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Genetic Diversity of Mastitis-Associated *Klebsiella pneumoniae* in Dairy Cows

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ABSTRACT

The objectives of this study were to determine the level of genetic diversity of *Klebsiella pneumoniae* isolated from clinical mastitis cases and to define genotypes most commonly associated with the disease. Individual quarter milk samples were collected from a single privately owned dairy herd over a 2-yr period and submitted to the Laboratory for Udder Health, Minnesota Veterinary Diagnostic Laboratory, University of Minnesota, for bacteriological culture. Eighty-four *K. pneumoniae* isolates were obtained and fingerprinted by repetitive DNA sequence PCR, 43 by pulsed-field gel electrophoresis (PFGE), and 29 by multilocus sequence typing (MLST). Significant genetic diversity was observed among the isolates regardless of the fingerprinting method used. Simpson's diversity index was 93.5, 96.1, and 97.0% when analyzed by repetitive DNA sequence PCR (n = 84), pulse field gel electrophoresis (n = 43), and MLST (n = 29), respectively. In some cases more than 1 genotype was obtained from a single milk sample originating from an individual quarter. The majority of infections were observed during the winter and accounted for 69.0% of *K. pneumoniae* mastitis cases. There was a negative correlation between a matrix of fingerprints similarity and a matrix of temporal distances. The MLST results revealed 5 new and novel allelic types, which have not been previously reported in the MLST database. Three isolates shared MLST types with human clinical isolates, raising the possibility that some *K. pneumoniae* isolates, of bovine origin, may be capable of causing disease in humans. There were 21 genotypes present within the herd, and there was no evidence for nonrandom distribution of genotypes uniquely associated with mastitis. We have shown, using 3 distinct genotyping methods, that *K. pneumoniae* isolated from clinical mastitis within a single dairy herd is caused by a genetically diverse population and that multiple genotypes can be isolated from a mastitic quarter. The data suggest that mastitis can be caused by a variety of *K. pneumoniae* genotypes. Diverse genotypes may have different levels of invasiveness and virulence and may originate from various sources within the dairy.
Key words: mastitis, *Klebsiella*, diversity

INTRODUCTION

Mastitis remains one of the most important diseases among dairy herds despite the widespread use of control programs including techniques such as teat dipping, dry cow therapy, and segregation of infected animals (Esslemont and Kossaibati, 1997). As a result of control programs for contagious mastitis pathogens (*Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma* species), mastitis caused by environmental pathogens (coliform bacteria, fecal streptococci, *Streptococcus dysgalactiae* and *uberis*) is now the primary disease in well-managed dairy herds with low SCC in bulk tank milk (Barkema et al., 1998).

Coliform bacteria can cause clinical or subclinical mastitis. Cows with clinical mastitis can have abnormal milk, swollen quarters, anorexia, and fever. In subclinical mastitis there are no visible signs of disease. Inflammation can only be monitored by in vitro diagnostic tests. Previously, coliform bacteria have been shown to be responsible for approximately half of the acute mastitis episodes (Erskine et al., 1991). The control and prevention of coliform mastitis remains a challenge to dairy producers. Control measures that are applied for contagious mastitis pathogens are often ineffective in controlling mastitis caused by coliform bacteria. Presently, the most efficient strategy for controlling coliform mastitis is prevention through appropriate management and prophylactic immunization (Bradley and Green, 2000).

The number of mastitis cases caused by coliform bacteria has increased worldwide (Schukken et al., 1989). Specifically, *K. pneumoniae* has been found to affect many dairy herds (Silva and Costa, 2001; Sampimon

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et al., 2006). Braman et al. (1973) found 33 capsular types of *K. pneumoniae* in 12 herds. Within each herd as many as 13 capsular types were found, indicating there were different variants of the same organism present. In another study, Kikuchi et al. (1995) reported diversity in plasmid profiles among 47 *K. pneumoniae* isolates from mastitic milk. However, plasmid profile typing is not as discriminatory as chromosomal DNA genotyping because plasmids may be lost, gained spontaneously, or exchanged between strains, making it difficult to determine the potential number of variants present.

Mastitis caused by *K. pneumoniae* can be particularly severe compared with mastitis caused by *Escherichia coli*, due to its poor response to antibiotic therapy, rapid evolution to toxic shock, and death (Silva and Costa, 2001). Infection of the gland with *Klebsiella* species has been reported to result in higher losses than infections due *E. coli* (Grohn et al., 2004). Thus, infection with *Klebsiella* spp. profoundly affects the profitability of dairy farmers due to discarded milk, costs of antibiotic treatment, and extra labor, death, or culling of infected animals and decreased fertility (Hansen et al., 2004). Genotyping of *K. pneumoniae* isolated from clinical mastitis is expected to increase our understanding of the epidemiology of this organism and perhaps assist in the development of appropriate strategies for the prevention and control of udder infections in dairy cows. The goal of this study was to measure the genetic diversity of *K. pneumoniae* from cases of clinical mastitis within a dairy herd, using 3 genotyping methods [repetitive DNA sequence genotyping-PCR (**rep-PCR**), pulsed-field gel electrophoresis (**PFGE**), and multilocus sequence typing (**MLST**)] to fingerprint and genotype these isolates.

MATERIALS AND METHODS

Klebsiella pneumoniae Isolates

During a 2-yr period of time (January 2003 to December 2004) *K. pneumoniae* isolates (n = 84) were obtained from cases of clinical mastitis from a privately owned 1,200 cow free-stall dairy herd in Wisconsin. Milk samples originating from infected quarters (swollen with abnormal milk) were sent to the Laboratory for Udder Health (University of Minnesota, St. Paul, MN) where they were processed using routine culture methods. All milk samples were cultured on MacConkey agar to isolate gram-negative bacteria and lactose positive colonies. If colonies had the gross morphologic appearance of *K. pneumoniae* they were restreaked on MacConkey agar. *Klebsiella pneumoniae* isolates included in the study were those in which it was the only organism isolated from the mastitic quarter. Following incubation at 37°C for 18 h, the identity of the isolates was verified by biochemical identification using the API 20E system (bioMérieux Vitek Inc., Hazelwood, MO). Three separate colonies from each clinical mastitis sample 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) and was preserved in a glycerol-blood solution and frozen at –80°C.

Repetitive DNA Sequence Genotyping

Bacterial genomic DNA was extracted using PrepMan Ultra Reagent (Applied Biosystems, Foster City, CA). Briefly, frozen cultures were streaked onto MacConkey agar plates and incubated. Two individual colonies were 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 placed in a heat block for 10 min at 100°C. After incubation, the solution was fractionated by centrifugation for 3 min at 7,500 × *g*, and the supernatant containing DNA and an equal amount of sterile DNase-free water was added. The rep-PCR fingerprints were obtained using a boxA1R primer (5′CTACGGCAAGGCGACGCTGACG 3′). The 25-μL PCR mixture contained 25 m*M* $MgCl_2$, 10× Buffer II, 25 pmol of boxA1R primer, 100 m*M* dNTP mix, AmpliTaq Gold DNA polymerase, and 2 μL of DNA template. Thermocycler PCR 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 an extension of 71°C for 15 min followed by storage at 4°C. These conditions were previously described by Goldberg et al. (2006). Samples were separated by electrophoresis using a 2% agarose gel at 4°C for 5 to 6 h at 62 V (6 V/cm) and stained in ethidium bromide/1× Tris-acetate-EDTA buffer. Gel images were electronically captured using Labworks 4.0 Image Acquisition and Analysis Software (UVP Inc., Upland, CA).

Pulsed-Field Gel Electrophoresis

The PFGE protocol was based on procedures previously described by Cho et al. (2006) in addition to those described in the CHEF Genomic DNA Plug Kit (BioRad, Hercules, CA). *Klebsiella pneumoniae* isolates were grown overnight in 10 mL of brain heart infusion broth at 37°C. Cells were pelleted by centrifugation and resuspended in 10 mL of cell suspension buffer (100 m*M* Tris:100 m*M* EDTA, pH 8.0) followed by centrifugation. The cells were washed a second time and resuspended in 5 mL of cell suspension buffer. Agarose plugs were prepared by adding 250 μL of 2% CleanCut agarose