NOTE *Bacteriology*

Asymptomatic Intramammary Infection with Multidrug-Resistant Gram-Negative Bacteria in a Research Dairy Farm: Incidence and Genetic Basis of Resistance

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ABSTRACT. Nonpathogenic and opportunistic bacteria are increasingly recognized as reservoirs of antibiotic resistance genes. However, nothing is known about the mechanisms of antibiotic resistance in such bacteria isolated from the udders of healthy animals. In this study, 150 Gram-negative strains isolated from milk samples of healthy dairy cows were screened for the presence of a large pool of antibiotic resistance markers. Strains carrying β-lactamase-resistance genes, including SHV-1, SHV-11, SHV-27, TEM-1, OXY-1, CTX-M-2 and class 1 integrons, were detected. Our findings give the first evidence that nonpathogenic and opportunistic bacteria carrying antibiotic resistance genes can asymptomatically invade healthy udders and suggest that they may play a role in the dissemination of antibiotic resistance genes to the other udder pathogens.
KEY WORDS: antibiotic resistance, Gram-negative bacteria, udder.

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The call for more successful udder health control programs is becoming more important in the context of animal health and public health. Even though it has been shown how well the Hazard Analysis and Critical Control Points (HACCP) approach [13] fits well in the udder health control concept, mastitis is still a major problem in dairy herds. Unfortunately, testing plans for monitoring multidrug-resistant opportunistic pathogens in samples from bulk tank milk, individual cows, and the dairy environment are still undefined in any HACCP program for the control of mastitis. Monitoring dairy farms for the presence of multidrug-resistant bacteria would assist in the development of appropriate strategies for the prevention and control of udder infections. The recent development of tools that allow the molecular epidemiological survey of antibiotic resistance mechanisms is likely to be important for directing treatments and to have worldwide economic implications.

Currently, the treatment choices for clinical mastitis can be based on the knowledge of the herd-level sensitivity patterns of the predominant bacterial strains [2]. Such herd-level knowledge can be obtained through sensitivity testing of clinical isolates after treatment has been initiated. However, *in vitro* susceptibility tests have been shown to be poor predictors of treatment outcomes [1]. Such clinical outcomes are likely to be either a complication of cases of mastitis by different multidrug-resistant opportunistic pathogens previously invading the udders or the evolution of resistance mechanisms in the causative agent during therapy through mutations or lateral dissemination of antibiotic resistance determinants.

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Molecular epidemiological studies together with application of strict hygienic measures are only now being implemented in dairy farms all over the world as a result of mastitis outbreaks [12]. Given that the penetration of coliforms into the bovine teat duct during intermilking periods has been well documented [4, 20], a better understanding of the antibiotic resistance mechanisms in the bacteria responsible for such asymptomatic cases is needed. In this study, we sought to determine the mechanisms of resistance in *Enterobacteriaceae* isolated from healthy dairy cows and to assess their genetic diversity.

Milk samples (n=46) were collected aseptically in sterile screw-capped bottles after discarding three streams of milk from 23 healthy Holstein dairy animals [3]. These animals were reared on an organic research farm in Japan. The system of raising the dairy cows in this farm includes use of organic feeds (grown without use of pesticides or synthetic fertilizers), no regular usage of antibiotics or growth hormone and emphasis on husbandry practices that 'limit stress and promote health.' Samples were collected in September 2007 and September 2009 (23 samples in each year). Records for this farm indicated that some animals were affected by mastitis, but there was no record of outbreaks of mastitis. The medical approach was restricted to dexamethasone as an anti-inflammatory, and in severe cases, kanamycin and cephazolin were used. Animals that were not cured were culled. None of the animals received antibiotics three months before collection of milk samples in 2007 and 2009. The results of a California mastitis test were negative for all milk samples [18]. The samples were transported immediately to the laboratory on ice.

Upon arrival at the laboratory, 25 m*l* of milk was added to 225 m*l* of buffered peptone water and incubated at 37°C with agitation for 24 hr. Following incubation, the enriched samples were subcultured onto MacConkey agar plates and

incubated for 24 hr at 35°C. Four colonies were picked up from each sample. An API-20E system (bioMérieux, Marcy-l'Éoile, France) was used for biochemical identification of isolated strains. When the phenotypes of isolates from the same milk sample were the same, the isolates were removed, since they could be the same clones.

Characterized bacterial strains were tested for their susceptibility to 23 antimicrobial agents (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) by the disk diffusion method, according to the guidelines of the Clinical and Laboratory Standards Institute [5]. It should be noted that according to M31-A3 of the Clinical and Laboratory Standards Institute, the break points for some antibiotics are not set. In such case, the guidelines of the company that produced the antibiotic disks were used as a reference. The antimicrobial agents tested were as follows (μg): amoxicillin-clavulanic acid (20 and 10), ampicillin (10), aztreonam (30), cefepime (30), cefoperazone (75), cefotaxime (30), cefotetan (30), cefoxitin (30), cefpirome (30), cefpodoxime (10), ceftazidime (30), ceftriaxone (30), chloramphenicol (30), ciprofloxacin (5), gentamicin (10), imipenem (10), kanamycin (30), nalidixic acid (30), norfloxacin (10), oxacillin (1), streptomycin (10), sulfamethoxazole-trimethoprim (23 and 75) and tetracycline (30).

Total DNA was prepared as previously described [7]. PCR amplification of the $bla_{\text{CTX-M}}$, bla_{CMY}, bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{OXY}, *qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib* genes was carried out for screening purposes using universal primers as previously described [8]. Additionally, PCR amplification of the variable regions of class 1 and 2 integrons was performed using 5'- and 3'-CS primers as previously described [8].

Genomic analyses of strains that carried the same antibiotic resistance gene(s) were performed by Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) using the primer ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') as previously described [6]. All PCR products produced during this study were sequenced on both strands with specific primers using an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). Sequence similarity analyses were performed using the BLAST algorithm available at the NCBI website (http://www.ncbi.nlm.nih.gov/blast/).

A total of 150 Gram-negative bacteria were isolated (75 nonrepetitive strains on each visit). In 2007, *Citrobacter freundii*, *C. koseri*, *Enterobacter aerogenes*, *E. cloacae*, *Escherichia coli*, *Klebsiella oxytoca*, *K. pneumoniae*, *Pseudomonas fluorescens* and *Serratia marcescens* were detected at 4, 13.3, 16, 6.6, 12, 10.6, 29.3, 6.6 and 1.3% of 75 isolates, respectively. In 2009, the same bacteria were detected at 2.6, 14.6, 10.6, 5.3, 13.3, 16, 24, 9.3 and 4% of 75 isolates, respectively. Previous studies that identified Gram-negative bacteria isolated from bulk tank milk of healthy animals attributed the source of contamination by these bacteria to be inefficient cleaning of the udder before milking or contamination from utensils and milking machines [9]. In our study, to exclude contamination from such sources and to give new insights regarding the bacterial community in healthy udders, we obtained milk samples manually after sterilization of the udders and surrounding tissue. *K. pneumoniae*, the most common *Klebsiella* species infecting dairy animals, had the highest prevalence on both visits in the present study. However, such a high prevalence of *K. pneumoniae* in milk samples may be because of its higher occurrence in the dairy environment compared with other species and/or its greater ability to invade and colonize the udder.

Previous studies have focused on the antibiotic resistance patterns of nonpathogenic bacteria isolated from healthy animals [14], but there is still a lack of data about the patterns of resistance in opportunistic or nonpathogenic bacteria that can invade the udder. In this study, antimicrobial susceptibility results showed that 16 of 75 isolates (21.3%) in 2007 expressed resistance phenotypes to two or more antimicrobial agents. However, even though only 4 of 75 isolates (5.3%) in 2009 were considered multidrug resistant, some isolates showed a resistance phenotype that extended to the third-generation oxyimino-cephalosporins (the same clones were isolated from different samples; therefore, only one strain represented each clone shown in Table 1).

Broad-spectrum β-lactamases, such as SHV-1, SHV-27, TEM-1 and OXY-1, were detected in 26, 4.3, 17.3 and 8.6% of the 23 milk samples collected in 2007, respectively. In 2009, SHV-11 and SHV-27 were detected in 8.6 and 4.3% of the 23 milk samples, respectively. Interestingly, decreased susceptibility to broad-spectrum cephalosporins in *E. coli* and *K. pneumoniae* clinical strains has been attributed to hyperproduction of such genes [19]. Of note, resistance to cefoperazone, one of the most common cephalosporin-based intramammary preparations, was observed in most strains carrying these genes, making its application as a therapeutic option in severe mastitis cases in this farm debatable (Table 1).

Extended-spectrum β-lactamases (ESBLs), such as CTX-M β-lactamases that have the ability to hydrolyze penicillins and narrow-spectrum and third-generation oxyimino-cephalosporins [15], were detected in 8.6% of animals in 2009. In Japan, in contrast to the high rate of isolation of CTX-M β-lactamase producers from humans, isolation of CTX-M β-lactamase producers from animals is still rare [21]. We believe that this may be related to the low impact of research in animals rather than their low prevalence. Nonetheless, this is the first record of a CTX-M-2-expressing *K. pneumoniae* from healthy dairy cows in Japan. Further studies may reveal how long such a strain can persist in this farm or whether it disseminates to other farms. However, a previous study has clearly shown that specific CTX-M-positive clones were able to persist for months in the farm [11].

Previous studies that have investigated the genetic diversity of certain species such as *K. pneumoniae* have concluded that more than one genotype can be isolated from the infected mammary gland within a herd at a given time point [16, 17]. The present study focused most attention on the genetic diversity of strains carrying specific antibiotic resis-

Table 1. Resistance phenotypes and prevalence of integrons and resistance genes in *Enterobacteriaceae* isolates

Isolate	Bacteria	Resistance phenotype[a)]	Resistance marker(s)
In 2007			
FM2-1	*K. pneumoniae*	MP, CFP, SXT	bla_{SHV-1}
FM3–1	*E. coli*	AMP, ATM, CFP, GEN, KAN, STR, SXT, TET	Class 1 (*dfrA1, aadA1*), bla_{TEM-1}
FM6-1	*K. pneumoniae*	AMP, KAN, STR, SXT, TET	Class 1 (*dfrA1, aadA1*)
FM6-2	*K. oxytoca*	AMP, KAN, STR, TET	bla_{OXY-1}
FM7-1	*K. pneumoniae*	AMP, GEN, STR, TET	bla_{SHV-1}
FM8-1	*C. koseri*	AMP, STR, TET	bla_{SHV-1}
FM9-1	*K. pneumoniae*	AMP, ATM, STR, TET	bla_{SHV-1}
FM9-2	*K. pneumoniae*	AMP, FOX, GEN, KAN, STR, TET	bla_{SHV-1}
FM13-1	*E. coli*	AMP, CFP, KAN, SXT	bla_{TEM-1}
FM13-2	*K. pneumoniae*	AMP, ATM, FOX, CFP, TET	bla_{SHV-27}
FM15-1	*E. coli*	AMP, ATM, CFP, CPD, GEN, KAN, STR, SXT, TET	bla_{TEM-1}
FM15-2	*K. pneumoniae*	AMP, CPD, STR, SXT	bla_{SHV-1}
FM17-1	*K. pneumoniae*	AMP, ATM, TET	bla_{SHV-1}
In 2009			
FM3-1	*K. pneumoniae*	AMP, GEN, KAN, STR	bla_{SHV-11}
FM10-1	*K. pneumoniae*	AMP, STR	bla_{SHV-27}
FM25-1	*K. pneumoniae*	AMP, CFP, CPD, CRO, CTT, CTX, STR, TET	Class 1 (*aadA2*), bla_{SHV-11}, $bla_{CTX-M-2}$

a) AMP, ampicillin; ATM, aztreonam; CFP, cefoperazone; CPD, cefpodoxime; CRO, ceftriaxone; CTT, cefotetan; CTX, cefotaxime; FOX, cefoxitin; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; SXT, sulfamethoxazole-trimethoprim; TET, tetracycline.

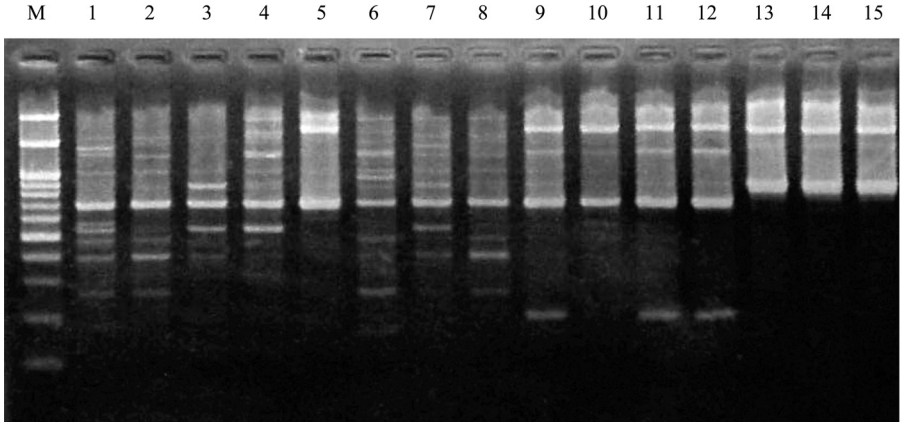

Fig. 1. Diversity of *Klebsiella* strains from milk samples based on banding patterns generated by random amplified polymorphic DNA typing with primer ERIC-2. Each strain is represented by a banding pattern. Lane M corresponds to the molecular size marker (100 bp ladder, SibEnzyme, Academtown, Russia). Lanes 1–8 represent *K. pneumoniae* strains that carried different SHV β-lactamases isolated in 2007. Lanes 9–12 represent *K. pneumoniae* strains that carried different SHV β-lactamases encoding genes isolated in 2009. Lanes 13–15 represent *Klebsiella oxytoca* strains that carried OXY β-lactamase encoding genes isolated in 2007.

tance genes. Genotyping by ERIC-PCR for antibiotic-resistant *K. pneumoniae* isolates obtained in 2007 and 2009 revealed that eight and three distinct genotypes of *K. pneumoniae* carried SHV β-lactamases, respectively (Fig. 1). In addition, clonal spread of antibiotic-resistant bacteria was observed. In 2007, one clone of *E. coli* (carried TEM-1 and a class 1 integron harboring gene cassettes *aadA2* and *dfr1*) was detected in two samples, and one clone of *K. oxytoca* (carried OXY-1) was detected in three samples. In 2009, one clone of *K. pneumoniae* carried SHV-1. CTX-M-2 and a class 1 integron harboring *aadA2* gene cassette could be detected in 2 samples (Fig. 1). This scenario has been frequently reported in different mastitis outbreaks, but in our case, the dissemination of multidrug-resistant opportunistic coliforms was more diverse and occurred in asymptomatic cows. Consequently, wide dissemination and colonization by these clones before an outbreak of mastitis would likely complicate treatment because of the poor response to antibiotic therapy.

In conclusion, this study provided the first evidence that

opportunistic pathogens carrying antibiotic resistance genes can asymptomatically colonize healthy udders and suggests that they may play a part in dissemination of antibiotic resistance genes to the other udder pathogens [10]. In addition, it is important for regular (at least yearly) molecular epidemiological surveys of antibiotic resistance mechanisms present in the dairy farms to be performed that will provide guidance in choosing the appropriate control measures before dissemination of multidrug-resistant bacteria.

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