitive DNA sequence PCR is not as discriminatory as other typing techniques such as pulsed-field gel electrophoresis and multilocus sequence typing and can lack interlaboratory reproducibility. We have addressed the reproducibility of rep-PCR by typing the same isolates several times and by comparing its discriminatory power with pulsed-field gel electrophoresis and multilocus sequence typing (Paulin-Curlee, 2007). On the basis of these studies, rep-PCR provides valuable and reproducible information regarding genetic diversity among *K. pneumonia* isolates.

If fecal shedding served as the source of *K. pneumoniae* on the dairy (Munoz et al., 2006), then a smaller number of animals may suggest lower overall environmental contamination and possibly a less diverse *K. pneumoniae* population. In this situation, only a few genotypes would have access to the mammary gland. Individual susceptibility of the host (age, stage of lactation, genetic resistance, and immune, nutritional, or metabolic status) may have played a role in the diversity of genotypes infecting the udder (Burvenich et al., 2003). Seasonality, topography, wind conditions, and the introduction of fewer cows into the milking rotation could also have played a role in the lower genetic diversity. Although all dairies in this study were not adding new animals, it does not rule out the possibility that a less diverse *K. pneumoniae* population could be present on dairies E and F.

The association between the genetic similarity matrix and the temporal distance matrix could provide more information on the dynamics of *K. pneumoniae* mastitis. The results showed a negative association between the 2 matrices in all dairies analyzed, indicating that isolates that were collected closer in time had higher similarity than the ones collected farther apart in time. Although the correlation was not very strong, it was significant for 2 dairy herds. The great genetic diversity may explain the low correlation observed. Similar results have been observed previously (Paulin-Curlee et al., 2007) and suggest that the environment may be changing over time, causing the *K. pneumoniae* population on the dairy also to change with time.

Nineteen genotypes were involved in 24 cases of reinfection in dairy A. Given the great genetic diversity observed, it is not surprising that most of the cows were infected with a different genotype each time. Similarly, the culled cows were infected with a different genotype each time. An interesting preliminary result, with a small sample size, is that similar *K. pneumoniae* genotypes were detected in clinical cases of mastitis, in the bedding, and in bulk tank milk. Although the directionality of pathogen spread cannot be determined conclusively from our study, this finding supports the contention that bedding may serve as one of the possible

sources of mastitis-causing microorganisms, and it highlights the impact of bedding quality and management on animal health and milk quality. To confirm this speculation, a prospective sampling of bedding and mastitis cases will be required to monitor genotypic similarities and differences from isolates derived from the 2 sample types.

Five out of 6 dairies used organic material as bedding. The relatively higher concentration and type of bacteria in bedding has been associated with bacterial concentration on the teat end and with the incidence of clinical mastitis (Zdanowicz et al., 2004). In the current study, recycled manure solids, with a high level of moisture and OM, were used as bedding material on one dairy. A high concentration of *Klebsiella* spp. and other coliform bacteria has been found in composted manure solids (Mote et al., 1988). Even if properly processed, coliform bacteria can quickly multiply to high numbers in recycled manure under favorable conditions (Zehner et al., 1986). Cows lying on highly contaminated bedding for long periods of time (Hogan et al., 1989) may have contributed to the higher incidence of *K. pneumoniae* mastitis on this dairy. Recycled manure solids were most likely the source of *K. pneumoniae* mastitis on dairy A. Dairies C, D, E, and F used either sawdust, wood shavings, or both as bedding. The use of wood by-products, which retain moisture and support the growth of *Klebsiella* spp., has been associated with mastitis outbreaks (Zdanowicz et al., 2004). Zehner et al. (1986) studied organic bedding materials as growth media for environmental pathogens and showed that *K. pneumoniae* had the highest growth in all bedding types evaluated. Despite the fact that dairy B used inorganic bedding (sand), which tends to retain less moisture at the surface and has lower OM, *Klebsiella* mastitis remained a problem. Munoz et al. (2006) detected a greater than 80% prevalence of *K. pneumoniae* fecal shedding in healthy dairy cattle and speculated that freshly voided feces could add bacteria, OM, and nutrients that could support growth in sand bedding. Thus, when recycling sand bedding material, it is important to remove as much of the manure and other organic material as possible to limit the potential growth of *K. pneumoniae.* In addition to the choice of bedding (recycled manure solids) and bedding management, many other variables (premilking udder preparation, proper attachment and handling of the milking unit, hygiene of installations, and age, lactation stage, and milk production of cows) could have influenced the high rate of *K. pneumoniae* mastitis on dairy A.

Distinct genotypes were isolated from the same mastitis case on 5 of the 6 dairies in this study. Such findings have been confirmed previously (Paulin-Curlee et al., 2007) and indicate that more than one *K. pneumoniae* genotype can be isolated from the infected mammary gland within a herd at a given time point. It is possible that exposure of the udder to a diverse *K. pneumoniae* population enabled more than one genotype to enter the teat canal concurrently, colonizing and infecting the mammary gland. The anatomical condition of the teat canal and teat end are the first barrier to preventing the entrance of invading pathogens (Elbers et al., 1998) and may have played a role in the isolation of distinct genotypes from the same clinical case. Equally critical is the preparation of the udder before milking, because it reduces bacterial populations at the teat orifice, thus reducing the probability of infection of the mammary gland (Pankey et al., 1987). The fact that cows were rarely infected twice with the same genotype and that more than one genotype pattern was isolated from the same clinical case suggests that *K. pneumoniae* populations were not only greatly diverse but appeared very dynamic. The dairy environment is continuously changing because of the introduction of new animals, changes in management practices, and the introduction of new bedding types or batches and feedstuffs, each with its own unique microbial populations and ability to support bacterial growth (Lynn et al., 1998).

Klebsiella pneumoniae had the highest growth in sterile bedding materials when compared with other environmental bacterial species (Zehner et al., 1986). Studies have shown that *Klebsiella* spp. has extended survival rates in fresh water and marine environments and is more resistant to solar radiation than *E. coli* (McCambridge and McMeekin, 1981; Lopez-Torres et al., 1987, 1988). Environmental *K. pneumoniae* under nutrient starvation were able to grow rapidly when transferred to high-nutrient media (Lappin-Scott et al., 1988) and were as virulent as clinical isolates in a mouse infection model (Struve and Krogfelt, 2004). The dairy environment is complex and is influenced by many factors that are difficult to control. These factors, combined with the presence of a wide range of OM and nutrients (dripping milk, feces, urine, feed, bedding, water, etc.), make the dairy facility a complex and nutrient-rich microbial habitat. Thus, it is possible that, in addition to the ubiquitous and hearty nature of *K. pneumoniae,* the particular conditions encountered in dairy facilities contribute to the higher levels of genetic diversity observed.

Similar seasonal findings were observed previously in our laboratory (Paulin-Curlee et al., 2007), which is contrary to what others have reported for udder infections caused by gram-negative bacteria (Todhunter et al., 1990). Animal crowding, poorly ventilated confinement barns, and high moisture levels associated with the fluctuation of temperature during fall and win-

ter could have supported the survival of bacteria. Such factors may have partially accounted for the increased rate of *K. pneumoniae* mastitis during the winter.

Although the sample size was limited, specific genotypes appeared to be associated with dairy herd, type of bedding, and state. The results of AMOVA showed that most of the genetic diversity (82%) was attributable to variation among genotypes within herds. Although the degree of difference given by the descriptive analysis and AMOVA varied, both methods consistently revealed that genetic diversity was associated with variation among herds within states and variation among genotypes within herds. Genetic diversity of fecal *K. pneumoniae* from various mammalian hosts has been reported to be partly associated with geographic location and taxonomic group of the host (Gordon and Lee, 1999). The exact reasons for the occurrence of unique genotypes in particular herds in the present study is unknown. Because specific genotypes also tended to occur with the use of a particular bedding material, it is possible that bedding was one of the risk factors. The characteristics of each dairy, such as the type of bedding and feed, the inherent intestinal microbial population of the animals in the herd, and the presence of other animals (domestic and wild) could have played a role in the observed associations.

Different management systems appeared to have an effect on the number and types of bacteria detected in clean and recycled sand bedding (Kristula et al., 2005). A similar effect may have been demonstrated regarding the genetic diversity of *K. pneumoniae* mastitis and the association of genotypes with herd and bedding type. Despite some common characteristics, each dairy is different in terms of herd size, geographic location, topography, installations, animal health status, and environment hygiene standards, all factors that could influence which genotypes of *K. pneumoniae* predominate in the herd. Conversely, when analyzing genetic diversity among all herds, genotype 1 was more nonrandomly distributed across dairies than were other genotypes. In addition, genotype 1 was associated with the largest number of reinfections and culling in dairy A. Whether this was due to the higher number of animals infected with this genotype remains unknown. Such findings raise the possibility of a prevalent genotype among herds or the existence of a common source of contamination. Alternately, it is possible that specific genotypes (e.g., genotype 1) may have converged into a pathogenic phenotype.

Molecular typing of *K. pneumoniae,* together with a detailed assessment of management practices, could be useful in identifying the major sources of *Klebsiella* within a herd. Detection of major genotypes causing mastitis can be useful to identify the source, which could then be targeted for management. This information could then be used to prevent an outbreak before it becomes a problem for the dairy. Considering the rapid progression and the high fatality rates of *K. pneumoniae* infection, the early detection of a contaminated source may prevent other cows from becoming ill and the dairy from sustaining major economic losses.

CONCLUSIONS

We showed that distinct *K. pneumoniae* genotypes can cause mastitis within a dairy herd. We were unable to demonstrate a nonrandom distribution of genotypes in causing mastitis at a specific time of the year in any of the dairies in our study. Udder reinfection and culling in dairy A was associated with 19 and 8 different genotypes, respectively. The same genotype was present in isolates from clinical mastitis, bedding, and bulk tank milk. The 2 dairies that showed lower genetic diversity had lower numbers of milking cows in the herd. It is possible that management practices and environmental hygiene standards, combined with geographic and weather factors, affected the *K. pneumoniae* genotypes found within the herd. The majority of genotypes were associated with the herd of origin, and only a few were detected in more than 2 dairies. Genotypes also tended to be associated with bedding type and dairy location (state). Genotype 1 was the most frequent across dairies and the one detected in the largest number of mastitis reinfection cases and culled cows. The frequent environmental contamination by feces and other OM may support the survival and growth of a diverse *K. pneumoniae* population within a dairy facility. Only the bedding was investigated as a possible source of *Klebsiella* mastitis in this study. This does not preclude feces as a source of *K. pneumoniae,* because bedding is likely to be contaminated with feces as the cows enter the stalls to rest. It is possible that other sources of contamination exist within a dairy herd, and additional studies are needed. Our findings reinforce the importance of keeping the dairy environment clean and dry at all times to reduce exposure of the teat end to bacteria. Genotyping is an essential tool to better understand the epidemiology of *Klebsiella* mastitis-causing microorganisms, and it may assist in mastitis control programs by identifying possible sources of infections and populations that most commonly cause mastitis.

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