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 ($10\text{ }\mu\text{L}$) 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 $100\times$ 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 $1,811\times g$ for 15 min at 4°C (model 5810R, Eppendorf; Fisher Scientific Inc., Pittsburgh, PA), washed twice with PBS (BD), and centrifuged again at $1,811\times g$ for 15 min at 4°C . Bacterial concentrations were determined by drop plating of serial dilutions and then adjusted to $1.5\times 10^7\text{ cfu/mL}$ in RPMI medium (Gibco, Carlsbad, CA) containing 5% fetal bovine serum (HyClone; Thermo Fisher Scientific, Waltham, MA) and a final concentration of 2 mM 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 characteristics¹

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 $\times$ Jersey	Ostrum et al. (2008)
QMP Z4-724	S						
QMP Z4-702	M	4					
QMP Z4-726	S						

¹Isolate identification, strain classification, farm, herd size, bedding and housing type, cattle breed, and references are provided.

²Random amplified polymorphic DNA (RAPD) type classification of strains from multiple cases of mastitis (M) and single case of mastitis (S).

Figure 1. Photomicrographs of polysaccharide capsule on *Klebsiella pneumoniae* bacteria. *Klebsiella pneumoniae* isolates grown in Luria-Bertani broth (A) had less distinct capsules compared with isolates grown in skim milk (B). Photomicrographs are representative of triplicate samples of 10 strains.

from bovine blood and analysis of bactericidal killings was conducted as previously described (Mullarky et al., 2001). In brief, opsonized bacteria ($100\ \mu\text{L}$; 1.5×10^7 cfu/mL) were combined with PMNL ($100\ \mu\text{L}$; 1×10^7 cells/mL) and incubated at 37°C for 1 h in a 96-well, sterile polystyrene flat-bottom tissue culture plate (Thermo Fisher, Atlanta, GA). As a reference for assay accuracy, *Staphylococcus aureus* ATCC 27217 (American Type Culture Collection, Manassas, VA) was run on a separate plate but not used in final calculations. After 1 h of incubation, 0.2% saponin ($50\ \mu\text{L}$; Sigma-Aldrich, St. Louis, MO) was added to each well to lyse PMNL. Thiazolyl blue tetrazolium bromide (MTT; $50\ \mu\text{L}$, 1 mg/mL; Alfa Aesar, Ward Hall, MA) was added to measure the number of bacteria remaining per well. After color development for approximately 20 min, freshly prepared extraction buffer ($100\ \mu\text{L}$) containing 10 mL of deionized distilled H_2O , 10 mL of *N,N*-dimethylformamide (Fisher Scientific, Pittsburgh, PA), and 4 g of SDS (J. T. Baker Inc., Phillipsburg, NJ) dissolved at 37°C was used to solubilize MTT-formazan generated by live bacteria. Plates were read at 595 nm on a BioTek μQuant microplate reader (BioTek Instruments Inc., Winooski, VT) and optical density was recorded. Quadruplicate wells were included on all plates for all samples and used in calculating percentage of bacteria killed at the end of the assay.

A paired *t*-test or repeated-measures one-way ANOVA with the Tukey post-hoc test was used to evaluate the association between strain (associated with mastitis in single or multiple cows) or growth media (LB or SM) and capsule size or killing by PMNL. Pearson correlation analysis was used to test the association between capsule size and killing by PMNL within strain within growth media. All analyses were conducted using

GraphPad Prism version 5.0c for Mac OS X software (GraphPad Software Inc., San Diego, CA). Significance was declared at $P < 0.05$.

Production of a capsule is indicative of changes in virulence in both murine and human *Klebsiella* infections (Podschun and Ullman, 1992; Favre-Bonté et al., 1999; Yoshida et al., 2000; Yeh et al., 2007). For visualization and analysis of capsule production, isolates were grown in either LB or SM (Figure 1). Capsule surface area (μm^2) was larger ($P < 0.01$) when isolates were grown in SM compared with LB media (Figure 2A). Capsule surface area was not significantly different ($P = 0.39$) between isolates that caused mastitis in single cows [96 ± 17.03 ($\pm\text{SEM}$)] compared with multiple cows [121.1 ± 30.97]. Isolates from single cases of mastitis produced a capsule that was $126.80 \pm 18.02\ \mu\text{m}^2$ in SM compared with $67.25 \pm 23.17\ \mu\text{m}^2$ in LB (Figure 2B). Isolates from multiple cases produced a capsule $130.20\ \mu\text{m}$ larger ($P < 0.05$) when grown in SM [186.80 ± 45.23] compared with LB (55.30 ± 10.47). No significant difference was observed in capsule production between isolates from single cases compared with multiple cases of mastitis when grown in similar media. However, the capsules produced in SM (186.8 ± 45.23) by isolates from multiple cases were significantly ($P < 0.05$) larger than the capsules produced in LB (67.3 ± 23.17) by isolates from single cases of mastitis. Differences in virulence gene expression have previously been described for *Escherichia coli*, another important mastitis pathogen, when comparing growth in broth versus milk (Lippolis et al., 2009). Furthermore, the competitive status of *K. pneumoniae* isolates to survive in the mammary gland in the presence of other environmental pathogens may be affected by capsule production. This has been shown to be the case for

Figure 2. Capsule polysaccharide expression by *Klebsiella pneumoniae* strains from single or multiple cases of mastitis. Strains were grown in Luria-Bertani broth (LB; gray) or skim milk (SM; white) and the thickness of the capsule was measured. (A) Growth in SM resulted in significantly larger capsule size compared with growth in LB (* $P < 0.005$). (B) Strains from multiple mastitis cases had significantly larger capsule size when grown in SM compared with LB (* $P < 0.05$). Data are presented as mean $\pm$ SEM.

Streptococcus pneumoniae (Lysenko et al., 2010), where capsule type affected evasion of PMNL and competitive growth with *Haemophilus influenzae*. The effect of capsule production on epithelial cell invasion (Oliver et al., 1998; Sahly et al., 2000), and thereby pathogenicity of *Klebsiella* spp. at mucosal sites, should be considered.

As PMNL are a primary host defense in the mammary gland, increased evasion by *K. pneumoniae* isolates would allow for enhanced survival in the mammary gland. Therefore, we evaluated the ability of strains to evade killing by PMNL. Similar to changes in capsule expression, isolates grown in SM (23.5 ± 4.7) were significantly ($P = 0.01$) better able to evade

Figure 3. Evasion of neutrophil killing by *Klebsiella pneumoniae* is media dependent. Strains of *K. pneumoniae* were grown in either Luria-Bertani broth (LB; gray) or skim milk (SM; white) and ability to evade killing by bovine neutrophils was measured. (A) Growth in SM resulted in significantly less percentage killing of *K. pneumoniae* compared with growth in LB (* $P < 0.05$). (B) Strains from single mastitis cases were significantly better able to evade killing when grown in SM compared with LB (* $P < 0.05$). Data are presented as mean $\pm$ SEM.

killing by PMNL compared with isolates grown in LB (42.6 ± 5.8 ; Figure 3A). Evasion of PMNL by *Klebsiella* spp. was not significantly different ($P = 0.38$) between isolates that caused mastitis in single cows (31.9 ± 6.4) compared with multiple cows (34.2 ± 6.0). Evasion of PMNL by *Klebsiella* spp. was significantly greater ($P < 0.05$; Figure 3B) when isolates that caused mastitis in single cows were grown in SM (19.0 ± 5.3) compared with LB (44.8 ± 8.4). However, no significant difference was observed in evasion of PMNL when comparing isolates that caused multiple cases of mastitis grown in

Figure 4. Correlation of capsule size and evasion of killing by PMNL by *Klebsiella pneumoniae* strains. For each strain, data represent isolates grown in Luria-Bertani broth (LB; gray circles) or skim milk (SM; black squares). Correlation between capsule size and percentage of bacteria killed is shown by media type for (A) strains isolated from single cases of mastitis and (B) strains isolated from multiple cases of mastitis.

SM or LB. Also, no significant differences were observed in the evasion of PMNL when comparing isolates that caused single cases compared with multiple cases of mastitis grown in similar media.

The correlation between capsule expression and ability of isolates to evade killing by PMNL for all strains and media types are shown in Figure 4. For strains associated with single cases of mastitis, the correlation between capsule size and killing by PMNL was nonsignificant in either LB ($r = -0.51$) or SM ($r = -0.33$; Figure 4A). For strains associated with multiple cases of mastitis, a positive correlation ($P = 0.06$) existed between capsule size and killing by PMNL when isolates were grown in LB ($r = 0.86$; Figure 4B), but not when

isolates were grown in SM ($r = 0.13$). Specifically, the greater the capsule sizes in LB, the less able the isolates causing multiple mastitis cases evaded killing by PMNL. For other mastitis pathogens (i.e., *Streptococcus* spp. and *Staph. aureus*), capsule presence and type have been linked to PMNL evasion and establishment of infection (Luong and Lee, 2002; Kampen et al., 2005; Barbuti et al., 2010). Together, these data indicate that although differences exist in capsule regulation and PMNL evasion by isolates that cause multiple as compared with single cases of mastitis, they do not explain differences in disease occurrence or prevalence.

In this study, despite the small number of strains evaluated, certain differences were observed in capacity to regulate virulence traits in isolates associated with single or multiple cases of mastitis. Specifically, growth media differentially affected virulence traits of isolates in vitro. Future studies may include study of additional virulence factors (e.g., cell wall receptors and endotoxins, as well as in vitro, ex vivo or in vivo assays and genomic comparisons) that may shed more light on the possibility of host adaptation of *K. pneumoniae* in dairy cattle.

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