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Short communication: Comparison of virulence factors in *Klebsiella pneumoniae* strains associated with multiple or single cases of mastitis

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ABSTRACT

Klebsiella pneumoniae mastitis in dairy cattle is generally due to an opportunistic infection from the environment, resulting in large heterogeneity among mastitis-causing strains within a herd. However, in mastitis outbreaks in 4 herds, several strains of *K. pneumoniae* were identified as the cause of infection in multiple cows, suggesting increased ability to either cause disease or evade host defenses. In this study, differences in capsule formation and immune evasion were compared in 5 pairs of *K. pneumoniae* strains, where one strain in each pair was associated with multiple cases of mastitis and the other with a single case of mastitis. Production of capsular polysaccharide, ability to evade killing by polymorphonuclear neutrophilic leukocytes (PMNL), and the relationship between the 2 were evaluated for each strain grown in broth or milk. Growth of isolates in skim milk increased capsule size and ability to evade killing by PMNL, depending on strain type. Specifically, strains associated with multiple cases of mastitis had increased capsule size in skim milk. Strains associated with single cases of mastitis were better able to evade killing by PMNL when grown in skim milk. Our results, although preliminary, suggest that the 2 groups of strains may constitute different subpopulations of *K. pneumoniae*. However, our findings do not indicate that capsule or evasions of killing by PMNL explain increased mastitis outbreaks with *Klebsiella*. Further work will explain the enhanced ability of some strains to cause mastitis in dairy cows.

Key words: *Klebsiella pneumoniae*, bovine mastitis, capsule, neutrophil

Short Communication

Klebsiella spp. are nonmotile gram-negative bacteria that are found in the environment and the gastrointestinal tract of humans and animals (Podschun and Ullmann, 1998; Munoz and Zadoks, 2007). These bacteria are an important cause of mastitis in dairy cows and generally lead to severe clinical symptoms and major economic losses (Munoz et al., 2007; Munoz and Zadoks, 2007; Schukken et al., 2012). Traditionally, *Klebsiella* spp. are considered environmental mastitis pathogens acquired through exposure to contaminated bedding, alleyways, and feces (Zadoks et al., 2011). Profiling of *Klebsiella pneumoniae* isolates by random amplified polymorphic DNA (**RAPD**) typing indicates diversity consistent with opportunistic environmental pathogens; however, recent outbreaks in New York farms provided isolates with identical profiles obtained from multiple cows, which may be suggestive of contagious transmission or enhanced virulence properties (Munoz et al., 2007). The purpose of this study was to examine this suggestion in more detail, with emphasis on known virulence factors of *Klebsiella* spp. (i.e., capsule formation and the ability to evade the phagocytic killing action of PMNL; Podschun and Ullmann, 1992; Schembri et al., 2005).

Ten isolates of *K. pneumoniae* from clinical mastitis cases were obtained through Quality Milk Production Services (QMPS) at Cornell University (Ithaca, NY). Isolates were identified to the species level using standard morphological and biochemical criteria, and citrate, motility, and indole testing (Munoz et al., 2006). Random amplified polymorphic DNA typing of multiple isolates per herd was used to determine whether strains were associated with a single cow or multiple cows within a herd (Munoz et al., 2007). Five pairs of isolates were obtained from 4 farms (Table 1). Within each pair, one RAPD type was isolated from a single animal, whereas the other RAPD type was isolated from multiple animals. Contemporaneous pairs of isolates within herds were selected so that the occur-

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rence of RAPD types in 1 or more animals could not be attributed to differences in season or management.

Isolates were stored in Trypticase soy broth with 15% glycerol at −80°C until needed. To detect bacterial capsules, an aliquot of overnight culture (10 μL) was combined with a drop of India ink (BD, Franklin Lakes, NJ) on a clean glass slide. A second slide was used to streak the mixture. For each strain, a minimum of 3 slides were evaluated from bacteria grown in Luria-Bertani broth (**LB**) and from bacteria grown in skim milk (**SM**). Slides were air dried, stained with crystal violet, and then rinsed with water. Once dry, the slides were observed under 100× oil immersion microscopy. Three micrographs of each slide were taken. The area (μm^2) occupied by the microbe, excluding its capsule, and the area occupied by the microbe and capsule combined were determined using Image Pro software (version 6.2; Media Cybernetics Inc., Bethesda, MD). The difference between the 2 areas was used to estimate capsule size.

For assays of killing by PMNL, bacteria were prepared by initial culture on esculin blood agar plates and subsequent culture of a single colony in 25 mL of LB (BD) or SM (BD) at 37°C for 15 to 18 h in an orbital plate shaker (New Brunswick Scientific Incubator Shaker; New Brunswick Scientific Co. Inc., New Brunswick, NJ). Bacteria were centrifuged at 1,811 × *g* for 15 min at 4°C (model 5810R, Eppendorf; Fisher Scientific Inc., Pittsburgh, PA), washed twice with PBS (BD), and centrifuged again at 1,811 × *g* for 15 min at 4°C. Bacterial concentrations were determined by drop plating of serial dilutions and then adjusted to 1.5×10^7 cfu/mL in RPMI medium (Gibco, Carlsbad, CA) containing 5% fetal bovine serum (HyClone; Thermo Fisher Scientific, Waltham, MA) and a final concentration of 2 m*M* L-glutamine (Gibco). Bacteria were stored at 4°C after drop plating. All bacterial concentrations were confirmed on the day of the experiment.

Blood (250 mL/cow) was collected from 4 cows, previously diagnosed with *Klebsiella* spp. mastitis, using jugular puncture and a blood collection kit (Kawasumi Laboratories America Inc., Tampa, FL). All animal use protocols were approved by the Virginia Tech Institutional Animal Care and Use Committee (Blacksburg). Blood was collected in a bottle containing 25 mL of PBS and allowed to clot at ambient temperature. Sera were pooled across cows and heat inactivated by incubation at 56°C for 30 min. One-milliliter aliquots of serum were stored at −80°C until use. The optimum concentration of sera required to opsonize each *K. pneumoniae* strain was determined as previously described (Aarestrup et al., 1994). For all strains tested, 6.25% serum was the optimal concentration for opsonization.

Bacterial resistance to killing by bovine PMNL was evaluated by the bactericidal assay. Isolation of PMNL

Table 1. Characteristics of *Klebsiella pneumoniae* isolates used in the evaluation of virulence characteristics[1]

Isolate	Strain[2]	Herd	Size	Housing	Bedding	Breed	Reference
QMP M1-199	M	1	110	Tie-stall	Straw	Holstein	Munoz et al. (2006) (cross-sectional study)
QMP M1-200	S						
QMP M1-222	M	2	1,200	Freestall	Sand	Holstein	Munoz et al. (2006) (longitudinal study)
QMP M1-428	S						
QMP M1-726	M	3	410	Freestall	Sawdust	Holstein	Munoz et al. (2007)
QMP M1-822	S						
QMP Z4-692	M	4	4,000	Freestall	Dried manure solids	Holstein and Holstein × Jersey	Ostrum et al. (2008)
QMP Z4-724	S						
QMP Z4-702	M	4					
QMP Z4-726	S						

[1]Isolate identification, strain classification, farm, herd size, bedding and housing type, cattle breed, and references are provided.
[2]Random amplified polymorphic DNA (RAPD) type classification of strains from multiple cases of mastitis (M) and single case of mastitis (S).