Ability of *Klebsiella* spp. mastitis isolates to produce virulence factors for enhanced evasion of bovine innate immune defenses.

Alicia Nedrow

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science
In
Dairy Science

I. K. Mullarky, Chair
R. N. Zadoks
C. S. Petersson-Wolfe
R. M. Akers

November 24, 2007
Blacksburg, VA

Keywords: *Klebsiella*, neutrophils, capsule, biofilm

Ability of *Klebsiella* spp. mastitis isolates to produce virulence factors for enhanced evasion of bovine innate immune defenses

Alicia Nedrow

Abstract

Klebsiella spp. are coliform bacteria that cause mastitis in dairy cattle and account for high mortality rates in infected cows leading to a significant financial loss. Recent outbreaks indicate that within farms a single strain can be responsible for clinical signs in multiple animals. Identification of the virulence of factors enabling *Klebsiella* spp. survival in the mammary glands of multiple animals may provide insight into host adaptation. In this study, *Klebsiella* spp. strains were evaluated for their ability to evade neutrophil killing, the primary immune defense in the bovine mammary gland. Our research focused on capsule and biofilm production by *Klebsiella* spp. when strains were grown in Luria Broth or skim milk to examine the effects on evasion of neutrophil killing. Biofilm production was not significantly related to the ability to resist neutrophil killing nor was capsule ($P = 0.29$). Farm ($P < 0.001$), media type ($P < 0.005$), and strain type by cow ($P < 0.001$) were found to play significant roles in neutrophil evasion. This suggests farm of origin, media type used, and cow all may play a role in evasion of neutrophils by *Klebsiella* spp. Further evaluation of virulence factor expression in different media types and the role of individual cow immune responses may provide insight into ability of *Klebsiella* spp. to cause outbreaks of mastitis in multiple animals.

Keywords: *Klebsiella*, mastitis, capsule, neutrophil, bactericidal

Acknowledgments

I would like to thank my advisor, Dr. Mullarky. The support, flexibility, advice, and encouragement she provided has been invaluable. Thank you to Dr. Wolfe for letting me know that my frustrations were normal and that the development of assays takes a significant amount of time to work correctly. I would like to thank Dr. Zadoks for giving me the opportunity to work on this project. Thanks also to Dr. Akers for explaining that “bad” data are not always useless or invalid data, and that such data always contributes to the scientific community’s knowledge base.

I would like to thank Becky for her help and friendship. Her help with troubleshooting problems, answering my barrage of questions, and reviewing ideas was priceless. Many thanks to Steph for making Toronto such a great experience! I would like to thank Mary for teaching me how to communicate with people who think in a different manner. Special thanks go to Logan for his photography and measurement assistance. To my other lab mates many thanks for the help and good times. Finally, thanks to former and current graduate students who assisted me with my project and shared their knowledge.

I would like to thank Wendy for all your amazing help. Words are insufficient for all the help you have provided.

A special thank you goes to my family for standing by me. Finally, thank you to my friends here, and at the “Fort,” for listening when I needed a sounding board.

Table of Contents

Abstract ii

Acknowledgments iii

List of Tables vii

List of Figures viii

Introduction ix

Chapter I 1

Literature Review 1

Human infections: 1

Klebsiella spp. as an environmental pathogen: 1

Dairy cow immunity and infection: 3

Virulence factors: 6

Capsule: 6

Biofilms: 8

References 9

Chapter II 14

Abstract 15

Introduction 16

Materials and Methods 18

Bacterial isolates 18

Biofilm detection 19

Capsule detection 19

Heat inactivated sera (Klebsiella serum) 20

Agglutination assay 20

Bovine blood neutrophil bactericidal assay 21

Statistical Analysis 22

Results 23

Biofilm 23

Capsule production 23

Evasion of PMN 23

Discussion 24

References 27

Chapter III 39

Overall summary: 39

References 42

Appendix A : Protocols 44

Detection of biofilm 44

Identification of Capsule 46

Isolation of PMN from Whole Blood for *Klebsiella* Bactericidal 47

Klebsiella Bactericidal Assay 49

Klebsiella dilutions (CFU) 53

Reduction of *Klebsiella* Capsule Production 55

Appendix B: SAS Code 57

Appendix C: SAS Output 59

List of Tables

Table 1. Characteristics of *Klebsiella* spp. isolates used in the evaluation of virulence characteristics. 30

Table 2. Biofilm production by *Klebsiella* spp. isolates. 31

Table 3. Results of statistical analysis for ability of *Klebsiella* spp. isolates collected from farms with multiple or unique cases of mastitis grown in Luria Broth (LB) or skim milk (SM) to evade neutrophil killing using the PROC MIXED in SAS (SAS Inst. Inc., Cary, NC). 32

List of Figures

Figure 1. Average capsule size in pixels between *Klebsiella* spp. isolates cultured in different media types. 33

Figure 2. Average capsule size of unique and multiple *Klebsiella* spp. isolates. 34

Figure 3. Photomicrographs of capsule production by *Klebsiella* spp. isolates using Image Pro version 6.2 (Media Cybernetics Inc. Bethesda, MD). 35

Figure 4. Bactericidal killing of *Klebsiella* spp. isolates. 36

Figure 5. Percent bactericidal killing of unique and multiple *Klebsiella* spp. isolates from different farms. 37

Figure 6. Percent bactericidal killing of unique and multiple *Klebsiella* spp. isolates from different cows. 38

Introduction

Klebsiella spp. are gram negative bacteria emerging as opportunistic and genetically diverse pathogens. *Klebsiella* spp. are typically found in the environment and on mucosal surfaces of multiple host species and cause a variety of infections including liver abscesses, pneumonia and urinary tract infections in humans. Virulent infections caused by *Klebsiella* spp. have been linked to capsule and biofilm formation. *Klebsiella* spp. are emerging as one of the leading gram negative pathogens causing severe clinical mastitis in dairy cows, and a commonly isolated gram negative mammary pathogen in the northeastern United States. In a clinical mastitis outbreak, a strain of *Klebsiella* spp. was found in multiple cows with the same random amplified banding pattern indicating a possible change in virulence factors (Munoz et al. 2007). An enhanced ability to resist host immune responses and survive in the mammary gland is suggested by one strain identified in multiple cows. The first line of defense against pathogens in the bovine mammary gland is the innate immune system composed predominantly of polymorphonuclear neutrophils (PMN) (Paape et al. 2003). PMN prevent the establishment of new intramammary infections (IMI) by phagocytizing and killing invading bacteria (Paape et al. 2003). Three *Klebsiella* spp. virulence factors evaluated in this study were increased ability to evade killing by PMN, ability to produce biofilm, and ability to produce capsule. We hypothesized that the increased ability to avoid destruction by PMN is enhanced by biofilm or capsule production. The effect of increased capsule production in the mammary gland was examined by growing the bacterial isolates in skim milk (SM) compared to the standard growth media of Luria Broth (LB). Knowledge of immune evasion mechanisms will help identify appropriate preventative methods and vaccine targets for *Klebsiella* spp. mastitis.

Chapter I

Literature Review

Human infections:

In humans *Klebsiella* spp. infections have become more common over the past two decades in Asian countries, particularly, Taiwan. In Taiwan, Singapore, Japan, and the United States pyrogenic liver abscesses caused by *Klebsiella* spp. (Podschun and Ullmann 1998; Ko et al. 2002; Cheng et al. 2007; Nadasy et al. 2007; Yu et al. 2007) are replacing previously predominant urinary tract and pneumonia infections (Wang et al. 1998; Ko et al. 2002). A mortality rate of 50% has been seen in people with liver abscesses (Cheng et al. 2007). Necrotizing fasciitis from *Klebsiella* spp. has been documented in approximately 11 cases from Asia and the Middle East. Sixty percent of these infections resulted in bacteremia (Kohler et al. 2007). As of 2007, in the United States 20 cases of liver abscesses, necrotizing fasciitis, and septic arthritis were attributed to *Klebsiella* spp. (Kohler et al. 2007; Nadasy et al. 2007).

When strains are compared from the same geographic region, genomic heterogeneity is seen suggesting an emergence of similar virulence factors. In fact, The bacteria infecting these individuals tend to show increased mucoviscosity and virulence (Wang et al. 1998). It is concluded that only bacterium with specific characteristics are able to cause virulent infections (Yeh et al. 2007). These altered and increased infection patterns warrant further investigation.

<u>Klebsiella</u> *spp. as an environmental pathogen:*

Klebsiella spp. are environmental pathogens, and the most common reservoirs are mucosal surfaces, the digestive tracts of mammals, vegetation, soil, and surface water (Paape et al. 2003). The preponderance of *Klebsiella* spp. are found in fecal matter and wood-based beddings (Munoz et al. 2006; Kristula et al. 2008). *Klebsiella* spp. are endogenous to the dairy cow's environment. In fact, fecal material is the largest contributor of *Klebsiella* spp. to the environment of dairy cattle (Munoz et al. 2006). Additionally, research has shown that bedding contaminated with milk supports greater growth of *Klebsiella* spp. than non-milk contaminated bedding (Nadasy et al. 2007). *Klebsiella* spp. are identified in 14.7% and 34.7% of mastitis cases when wood and recycled manure beddings, respectively are used (Paulin-Curlee et al. 2007; Paulin-Curlee et al. 2008). Wood based beddings can be contaminated before introduction into the cow's environment, but all types of bedding are considered contaminated once exposed to fecal matter (Munoz et al. 2006; Nadasy et al. 2007).

A study conducted by Munoz et. al. 2006, found that 80% of fecal samples taken from 100 cows over a five month period tested positive for *Klebsiella* spp. Approximately the same percent of samples tested positive when 10 cows on 10 different farms were tested all at one time (Munoz et al. 2007). Certain beddings, such as inorganic sand is a better choice than others since the growth of *Klebsiella* spp. cannot be supported until organic matter is introduced. Finally, most documentation has shown an increase in *Klebsiella* spp. infections during the summer months (Todhunter et al. 1991) due to hot and humid conditions that promote growth of environmental pathogens.

Traditionally, *Klebsiella* spp. are considered environmental rather than contagious pathogens, which are communicably spread. However, recent outbreaks suggest that *Klebsiella* spp. may have developed characteristics similar to contagious pathogens. Munoz et. al. 2007,

reported two outbreaks of *Klebsiella* spp. on a single dairy farm. During the first outbreak all but one of the isolates had an identical rapid amplified polymorphic DNA (RAPD) banding pattern (Munoz et al. 2007) The same dairy farm had a second outbreak of *Klebsiella* spp. mastitis cases two months later. These strains did not have the same RAPD banding pattern, as those previously isolated (Munoz et al. 2007) The results point to a single origin of the *Klebsiella* spp. isolates for the first outbreak, and a diverse origin in the second outbreak (Munoz et al. 2007). This suggests a possible mutation of *Klebsiella* spp. having enhanced virulence in the first outbreak, but not the second. The single strain predominance likely resulted from one or more of the following factors: transmission between cows, a predominant strain within the environment, or increased ability of a specific strain in the environment to cause mastitis. The second outbreak was typical of an environmental pathogen.

Dairy cow immunity and infection:

Staphylococcus aureus and *Streptococcus agalactiae* have been identified as the most commonly isolated contagious mammary pathogens by The National Mastitis Council (NMC). Good management practices and implementation of NMC recommendations have reduced the number of infections caused by these two pathogens (Hogan 1999). Incidence of gram negative bacterial infections is on the rise because of a decrease in incidence of contagious mastitis and therefore decreased competition by contagious Gram positive pathogens. In the past few years up to 40% of mastitis causing organisms isolated from intramammary infections (IMI) have been gram negative (Erskine et al. 1991; Munoz et al. 2007). Current control and prevention programs recommended by NMC for contagious mastitis pathogen are not as effective in preventing environmental as they are contagious IMI (Bannerman et al. 2004).

In well managed dairy herds within the United States, *Escherichia coli* is the most frequently isolated gram negative mammary pathogen. However, *Klebsiella* spp. are increasingly being isolated (Kikuchi et al. 1995; Munoz et al. 2006; Munoz et al. 2007; Paulin-Curlee et al. 2007) . *Klebsiella* spp. account for 39.4% of acute cases of gram negative mastitis in the United States (Paulin-Curlee et al. 2008). Isolation of *Klebsiella* spp. has increased in New York State (Munoz et al. 2007). Research shows *Klebsiella* spp. causes longer, more severe infections when compared to *E. coli*, and antibiotic treatment is less effective (Erskine et al. 2002; Munoz et al. 2007). The J5 vaccine has been developed to reduce the severity of gram negative bacterial infections. J5 does not reduce the symptoms of *Klebsiella* spp. mastitis as much as for other gram negative infections, but it does reduce culling of infected animals (Wilson et al. 2007; 2008).

During times of stress, such as, early stages of lactation or nutritional imbalances the cow is more susceptible to mastitis infection. Infection of the mammary gland has three stages invasion, infection, and inflammation (Oviedo-Boyso et al. 2007). The first physical barrier for protection against invading organisms in the dairy cow is the teat end, which is the point of entry for all pathogens into a healthy gland (Sordillo and Streicher 2002; Hogan 2003). The infectious organisms then pass through the teat sphincter to the teat canal. Within the teat canal, keratin, believed to have bacteriostatic properties, must be bypassed. The innate immune response is characterized by macrophages phagocytizing bacteria in the gland cistern. The infectious organism is recognized due to expression of Pathogen Associated Molecular Patterns (PAMPs) such as lipopolysaccharide (LPS). Toll Like Receptors (TLR) on host cells recognize PAMPs causing the synthesis and release of proinflammatory cytokines and resulting in the epithelial cells to express adhesion molecules that recruit polymorphonuclear neutrophils (PMN) to the

site of infection . Clinical signs of *Klebsiella* spp. infections include abnormal milk, fever, swelling of the gland, reduced milk production, and anorexia (Pinna et al. 2005). Experimentally infected mammary glands examined post mortem show massive inflammation and extensive tissue necrosis (Bannerman et al. 2004).

A very important part of the innate immune response is the PMN. These cells are activated in a nonspecific manner and quickly arrive at the site of infection (Sordillo and Streicher 2002). PMN are the first line of immune defense in the mammary gland once recruited. The primary job of PMN is to target and destroy invading organisms (Paape et al. 2003). This results in an inflammatory response with an increase in the number of PMN entering the gland, and a subsequent increase in somatic cell count (SCC). PMN account for over 90% of somatic cells in the bovine mammary gland during infection (Bannerman et al. 2004). Unfortunately, due to their nonspecific nature, these cells also cause damage to host tissue.

The release of toxins contained in the cell wall of *Klebsiella* spp. during antibiotic treatment suggests that the use of antibiotics is detrimental to the cow. Therefore, the best therapy for coliform mastitis is fluids and anti-inflammatory drugs (Hogan 2003). As the SCC number increases in the mammary gland, the quality and quantity of milk produced is decreased. Approximately 80% of the economical loss associated with mastitis is discarded milk and lowered production. *Klebsiella* spp. mastitis infections can be severe, with infected cows having a increased culling and death rate compared to cows infected with other gram negative bacterial infections (Erskine et al. 2002). Within 60 days, 25% of dairy cows identified with *Klebsiella* spp. mastitis may not be in the herd (Oviedo-Boyso et al. 2007). This loss of production and possibly the cow results in a large economic loss to the farmer. Therefore, finding effective treatment or prevention is paramount to negate this loss.

Dry period secretions contain lactoferrin, an iron binding compound, in the mammary gland that reduces the amount of free iron available for bacterial use. However, lactoferrin has less of an antimicrobial effect on *Klebsiella* spp. than other bacteria during the dry period (Hogan 2003). Due to immune system depression associated with calving and the transition period, dairy cows are more susceptible to mastitis during this time. Additionally, leakage of secretions prior to calving results in teat canal relaxation, which opens up a pathway for bacteria to enter the gland (Sordillo and Streicher 2002). During lactation, the concentration of lactoferrin drops causing an increase in iron availability to *Klebsiella* spp. (Rainard and Riollet 2006).

Virulence factors:

Currently, little information is reported in the literature about *Klebsiella* spp. pathogenicity in IMI. Factors identified in human infections which may contribute to the virulence of *Klebsiella* spp. include capsule type, biofilm production, and evasion factors. This study focused on capsule and biofilm production, and PMN evasion factors of the innate immune system.

Capsule:

Capsule production has been the most extensively studied virulence factor. Capsule formation is believed to cause bacterial surfaces not to be recognized by the innate immune system. Capsule has unique properties including the presence of a complex acidic polysaccharide sheet forming thick bundles of fibrillous structures protecting the bacterium from phagocytosis. The specific composition of a capsule tends to be variable (Schembri et al. 2005). Conjectures have been made that different capsule properties resulting from variation in the high mannose and fructose content contribute to differences in virulence (Pinna et al. 2005). Thick

capsules of *Klebsiella* spp. have shown poor adhesion to and internalization by host cells, but decreased recognition by the host immune system. The opposite is true for strains with little or no capsule; bacteria are able to adhere and invade the cell, but are more susceptible to the defenses of the innate immune system (Struve and Krogfelt 2004). The possibility of capsule polysaccharides acting as an adhesion by binding to host cells has been suggested (Donlan and Costerton 2002). While capsule has shown to be both protective and inhibitory once they are internalized by host cells the encapsulated isolates are able to maintain a presence, but noncapsulated variants decline significantly within the host cell (Sahly et al. 2000).

Capsule can both contribute to and hinder pathogenicity once the bacterium is within the host. The, K1, capsule type is composed of a linear homopolymer of sialic acid residues, and is more virulent than the K2 capsule type (Lau et al. 2007). Increased virulence of K1 can be contributed to the mucoviscosity associated gene A (*mapA*) and regulator of muciod phenotype A (*rmpA*) (Nadasy et al. 2007). Approximately 87% of *Klebsiella* spp. liver abscesses contain the *rmpA* gene (Yeh et al., 2007). The K1 serotype is provided resistance against host immune responses by *magA* (Wu et al. 2008). These two capsule types have been identified in strains isolated from cases of liver abscesses in Taiwan. Combined K1 and K2 capsule types account for 78% of Taiwanese liver abscesses, and capsule type seems more important in determining the virulence of the strain than presence of the *rmpA* and *magA* genes (Yeh et al. 2007).

According to Farve-Bonte et al., (1999) three adhesion phenotypes exist. The first phenotype is a diffuse pattern where bacteria spread over the host cell's surface. The second is an aggregative phenotype where bacteria clump together onto the host cells. The third is a localization pattern associated with microcolonies. Pili are not flagella, but are smaller filamentous projections composed of proteins which enhance recognition of specific tissues in

the host. Type 1 pili are thick, rigid, adhesive surface organelles found on most members of the Enterbacteriaeae family. They have non-specific binding to mucoidal and epithelial cells helping internalization of the bacteria (Schembri et al. 2005). Type 1 fimbrae recognizes the mannose containing glycol proteins targeting and attaching to the host cell. The lack of capsule enhances type 1 pili functionality (Sahly et al. 2000). Inside the host cell production of type 1 pili is turned off to prevent intracellular killing. Understanding of how pili mechanisms work is limited (Pinna et al. 2005). Other adhesions are unable to extend beyond the capsule, rendering them nonfunctional, but pili are able to protrude beyond the capsule (Schembri et al. 2005). Capsule deters pili formation by interfering with assembly, but does not affect the number of pili produced. Type 3 pili are composed of adhesions, mrkA and mrkD which facilitate binding to epithelial and endothelial cell (Schembri et al. 2004).

Biofilms:

Biofilms are a polymeric matrix that improves the capacity of bacteria to adhere to the surface of tissues and biomaterials. Biofilm is a protective mechanism allowing the bacteria to survive in environments which could be suboptimal. Biofilms are held together by an exopolysaccharide layer surrounded by water filled channels allowing nutrient and waste exchange (Pinna et al. 2005). The key component of the matrix are polysaccharides made up of fructose and glucose chains (Vuong et al. 2004). Bacteria which produce biofilms are significantly less susceptible to antibiotics, and the host's innate immune defense (Vuong et al. 2004). Hypotheses surmise bacteria within the biofilm grow slower due to decreased nutrient availability explaining slower antibiotic uptake (Donlan and Costerton 2002).

References

Bannerman, D.D., Paape, M.J., Hare, W.R. and Hope, J.C. (2004) Characterization of the bovine innate immune response to intramammary infection with Klebsiella pneumoniae. *J Dairy Sci* **87**, 2420-2432.

Cheng, K.S., Tang, H.L., Hsu, C.H., Lai, H.C., Yu, C.J. and Chou, F.T. (2007) A clinical survey of Klebsiella pneumoniae virulence and genotype in pyogenic liver abscess. *Adv Ther* **24**, 589-593.

Donlan, R.M. and Costerton, J.W. (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* **15**, 167-193.

Erskine, R.J., Bartlett, P.C., VanLente, J.L. and Phipps, C.R. (2002) Efficacy of systemic ceftiofur as a therapy for severe clinical mastitis in dairy cattle. *J Dairy Sci* **85**, 2571-2575.

Erskine, R.J., Tyler, J.W., Riddell, M.G., Jr. and Wilson, R.C. (1991) Theory, use, and realities of efficacy and food safety of antimicrobial treatment of acute coliform mastitis. *J Am Vet Med Assoc* **198**, 980-984.

Hogan, J.S., K. L. (2003) Coliform mastitis. *Vet Res* **34**, 507-519.

Hogan, J.S., R. N. Gonzalez, R. J. Harmon, S. C. Nickerson, S. P. Oliver, J. W. Pankey, and K. L. Smith (1999) *Laboratory Handbook on Bovine Mastitis.* . Madison WI: National Mastitis Council.

Kikuchi, N., Kagota, C., Nomura, T., Hiramune, T., Takahashi, T. and Yanagawa, R. (1995) Plasmid profiles of Klebsiella pneumoniae isolated from bovine mastitis. *Vet Microbiol* **47**, 9-15.

Ko, W.C., Paterson, D.L., Sagnimeni, A.J., Hansen, D.S., Von Gottberg, A., Mohapatra, S., Casellas, J.M., Goossens, H., Mulazimoglu, L., Trenholme, G., Klugman, K.P., McCormack,

J.G. and Yu, V.L. (2002) Community-acquired Klebsiella pneumoniae bacteremia: global differences in clinical patterns. *Emerg Infect Dis* **8**, 160-166.

Kohler, J.E., Hutchens, M.P., Sadow, P.M., Modi, B.P., Tavakkolizadeh, A. and Gates, J.D. (2007) Klebsiella pneumoniae necrotizing fasciitis and septic arthritis: an appearance in the Western hemisphere. *Surg Infect (Larchmt)* **8**, 227-232.

Kristula, M.A., Dou, Z., Toth, J.D., Smith, B.I., Harvey, N. and Sabo, M. (2008) Evaluation of Free-Stall Mattress Bedding Treatments to Reduce Mastitis Bacterial Growth. *J Dairy Sci* **91**, 1885-1892.

Lau, H.Y., Clegg, S. and Moore, T.A. (2007) Identification of Klebsiella pneumoniae genes uniquely expressed in a strain virulent using a murine model of bacterial pneumonia. *Microb Pathog* **42**, 148-155.

Munoz, M.A., Ahlstrom, C., Rauch, B.J. and Zadoks, R.N. (2006) Fecal shedding of Klebsiella pneumoniae by dairy cows. *J Dairy Sci* **89**, 3425-3430.

Munoz, M.A., Welcome, F.L., Schukken, Y.H. and Zadoks, R.N. (2007) Molecular epidemiology of two Klebsiella pneumoniae mastitis outbreaks on a dairy farm in New York State. *J Clin Microbiol* **45**, 3964-3971.

Nadasy, K.A., Domiati-Saad, R. and Tribble, M.A. (2007) Invasive Klebsiella pneumoniae syndrome in North America. *Clin Infect Dis* **45**, e25-28.

Oviedo-Boyso, J., Valdez-Alarcon, J.J., Cajero-Juarez, M., Ochoa-Zarzosa, A., Lopez-Meza, J.E., Bravo-Patino, A. and Baizabal-Aguirre, V.M. (2007) Innate immune response of bovine mammary gland to pathogenic bacteria responsible for mastitis. *J Infect* **54**, 399-409.

Paape, M.J., Bannerman, D.D., Zhao, X. and Lee, J.W. (2003) The bovine neutrophil: Structure and function in blood and milk. *Vet Res* **34**, 597-627.

Paulin-Curlee, G.G., Singer, R.S., Sreevatsan, S., Isaacson, R., Reneau, J., Foster, D. and Bey, R. (2007) Genetic diversity of mastitis-associated Klebsiella pneumoniae in dairy cows. *J Dairy Sci* **90**, 3681-3689.

Paulin-Curlee, G.G., Sreevatsan, S., Singer, R.S., Isaacson, R., Reneau, J., Bey, R. and Foster, D. (2008) Molecular subtyping of mastitis-associated Klebsiella pneumoniae isolates shows high levels of diversity within and between dairy herds. *J Dairy Sci* **91**, 554-563.

Pinna, A., Sechi, L.A., Zanetti, S. and Carta, F. (2005) Detection of virulence factors in a corneal isolate of Klebsiella pneumoniae. *Ophthalmology* **112**, 883-887.

Podschun, R. and Ullmann, U. (1998) Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin Microbiol Rev* **11**, 589-603.

Rainard, P. and Riollet, C. (2006) Innate immunity of the bovine mammary gland. *Vet Res* **37**, 369-400.

Sahly, H., Podschun, R., Oelschlaeger, T.A., Greiwe, M., Parolis, H., Hasty, D., Kekow, J., Ullmann, U., Ofek, I. and Sela, S. (2000) Capsule impedes adhesion to and invasion of epithelial cells by Klebsiella pneumoniae. *Infect Immun* **68**, 6744-6749.

Schembri, M.A., Blom, J., Krogfelt, K.A. and Klemm, P. (2005) Capsule and fimbria interaction in Klebsiella pneumoniae. *Infect Immun* **73**, 4626-4633.

Schembri, M.A., Dalsgaard, D. and Klemm, P. (2004) Capsule shields the function of short bacterial adhesins. *J Bacteriol* **186**, 1249-1257.

Sordillo, L.M. and Streicher, K.L. (2002) Mammary gland immunity and mastitis susceptibility. *J Mammary Gland Biol Neoplasia* **7**, 135-146.

Struve, C. and Krogfelt, K.A. (2004) Pathogenic potential of environmental Klebsiella pneumoniae isolates. *Environ Microbiol* **6**, 584-590.

Todhunter, D.A., Smith, K.L., Hogan, J.S. and Schoenberger, P.S. (1991) Gram-negative bacterial infections of the mammary gland in cows. *Am J Vet Res* **52**, 184-188.

Vuong, C., Kocianova, S., Voyich, J.M., Yao, Y., Fischer, E.R., DeLeo, F.R. and Otto, M. (2004) A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. *J Biol Chem* **279**, 54881-54886.

Wang, J.H., Liu, Y.C., Lee, S.S., Yen, M.Y., Chen, Y.S., Wann, S.R. and Lin, H.H. (1998) Primary liver abscess due to Klebsiella pneumoniae in Taiwan. *Clin Infect Dis* **26**, 1434-1438.

Wilson, D.J., Grohn, Y.T., Bennett, G.J., Gonzalez, R.N., Schukken, Y.H. and Spatz, J. (2007) Comparison of J5 vaccinates and controls for incidence, etiologic agent, clinical severity, and survival in the herd following naturally occurring cases of clinical mastitis. *J Dairy Sci* **90**, 4282-4288.

Wilson, D.J., Grohn, Y.T., Bennett, G.J., Gonzalez, R.N., Schukken, Y.H. and Spatz, J. (2008) Milk Production Change Following Clinical Mastitis and Reproductive Performance Compared Among J5 Vaccinated and Control Dairy Cattle. *J Dairy Sci* **91**, 3869-3879.

Wu, J.H., Wu, A.M., Tsai, C.G., Chang, X.Y., Tsai, S.F. and Wu, T.S. (2008) Contribution of fucose-containing capsules in Klebsiella pneumoniae to bacterial virulence in mice. *Exp Biol Med (Maywood)* **233**, 64-70.

Yeh, K.M., Kurup, A., Siu, L.K., Koh, Y.L., Fung, C.P., Lin, J.C., Chen, T.L., Chang, F.Y. and Koh, T.H. (2007) Capsular serotype K1 or K2, rather than magA and rmpA, is a major virulence determinant for Klebsiella pneumoniae liver abscess in Singapore and Taiwan. *J Clin Microbiol* **45**, 466-471.

Yu, V.L., Hansen, D.S., Ko, W.C., Sagnimeni, A., Klugman, K.P., von Gottberg, A., Goossens, H., Wagener, M.M. and Benedi, V.J. (2007) Virulence characteristics of Klebsiella and clinical manifestations of K. pneumoniae bloodstream infections. *Emerg Infect Dis* **13**, 986-993.

Chapter II

Affect of virulence factors on the ability of *Klebsiella* spp. isolates to evade host immune defense

A.J. Nedrow[1], W. Wark[1], M. Dickenson[1], R.N. Zadoks[2], and I.K. Mullarky[1*]

[1] Department of Dairy Science, Virginia Polytechnic Institute and State University, Blacksburg VA 24061

[2] Quality Milk Production Services, New York State College of Veterinary Medicine, Cornell University, 22 Thornwood Drive, Ithaca, New York 14850; current address Royal (Dick) School of Veterinary Studies, The University of Edinburgh, Easter Bush Veterinary Centre, Roslin, Scotland, UK.

Formatted for Journal of Applied Microbiology

Key words: *Klebsiella*, capsule, bactericidal, mastitis

mullarky@vt.edu

Department of Dairy Science (0315)

2050 Litton-Reaves Hall

Virginia Tech

Blacksburg, VA 24061

(540) 231-2410

(540) 231-5014 (fax)

Abstract

Klebsiella spp. are coliform bacteria that frequently cause mastitis in dairy cattle, and produce significant financial loss for the producer. Recent outbreaks indicate that a single strain can be responsible for intramammary infection in multiple cows on a farm. Identification of virulence factors found in these isolates may enable it to survive in the mammary gland of multiple animals. This will provide insight into host adaptation and the increased virulence of *Klebsiella* spp. In this study, *Klebsiella* spp. isolates infecting multiple or single dairy cows were identified. These isolates were evaluated for biofilm and capsule production and the ability to evade neutrophil killing. Biofilm was not produced above the cutoff value of 0.1 OD for any of the isolates tested. Capsule size was not significantly different ($P = 0.29$) between isolates found in multiple animals when compared to isolates found in single cases of bovine mastitis. Additionally there was no difference in ability to evade neutrophil killing between the two strain types ($P = 0.47$). This suggests that other factors, such as iron uptake systems and serotype may contribute to the abilities of *Klebsiella* spp. to evade the bovine innate immune system defenses. Identification of these factors will provide potential routes for prevention and treatment of this infection.

Keywords: *Klebsiella*, mastitis, capsule, PMN, bactericidal

Introduction

Klebsiella spp. are gram negative bacteria emerging as opportunistic and genetically diverse pathogens. *Klebsiella* spp. are typically found in the environment and on mucosal surfaces of multiple host species. *Klebsiella* spp. have been associated with a variety of infections in humans including liver abscesses, pneumonia and urinary tract infections (Kohler et al. 2007). *Klebsiella* spp. have emerged as a leading cause of severe gram negative clinical mastitis in dairy cows and as one of the most commonly isolated gram negative mammary pathogens the United States (Ko et al. 2002; Cheng et al. 2007; Munoz et al. 2007).

In human *Klebsiella* spp. infections, capsule and biofilm production have been identified as virulence factors (Podschun and Ullmann 1998). Biofilm is a polymeric matrix produced by bacteria allowing adherence to the surface of tissues and biomaterials. The primary components of the matrix are polysaccharides such as repeating units of fructose and glucose (Vuong et al. 2004). Biofilm production protects bacterial strains from destruction by forming a protective polymeric matrix layer around colonies (Podschun and Ullmann 1998). Biofilm production has been shown to play a role in colonization of the cornea (Pinna et al. 2005) and in the gastro-intestinal tract of humans (Hennequin and Forestier 2009). Changes in biofilm production and therefore virulence of *Klebsiella* spp. has been recently observed in the Eastern hemisphere (Vuong et al. 2004).

Capsule production by *Klebsiella* spp. has been studied extensively and is considered a dominant virulence factor. The capsule is comprised of complex acidic polysaccharides forming thick bundles of fibrillous structures which protect the bacterium from phagocytosis. Specific composition of capsules tend to be strain dependent (Schembri et al. 2005), and a major virulence factor for *Klebsiella* spp. (Yeh et al. 2007). Changes in capsule expression among

strains suggests an improvement by strains to conceal bacterial surfaces from recognition by the innate immune system (Podschun and Ullmann 1998). This lack of recognition, specifically by polymorphonuclear neutrophils (PMN) results in diminished immune response to the infection.

In dairy cattle, a first line of defense against pathogens in the bovine mammary gland is the innate immune system, composed predominantly of PMN. PMN make up 90% of the immune cells found in the infected mammary gland. These cells have the ability to phagocytize and kill invading bacteria. In this study, two virulence factors, biofilm and capsule production, were evaluated for increased ability to evade killing by PMN. *Klebsiella* spp. isolates identified as causing a unique or multiple intramammary infections (IMI) were evaluated for biofilm and capsule production and the ability to evade killing by bovine PMN. We hypothesized that *Klebsiella* spp. virulence is enhanced by increased biofilm or capsule production, thereby providing multiple isolates with an increased ability to evade innate immune defenses compared to unique isolates. Knowledge of mechanisms associated with immune evasion will contribute to the development of appropriate preventative methods and vaccine targets for *Klebsiella* spp. mastitis.

Materials and Methods

Bacterial isolates

In this study 10 *Klebsiella pneumoniae* isolates cultured from bovine clinical mastitis milk samples were obtained from the Quality Milk Production Services, Cornell University, Ithaca NY. Isolates were identified to the genus level using standard biochemical tests (Hogan 1999) and to the species level using random amplified polymorphic DNA-typing by sequencing of the *rpoB* gene (Munoz et al., 2007). The *K. pneumoniae* isolates represented five pairs of isolates obtained from four dairy farms. For each pair one strain was isolated from a single cow and one strain from multiple cows within the same herd. Pairs of isolates within herds were chosen so occurrence in single or multiple infections could be attributed to isolates and not herd. Table 1 identifies the origin of isolates, farm size, bedding type, housing type, breed, and scientific papers where the specific infection characteristics are further described (Zadoks 2009).

Isolates were stored in trypticase soy broth with 15% glycerol at -80º C until needed. Bacteria were prepared by initial culture on esculin blood agar plates and subsequent culture of a single colony in Luria broth (LB) (BD Franklin Lakes, New Jersey, USA) or skim milk (SM) (BD Franklin Lakes, New Jersey, USA) (25 ml) at 37ºC for 15 to 18 hours in an orbital plate shaker (Model I2400, New Brunswick Scientific Incubator Shaker, New Brunswick, NJ, USA). After culturing, bacteria were centrifuged (Model number 5810R, Eppendorf, Fisher Scientific, Inc., Pittsburgh, PA, USA) at 3,000 rpm at 4ºC for 15 min, washed two times with phosphate buffered saline (PBS) (BD Franklin Lakes, New Jersey, USA) and centrifuged at 3,000 rpm at 4ºC for 15 min. Bacterial concentrations were determined using serial dilutions and adjusted to 1.5×10^7 CFU/ml in Roswell Park Memorial Institute (RPMI) media (Gibco, Carlsbad, CA, USA)

containing 5% fetal bovine serum (Hyclone Thermo Fisher Scientific, Waltham, MA, USA) and 1% L-glutamine (Gibco, Carlsbad, CA, USA).

Biofilm detection

In polyvinyl chloride (PVC) flat bottom 96 well plates (BD, Franklin Lakes, New Jersey, USA) a 100 µl dilution of 1:10 LB to PBS and 100 µl standardized overnight bacterial cultures were combined into eight wells for each isolate. A single replicate was completed for each of the 5 unique and 5 multiple isolates. Serial dilutions were completed to determine CFU/ml of cultures. Concentra ration of cultures did not impact biofilm production and therefore was not included in statistical analysis. Additionally CFU concentrations were adjusted to 1.5×10^7 for the bactericidal assay. Plates were incubated for 8 hours at 37ºC at 5% CO_2 air atmosphere. After incubation, wells were tapped out, washed twice with PBS (200µl), and stained with 1% gram's crystal violet (CV) for 15 min at room temperature (RT). Washing of the wells was repeated, and the plates were dried overnight in a 37ºC incubator. After drying, CV was eluted with 95% ethanol (200µl) and the plates were read at 595 nm on a Bio-Teck µQuant Microplate reader (Bio-Tek Inc., Winooski, VT, USA). An isolate was considered positive for biofilm formation when an OD reading was greater than 0.1 as previously described (Maldonado et al. 2007).

Capsule detection

Bacterial capsule was detected as previously described (Claus 1989). An aliquot of overnight culture (10 µl) grown from the same colony as for biofilm was combined with one drop of India ink (BD, Franklin Lakes, New Jersey, and USA) on a clean glass slide. Concentration (CFU/ml) of overnight cultures were determined, but were found to be not

significant in the statistical model. A second slide was used to streak the mixture across the slide. Slides were air dried, stained with CV, and rinsed with water (Claus 1989). Once dry the slides were then observed under 100x oil immersion microscopy. Three random micrographs of each slide were taken. The area occupied by the microbe and the entire area including the capsule was determined. The difference between the two areas measured was used to estimate the area of the capsule. Image Pro software version 6.2 (Media Cybernetics Inc. Bethesda, MD) was used to take measurements. For each isolate a minimum of three bacteria were measured for LB and SM.

Heat inactivated sera (Klebsiella serum)

Blood was collected (250 ml) in a bottle containing 25 ml PBS via jugular puncture using a blood collection kit (Kawasumi Laboratories, Tampa Florida, USA) from four cows, previously diagnosed with *Klebsiella* spp. mastitis. The blood was allowed to clot at RT, sera was removed, pooled from the four cows, and heat-inactivated by incubation at 56°C for 30 minutes. One ml aliquots of the serum were stored at -80ºC.

Agglutination assay

Bovine *Klebsiella* spp. sera were obtained from four lactating Holstein dairy cows previously diagnosed with *Klebsiella* spp. mastitis. The optimum concentration of sera required to opsonize the different isolates of *Klebsiella* spp. was determined. Two fold serial dilutions of serum in PBS were completed in a 96-well U-bottom plate (BD, Franklin Lakes, New Jersey, USA). Once sera was diluted *Klebsiella* spp. (100 µl, $1x10^7$cfu/ml) were added to each well. The plate was mixed for 5 min and incubated at RT overnight. The first dilution that did not

agglutinate was used to opsonize *Klebsiella* spp. bacteria for the bovine blood PMN bactericidal assay (Aarestrup et al. 1994).

Bovine blood neutrophil bactericidal assay

Bovine whole blood (100 ml) was collected via jugular puncture using a blood collection kit (Kawasumi Laboratories, Tampa Florida, USA) in a blood bottle containing 10% (vol/vol) 40 mM EDTA. PMN were isolated as previously described (Mullarky et al. 2001). In brief, blood was transferred to 50 ml conical tubes and centrifuged for 30 minutes 15ºC at 2000 rpm. Plasma and buffy coats were discarded. The remaining red blood cells (RBC) and PMN were mixed, and 7 ml of the mixture was resuspended in 20 ml ddH_2O to lyse the RBC. To stop the lysing process, 3 x Modified Eagle's Medium (MEM) was added (10ml) to neutralize the ddH_2O. The tubes were brought up to a final volume (45ml) with PBS containing 5 mM EDTA (PBSE) and centrifuged for five min at 15ºC 2000 rpm. Supernatants were poured off, and remaining cell pellet was rinsed with PBSE (45 ml) and centrifuged for 5 min at 15ºC 1000 rpm. This procedure was repeated until RBC were no longer visible. Isolated PMN were counted and resuspended to a final concentration of $1x10^7$ cells/ml in Roswell Park Memorial Institute (RPMI) media (Gibco, Carlsbad, CA, USA) containing 5% fetal bovine serum (Hyclone) and 1% L-glutamine (Gibco) for use in the bactericidal assay.

Bacterial resistance to bovine PMN was evaluated by the bactericidal assay as previously described (Mullarky et al. 2001). In brief, bacteria ($1.5x10^7$ CFU/ml) were opsonized in 6.25% *Klebsiella* serum for 30 min at 37ºC. Using 96-well tissue culture plates (BD, Franklin Lakes, New Jersey, USA). Opsonized bacteria (100µl, $1.5x10^7$ CFU/ml) were combined with PMN (100µl, $1x10^7$ cells/ml) and incubated at 37 ºC for 1 hr. As a control for assay conditions,

Staphylococcus aureus ATCC 29217 (American Type Culture Collection, Manassas VA, USA) was run on a separate plate. Wells with bacteria, media or PMN only were included on all plates and used in calculating percentage bacteria killed at the end of the assay. After one hour of incubation, 0.2% saponin (50µl) (Sigma-Aldrich St. Louis, MO) was added to each well to lyse PMN. Thiazolyl Blue Tetrazolium Bromide (50µl, 1mg/ml) (Alpha Aesar, Ward Hall, MA, USA) was added to measure the remaining number of bacteria within the wells. After color development, approximately 20 min, the bacteria were lysed with the addition of freshly prepared extraction buffer (100µl) containing10ml ddH_2O, 10ml N,N-Dimethylformamide (Fisher Scientific, Pittsburg, PA, USA), and 4 g SDS (J. T. Baker, Phillsburg, NJ,USA) dissolved at 37°C. Plates were read at a wavelength of 595 nm on a Bio-Teck µQuant microplate reader (Bio-Tek Inc., Winooski, VT, USA) and OD was recorded.

Statistical Analysis

Data was analyzed using SAS Software, Cary, NC. Descriptive statistics were derived using the frequency procedure in SAS software. Using the PROC MIXED model (SAS system for Windows ver. 9.2) media type, strain type, farm, capsule, cow, and all plausible two way interactions were included in the original model and tested for significance. Variables were offered into the model and non significant variables and two way interactions were removed from the model by stepwise backwards elimination. The final model included media type, strain type, farm, cow, strain type by cow, plus capsule. For the final model least squares means and standard errors were determined for significant variables. To adjust for multiple comparisons within each model, Tukey's adjusted P-values were calculated for each variable. Significance was declared at $P < 0.05$.

Results

Biofilm

None of the tested *K. pnuemoniae* isolates were above the positive cut off value of 0.1 OD for biofilm production, and were not included in the statistical analysis (Table 2). Average biofilm expression for multiple isolates in LB was 0.07±0.001 as compared to SM 0.06±0.002. For unique isolates, average biofilm expression was 0.07±0.006 in LB and 0.06±0.002 in SM.

Capsule production

Capsule production did not significantly ($P = 0.29$) impact bactericidal killing. There was a trend (23.6 ±6.7) for lower evasion of PMN killing by *Klebsiella* spp. grown LB (45.31±3.7) as compared to SM (21.70±4.74). Average capsule size in pixels for isolates grown in LB (2465±409.2) and SM (3481± 442.2) are represented in Figure 1. CFU for capsule size measurements was not included in the statistical model. Though not significant, multiple isolates expressed less capsule when grown in LB (2632± 566.7) as compared to SM (3641±784.1). Similarly, there was less capsule expression for unique isolates grown in LB (2297±646.8) as compare to SM (3321±503.1). Average capsule size in pixels for multiple and unique isolates grown in either LB or SM are represented in Figure 2. A representative micrograph of capsule production by unique and multiple isolate grown in LB or SM is shown in Figure 3.

Evasion of PMN

Evasion of PMN by *Klebsiella* spp. was not significantly different ($P = 0.47$) between isolates that caused mastitis in single cows (31.60±3.7) as compared to isolates from multiple

(35.4±3.8) cases of mastitis. However, the overall effect of media type played a significant role on bactericidal killing percent with bacterial killing of isolates grown in LB being 23.6± 6.7% higher than killing in SM media (*P* < 0.001; Figure 4). Farm proved to be a significant factor (*P* < 0.001) in bactericidal killing. Specifically, isolates from farm 1 (11.86± 4.8) were 37.8% better at evading PMN killing as compared to isolates from farm 4 (49.6±5.8) (*P* < 0.001, Figure 5). Similarly, isolates from farm 1 were 29.1 ±7.6 % better at evading PMN killing as compared to isolates from farm 3 (40.9±5.8) (*P* < 0.01; Figure 5). Strain type and cow interaction was significant (*P* = 0.001) in the evasion capabilities of *Klebsiella* spp. isolates. Bactericidal killing percentages of isolates by cow are depicted in Figure 6. No significant effect on percent killing was observed with media type by strain type, media type by farm, strain type by farm, media type, strain type, farm, CFU, or media type by cow.

Discussion

Klebsiella spp. isolates that were associated with single or multiple intramammary infections have been shown to produce biofilm and capsule and to evade killing by bovine PMN. In our study, *Klebsiella* spp. isolates did not exhibit significant biofilm production. Therefore, biofilm production may not contribute to enhanced survival in the mammary gland. However, further evaluation of biofilm production in SM may be warranted including testing of additional isolates and increasing incubation time from 8 hours to 12 hours to represent another common milking interval.

Mannose and fructose content are capsule properties that contribute to virulence (Pinna et al. 2005). The thick capsule of *Klebsiella* spp. has shown poor adhesion to host cells, and decreased recognition by the innate immune system. However isolates with little or no capsule

are not better able to evade the innate immune system, but show better adhesion abilities to host cells (Struve and Krogfelt 2004). While difference between capsule sizes in the tested media was not statistically significant, biologically some of the isolates could be using this mechanism for evasion.

The farm of isolate origin and the cow from which PMN were isolated had significant role in evasion abilities. Significance of farm was surprising since farm was controlled for by using two types of isolates, unique and multiple, from each farm. Significant of cow can be explained more easily since the function of PMN is influenced by the cow from which blood was collected. Specifically, though cows were under the same environmental conditions, they varied in age, stage of lactation, had differing SCC scores and were not genetically identical. Strain by cow was examined, and while overall the interaction was significant no difference was seen in killing ability of multiple as compared to unique isolates by PMN from a single cow.

Media type in bactericidal killing remained significant in the final model because of the differences in composition of SM and LB. SM has increased concentrations of lactose, and iron suggesting that LB may not provide the optimal carbohydrates and that it may be limiting for optimal bacterial growth. If the *Klebsiella* spp. isolates used in this study are host adapted then SM media would be a more ideal growth medium as it more similarly mimics the mammary gland secretions.

In conclusion, our studies indicate that farm, media type, and strain type by cow play a role in an enhanced ability to evade PMN killing. The enhanced ability of isolates grown in SM was not attributed to capsule or biofilm and therefore may be due to other virulence factors. Other virulence factors to be investigated include iron acquisition systems and pili expression.

In addition, future studies should evaluate differences in individual cows' immune systems, and their abilities to effectively eliminate invading pathogens.

References

Aarestrup, F.M., Scott, N.L. and Sordillo, L.M. (1994) Ability of Staphylococcus aureus coagulase genotypes to resist neutrophil bactericidal activity and phagocytosis. *Infect Immun* **62**, 5679-5682.

Cheng, K.S., Tang, H.L., Hsu, C.H., Lai, H.C., Yu, C.J. and Chou, F.T. (2007) A clinical survey of Klebsiella pneumoniae virulence and genotype in pyogenic liver abscess. *Adv Ther* **24**, 589-593.

Claus, G.W. (1989). In *Understanding microbes: A laboratory textbook for microbiology* ed. Balkwill, D. New York: W. H. Freeman.

Hennequin, C. and Forestier, C. (2009) oxyR, a LysR-type regulator involved in Klebsiella pneumoniae mucosal and abiotic colonization. *Infect Immun* **77**, 5449-5457.

Hogan, J.S., R. N. Gonzalez, R. J. Harmon, S. C. Nickerson, S. P. Oliver, J. W. Pankey, and K. L. Smith (1999) *Laboratory Handbook on Bovine Mastitis.* . Madison WI: National Mastitis Council.

Ko, W.C., Paterson, D.L., Sagnimeni, A.J., Hansen, D.S., Von Gottberg, A., Mohapatra, S., Casellas, J.M., Goossens, H., Mulazimoglu, L., Trenholme, G., Klugman, K.P., McCormack, J.G. and Yu, V.L. (2002) Community-acquired Klebsiella pneumoniae bacteremia: global differences in clinical patterns. *Emerg Infect Dis* **8**, 160-166.

Kohler, J.E., Hutchens, M.P., Sadow, P.M., Modi, B.P., Tavakkolizadeh, A. and Gates, J.D. (2007) Klebsiella pneumoniae necrotizing fasciitis and septic arthritis: an appearance in the Western hemisphere. *Surg Infect (Larchmt)* **8**, 227-232.

Maldonado, N.C., Silva de Ruiz, C., Cecilia, M. and Nader-Macias, M.E. (2007) A simple technique to detect Klebsiella biofilm-forming-strains. Inhibitory potential of Lactobacillus

fermentum CRL 1058 whole cells and products. In *Communicating Current Research and Educational Topics and Trends in Applied Microbiology* ed. Méndez-Vilas, A. pp.52-59. Badajoz: Formatex.

Mullarky, I.K., Su, C., Frieze, N., Park, Y.H. and Sordillo, L.M. (2001) Staphylococcus aureus agr genotypes with enterotoxin production capabilities can resist neutrophil bactericidal activity. *Infect Immun* **69**, 45-51.

Munoz, M.A., Welcome, F.L., Schukken, Y.H. and Zadoks, R.N. (2007) Molecular epidemiology of two Klebsiella pneumoniae mastitis outbreaks on a dairy farm in New York State. *J Clin Microbiol* **45**, 3964-3971.

Pinna, A., Sechi, L.A., Zanetti, S. and Carta, F. (2005) Detection of virulence factors in a corneal isolate of Klebsiella pneumoniae. *Ophthalmology* **112**, 883-887.

Podschun, R. and Ullmann, U. (1998) Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin Microbiol Rev* **11**, 589-603.

Schembri, M.A., Blom, J., Krogfelt, K.A. and Klemm, P. (2005) Capsule and fimbria interaction in Klebsiella pneumoniae. *Infect Immun* **73**, 4626-4633.

Struve, C. and Krogfelt, K.A. (2004) Pathogenic potential of environmental Klebsiella pneumoniae isolates. *Environ Microbiol* **6**, 584-590.

Vuong, C., Kocianova, S., Voyich, J.M., Yao, Y., Fischer, E.R., DeLeo, F.R. and Otto, M. (2004) A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. *J Biol Chem* **279**, 54881-54886.

Yeh, K.M., Kurup, A., Siu, L.K., Koh, Y.L., Fung, C.P., Lin, J.C., Chen, T.L., Chang, F.Y. and Koh, T.H. (2007) Capsular serotype K1 or K2, rather than magA and rmpA, is a major

virulence determinant for Klebsiella pneumoniae liver abscess in Singapore and Taiwan. *J Clin Microbiol* **45**, 466-471.

Zadoks, R.N. (2009) ed. Nedrow, A.

<table>
<tr><th colspan="2">Isolate ID</th><th>Herd</th><th>Bedding type</th><th>Herd Size</th><th>Housing Type</th><th>Breed</th><th>Reference</th></tr>
<tr><td>1 (M)</td><td>QMP M1-199</td><td rowspan="2">4</td><td rowspan="2">Not reported</td><td rowspan="2">109 to 1500 cows</td><td rowspan="2">Not reported</td><td rowspan="2">Not reported</td><td rowspan="2">Munoz et al., 2006 (cross-sectional study)</td></tr>
<tr><td>2 (U)</td><td>QMP M1-200</td></tr>
<tr><td>3 (M)</td><td>QMP M1-222</td><td rowspan="2">3</td><td rowspan="2">Sand</td><td rowspan="2">1200 cows</td><td rowspan="2">Not reported</td><td rowspan="2">Not reported</td><td rowspan="2">Munoz et al., 2006 (longitudinal study)</td></tr>
<tr><td>4 (U)</td><td>QMP M1-428</td></tr>
<tr><td>5 (M)</td><td>QMP M1-726</td><td rowspan="2">1</td><td rowspan="2">Sawdust</td><td rowspan="2">410 cows</td><td rowspan="2">Free Stalls</td><td rowspan="2">Holstein</td><td rowspan="2">Munoz et al., 2007</td></tr>
<tr><td>6 (U)</td><td>QMP M1-822</td></tr>
<tr><td>7 (M)</td><td>QMP Z4-692</td><td rowspan="4">2</td><td rowspan="4">Sand</td><td rowspan="4">4000 cows</td><td rowspan="4">Free stalls</td><td rowspan="4">Holstein, Holstein/Jersey crosses</td><td rowspan="4">Oostrum et al., 2008</td></tr>
<tr><td>8 (M)</td><td>QMP Z4-702</td></tr>
<tr><td>9 (U)</td><td>QMP Z4-724</td></tr>
<tr><td>10 (U)</td><td>QMP Z4-726</td></tr>
</table>

Table 1. Characteristics of *Klebsiella* spp. isolates used in the evaluation of virulence characteristics. Farms of origin, bedding type, housing type, and animal breeds are provided. Isolate identification (ID) is provided along with multiple (M) or unique (U) classification.

Isolate	LB	SM	Multiple or Unique
1	0.068	0.068	Multiple
2	0.074	0.068	Unique
3	0.075	0.068	Multiple
4	0.092	0.067	Unique
5	0.069	0.062	Multiple
6	0.066	0.062	Unique
7	0.067	0.064	Multiple
8	0.071	0.06	Multiple
9	0.058	0.059	Unique
10	0.08	0.063	Unique
Control	0.07	0.07	

Table 2. Biofilm production by *Klebsiella* spp. isolates. Level of biofilm produced by isolates grown in Luria Broth (LB) or skim milk (SM) represented as optical density (OD, measured at 595nm). The cutoff value for positive growth was 0.1 OD which none of the isolates achieved.

Dependant variable	**Variable description**	**Estimate**	**SE**	**Adjusted *P*- value**
Intercept		10	9.1	0.27
Media Type				
	LB vs. SM	23.6	9.7	0.0008
Farm				
	4 vs. 2	37.8	7.1	< 0.0001
	3 vs. 2	29.1	7.6	0.0019
Strain Type by cow				
	Multiple 4184 vs. Unique 4168	34.7	10.4	0.0183
	Unique 4136 vs. Unique 4168	53.2	10.8	0.0001
	Unique 4168 vs. Unique 4184	-25.68	9.9	0.0312
	Multiple 4168 vs. Unique 4136	-25.5	8.6	0.0502

Table 3. Results of statistical analysis for ability of *Klebsiella* spp. isolates collected from farms with multiple or unique cases of mastitis grown in Luria Broth (LB) or skim milk (SM) to evade neutrophil killing using the PROC MIXED in SAS (SAS Inst. Inc., Cary, NC).

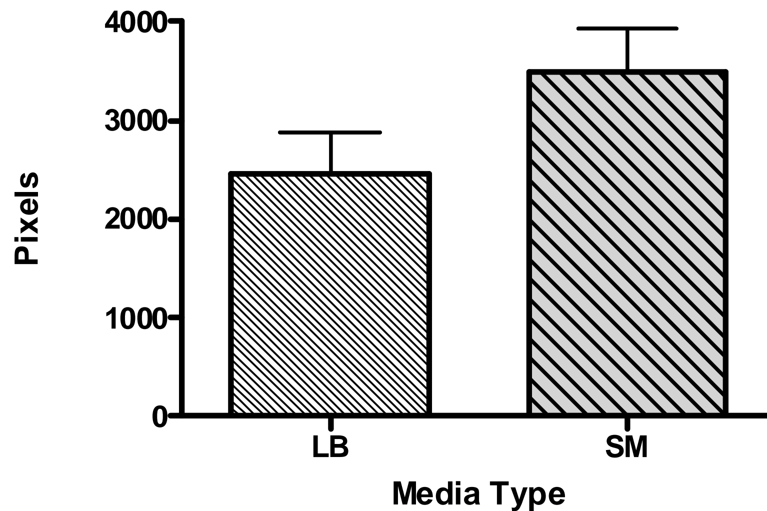


Figure 1. Average capsule size in pixels between *Klebsiella* spp. isolates cultured in different media types. *Klebsiella* spp. isolates were culture in Luria broth (LB; n=10) or skim milk (SM; n=10). Overnight cultures were streaked on glass slides, stained with India ink, counterstained with crystal violet and capsule expression was measured using Image Pro software version 6.2 (Media Cybernetics Inc. Bethesda, MD). No significant difference in capsule size on bacteria grown in different media types was found (P = 0.29). Least square means or SEM were not completed.

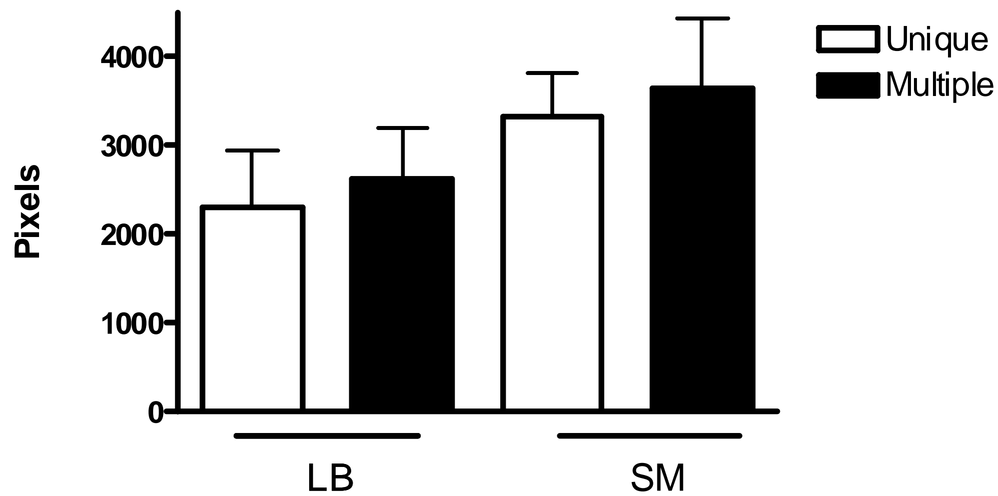


Figure 2. Average capsule size of unique and multiple *Klebsiella* spp. isolates. Capsule size of unique (white bars, n=5) and multiple (black bars n=5) *Klebsiella* spp. isolates grown in Luria broth (LB) or skim milk (SM) are represented in pixels measured with Image Pro software version 6.2 (Media Cybernetics Inc. Bethesda, MD). There was no significant difference between media types or isolate types for capsule production by bacteria (P = 0.29), so no LS means or SEM are available.

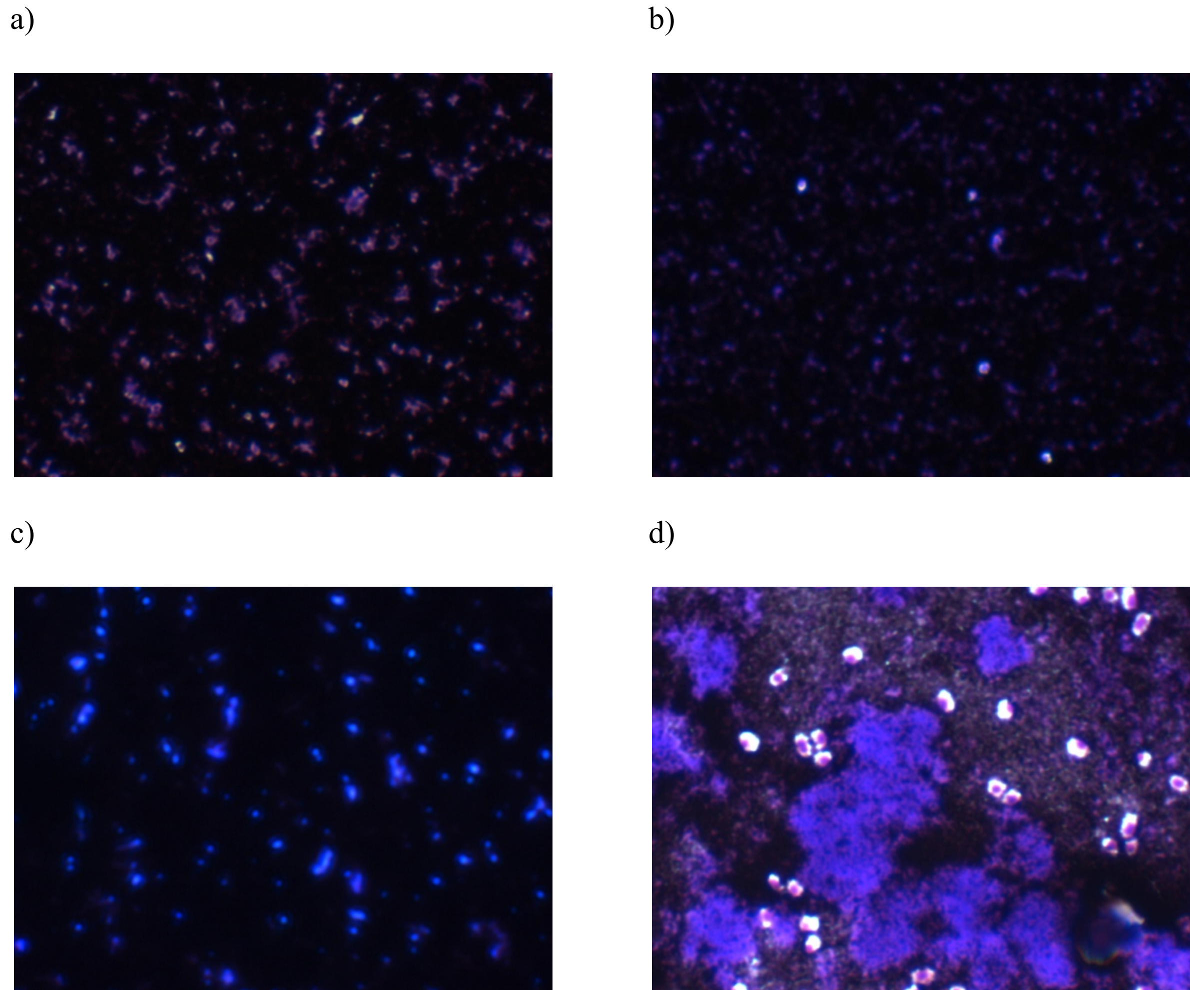


Figure 3. Photomicrographs of capsule production by *Klebsiella* spp. isolates using Image Pro version 6.2 (Media Cybernetics Inc. Bethesda, MD). Capsule production is represented for a) a multiple strain grown in Luria broth, b) a multiple strain grown in skim milk, c) a unique strain grown in Luria broth, and d) a unique strain grown in skim milk.

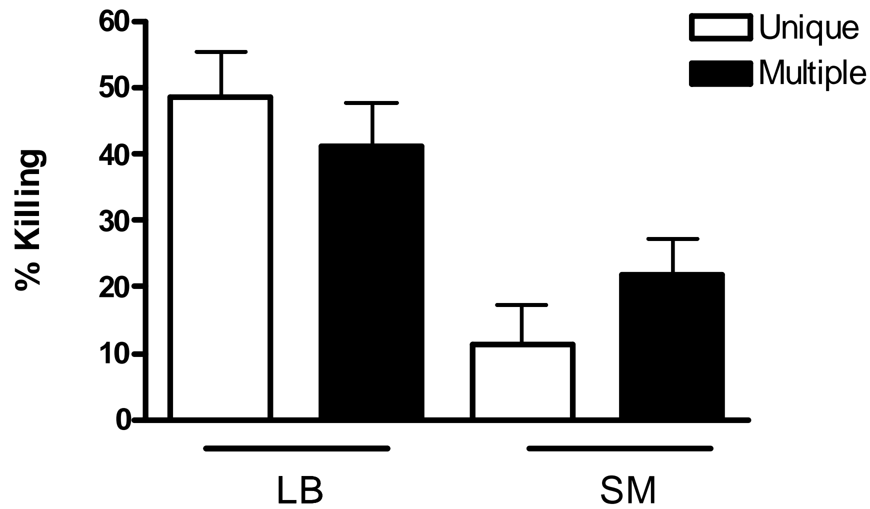


Figure 4. Bactericidal killing of *Klebsiella* spp. isolates. Unique (white bars, n=5) and multiple (black bars, n=5) isolates of *Klebsiella* spp. were grown in Luria broth (LB) or skim milk (SM) and percent of bacteria killed by bovine neutrophils was determined. Isolates grown in LB had a higher percent bacteria killed (23.6 ± 6.70) compared to SM isolates regardless of isolate type ($P < 0.001$).

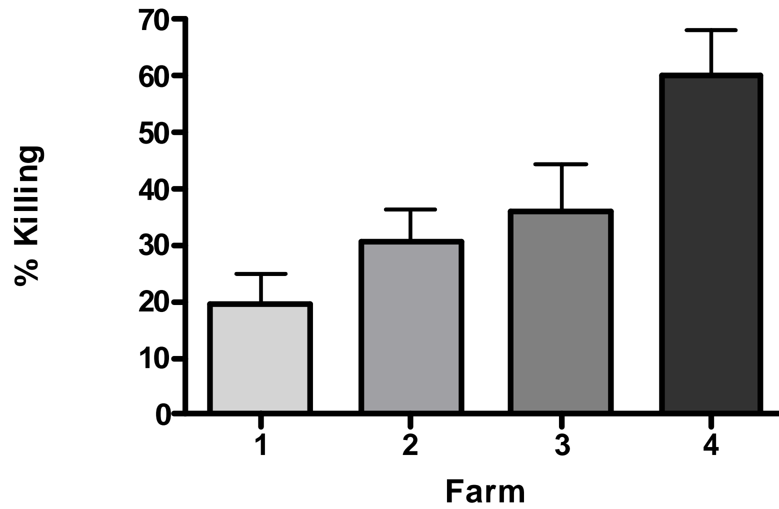


Figure 5. Percent bactericidal killing of unique and multiple *Klebsiella* spp. isolates from different farms. Isolates originating from different farms showed a significant difference ($P < 0.001$; n=2 for farms 1, 2, and 3; n=4 for farm 4) in evasion of PMN after one hour incubation independent of media type. Specifically isolates from farm 1 were significantly ($P < 0.001$) better able to evade PMN killing than isolates from farm 4 (37.8 ± 7.1) and farm 3 (29.1 ± 7.6).

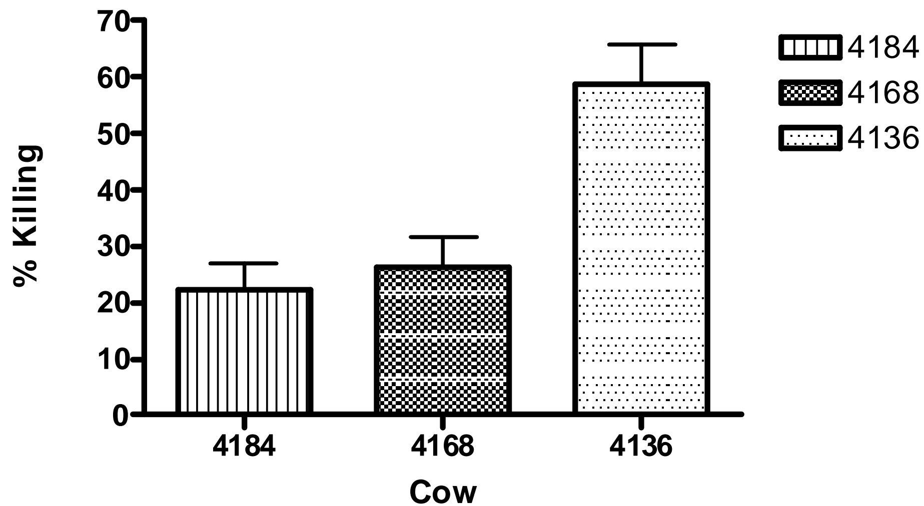


Figure 6. Percent bactericidal killing of unique and multiple *Klebsiella* spp. isolates from different cows. Ability of neutrophils to kill *Klebsiella* spp. isolates (n=10) was significantly impacted by cow (P <0.0018). Neutrophil killing ability was increased when cells were isolated from cow 4136 (27.54 ±8.8; P < 0.001) and lower when isolated from cow 4168 (-25.68 ± 9.9; P=0.01). Significance was not seen for unique and multiple isolates incubated with neutrophils from the same cow.

Chapter III

Overall summary:

This study focused on the ability of *Klebsiella* spp. mastitis isolates to produce the virulence factors biofilm and capsule and to evade PMN killing. Previous research in humans has shown biofilm and capsule production to have significant impacts on the virulence of an infection (Ko et al. 2002; Pinna et al. 2005; Kohler et al. 2007). *Klebsiella* spp. are environmental pathogens which are endogenous to mammalian mucosal surfaces and most importantly the digestive tract. This results in large quantities of *Klebsiella* spp. shed in fecal matter (Munoz et al. 2006) and almost always present in the cow's environment.

In this study biofilm production was tested for only 8 hours to represent a commonly used milking interval. Biofilm was produced, but not at a level considered positive (Maldonado et al. 2007). The incubation time could be extended to 12 hours to represent another common milking interval. Current research of biofilm production in medical devices shows increased biofilm production when organisms are grown in a high shear environment (Donlan and Costerton 2002). While shear force within the mammary gland may or may not be applicable to the dairy cow, it is an avenue which warrants further exploration.

Traditionally, *Klebsiella* spp. isolates are grown in Luria broth (LB) media. The isolates examined in this study were mammary pathogens, and in the mammary gland lactose is the main carbohydrate source (Vangroenweghe et al. 2002). An increased ability to evade PMN killing was expected from the isolates grown in skim milk (SM) compared to (LB) because SM has an increased lactose and iron content. Isolates cultured in SM may have increased capsule size and therefore improved evasion capabilities of PMN killing. However that was not found to be the

case in this study. Furthermore, if some of these isolate in this study are host adapted, LB would serve as a growth limiting media, therefore SM would be a more ideal medium. In this study, SM was found to improve the ability of *Klebsiella* spp. to evade PMN killing, however capsule was not the virulence factor that imparted this increased virulence. Further study of other virulence factors unregulated in SM is warranted. Analysis of a greater number of isolates may be required to conclusively show SM improves evasion of PMN killing. In addition, control of variables such as, cow and farm should be considered and may allow for differences between unique and multiple isolates to emerge.

Capsule suppression was attempted, though unsuccessfully, by culturing isolates in bismuth subsalicylate (BSS), a compound that inhibits capsule production (Domenico et al. 1992). The isolates did not grow substantially enough (CFU/ml) in the BSS media to be used in the bactericidal assay. If suppression and enhancement had both been effective, we would have been able to further analyze the role of capsule in evasion of PMN killing. If the BSS media could be successfully used to grow *Klebsiella* spp. capsule's effect could be further explored.

Specific serotypes types have been shown to be more virulent than others, especially those in the Eastern Hemisphere (Yeh et al. 2007). Serotyping would provide vital information to ascertain if the increasing rates of mastitis cases identified are resulting from specific serotypes. Increased virulence has been observed in serotypes K1 and K2 in human infections (Yeh et al. 2007). Approximately 80 other serotypes are in existence, but not all mechanisms associated with them have been explored (Yeh et al. 2007). Had the serotype been known for each of the isolates in this study, possible identification of serotypes specific to intramammary infection could have been identified thereby leading to easier identification of possible vaccine targets.

The research conducted here is a good starting point to explore if *Klebsiella* spp. isolates are becoming host adapted, and able to better evade the dairy cow's immune system. The possibility does exist that something within the environment of these isolates contributed to the increased infection rate. The variability in cows from which PMN were isolated hindered our ability to obtain the answer to the original question; if isolates isolated from multiple intramammary infections are better able to evade the innate immune system as compared to isolates isolated from only single intramammary infections. However, this research does provide exciting data about the effects of host immune responses of PMN evasion rates by *Klebsiella* spp. isolates causing mastitis.

References

Domenico, P., Salo, R.J., Straus, D.C., Hutson, J.C. and Cunha, B.A. (1992) Salicylate or bismuth salts enhance opsonophagocytosis of Klebsiella pneumoniae. *Infection* 20, 66-72.

Donlan, R.M. and Costerton, J.W. (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 15, 167-193.

Ko, W.C., Paterson, D.L., Sagnimeni, A.J., Hansen, D.S., Von Gottberg, A., Mohapatra, S., Casellas, J.M., Goossens, H., Mulazimoglu, L., Trenholme, G., Klugman, K.P., McCormack, J.G. and Yu, V.L. (2002) Community-acquired Klebsiella pneumoniae bacteremia: global differences in clinical patterns. *Emerg Infect Dis* 8, 160-166.

Kohler, J.E., Hutchens, M.P., Sadow, P.M., Modi, B.P., Tavakkolizadeh, A. and Gates, J.D. (2007) Klebsiella pneumoniae necrotizing fasciitis and septic arthritis: an appearance in the Western hemisphere. *Surg Infect (Larchmt)* 8, 227-232.

Maldonado, N.C., Silva de Ruiz, C., Cecilia, M. and Nader-Macias, M.E. (2007) A simple technique to detect Klebsiella biofilm-forming-strains. Inhibitory potential of Lactobacillus fermentum CRL 1058 whole cells and products. In *Communicating Current Research and Educational Topics and Trends in Applied Microbiology* ed. Méndez-Vilas, A. pp.52-59. Badajoz: Formatex.

Munoz, M.A., Ahlstrom, C., Rauch, B.J. and Zadoks, R.N. (2006) Fecal shedding of Klebsiella pneumoniae by dairy cows. *J Dairy Sci* 89, 3425-3430.

Pinna, A., Sechi, L.A., Zanetti, S. and Carta, F. (2005) Detection of virulence factors in a corneal isolate of Klebsiella pneumoniae. *Ophthalmology* 112, 883-887.

Vangroenweghe, F., Dosogne, H. and Burvenich, C. (2002) Composition and milk cell characteristics in quarter milk fractions of dairy cows with low cell count. *Vet J* 164, 254-260.

Yeh, K.M., Kurup, A., Siu, L.K., Koh, Y.L., Fung, C.P., Lin, J.C., Chen, T.L., Chang, F.Y. and Koh, T.H. (2007) Capsular serotype K1 or K2, rather than magA and rmpA, is a major virulence determinant for Klebsiella pneumoniae liver abscess in Singapore and Taiwan. *J Clin Microbiol* 45, 466-471.

Appendix A: Protocols

Detection of biofilm

Materials:

PVC plates flat bottom (2)
1% Crystal violet
Klebsiella culture
Positive culture
10% LB broth
95% ethanol

Procedure:

1. Load each well with 90 µl LB broth
2. Load each well with 10 µl of culture out of the tube used for opsinization
3. Incubate for 8 hours at 37°C
4. Invert plate and tap out media until most is removed
5. Wash with 200 µl using PBS two times
    a. Tap out after each rinse
6. Fill wells with 200 µl crystal violet
7. Incubate at room temperature for 15 min
8. Invert plate and tap out dye until most is removed
9. Wash with 200 µl PBS
10. Invert plate and tap out PBS until most is removed
    a. Repeat 2 times
11. Invert and incubate at 37°C for 30 min without lid
12. Add 200 µl 95% ethanol to wells and mix

13. OD read of plate with ethanol with µQuant @590nm

Identification of Capsule

Materials:

18 hour culture grown overnight in orbital shaker
Dropper bottle of India ink
Slides
Gram Crystal violet

Procedure:

1. Clean glass slide
2. Place a drop of India ink on one end of the slide
3. Using a sterile loop pick up a small amount of culture, and mix with the India ink
4. Taking a second slide hold at an acute angle with the short side pulling the ink mixture across the slide
5. Air dry the slide
6. Stain the dry slide with crystal violet for 1 min
7. Gently rinse the slide with water
8. Air dry
9. Read under oil immersion
10. Cells are purple, India ink is the grey/black area, and clear area between the bacteria and India ink is the capsule

From Understanding Microbes by W. G. Claus

Isolation of PMN from Whole Blood for *Klebsiella* Bactericidal

Purpose: To isolate neutrophils from whole blood samples for use in functional assays.

Materials:

PBSE@5mM concentration
dd H_2O
3 X Minimum Essential Medium (3 X MEM, from 10 X MEM, Sigma M0275) (pH 7.0, 20°C)

Procedure:

Start with blood collected with 10% (vol/vol) 40mM EDTA.

1. Transfer blood to 50 ml tubes.
2. Spin for 30 min, 2000 RPM, 15°C (no brake).
3. Discard plasma and buffy coat layer.
4. Resuspend blood and neutrophils by pipetting up and down.
    a. Add no more than 7 ml of blood to 20 ml sterile ddH_2O (20°C) in a 50 ml centrifuge tube. Pipette up and down 4 X for a total of 30 sec.
5. Add 10 ml 3 X MEM to the tubes.
6. Top off the tube with PBSE if there is any room left.
7. Spin for 5 min, 2000 RPM, 15°C.
8. Rinse cells with PBSE. Spin at 1000 rpm, 5 min, and 15°C.
    a. If RBC are still present in pellet, follow steps 10-16. If RBC are not present go to step 16.
9. Discard supernatant and resuspend in remaining volume.
10. Add 10 ml sterile ddH_2O. Pipette up and down 4 X (30-40sec).

11. Add 5 ml 3 X MEM, and mix well. Top off tube with PBSE
12. Spin for 5 min, 1000 RPM, 15°C.
13. Rinse cells one more time with PBSE (see step 9).
14. Spin for 5 min, 1000 RPM, 15°C.
15. Pour off supernatant and resuspend (rinse all, but one tube with 5mls media then rerinse all but one tube with an additional 5ml media to gather all cells into one tube with 10ml media total) cells in 10 ml bactericidal media.
16. Neutrophils are ready to count. Remove aliquot and count using hemocytometer.
17. Spin Neutrophils for 5 min, 1000 RPM, 15°C while counting.
18. Cells should be diluted to $1x10^7$ cell/ml final concentration

Klebsiella Bactericidal Assay

Purpose: To determine the extent of bacterial killing by bovine neutrophils.

Reagents:

Heat inactivated sera (*Klebsiella* antiserum):
-The *Klebsiella* antiserum is obtained from cows infected with *Klebsiella*. Pooled antiserum from the eight animals was heat-inactivated by incubating at 56 °C for 30 min. The antiserum is then aliquot and stored at -70°C.
2% Saponin stock solution in PBS (filter sterilized)
2 mg/ml MTT solution in PBS (filter sterilized, protect from light, stores for 1 month)
Bacteria (*Klebsiella*)
Antibiotic-free RPMI (SIGMA, R5886) + 5% Fetal Bovine Serum (FBS, Hyclone) + 1% l-g (assay media)
LB broth
PBS
96 well flat bottom plates
Extraction Buffer:
10ml ddH_2O
10ml DMF (*N,N*-Dimethylformamide)
4 g SDS
Dissolve at 37°C

NOTE: Use aseptic technique throughout, conduct in biosafety cabinet.

Part I – *Preparation of bacteria*

Purpose: To identify CFU/ml in *Klebsiella* cultures.

Procedure:

1. Inoculate 25 ml LB using one colony of Bacteria from isolation plate
2. Incubate overnight (~12 hrs) at 37°C on shaker
3. Transfer culture to 50 ml centrifuge tube
4. Centrifuge 4000 rpm, 15 min, 4°C.
5. Removed sup with vacuum
6. Resuspend pellet in 25 ml PBS, centrifuge and repeat

7. Resuspend pellet in 20 ml PBS.
8. From culture make dilutions:

tube label	Dilution	ml of culture	ml of media
A	10	0.5	4.5
B	100	0.5	4.5
C	10^3	0.5	4.5

9. Continue making dilutions in a 96 well plate:
10. Add 100 µl of bacterial cultures from <u>TUBE B</u> to Row A (1:2 dilution), mix
11. Plate appropriate dilutions (10^4 to 10^7) by dropping 3 x 25 µl drops onto blood plate.
12. Incubate blood plate overnight at 37°C.
13. Count number of colonies in 3 drops: (colony count/3) x 40 x final dilution = CFU/ml
14. Adjust closest dilution to 1 x 10^7 CFU/ml.
15. Opsonize bacteria by incorporating the correct percentage of *Klebsiella* anti-sera (determined by agglutination assay) into the bacterial dilution and mixing on nutator for 30 min.
    a. 6.24% antisera for *Klebsiella* and load bacteria last*
    b. To double check *Klebsiella* concentration, make 5 10-fold dilutions from opsonized stock. Plate 25-µl each of dilutions 4,5,6 and 7 in triplicate on TSB plates and incubate at 37C for 24 hrs
    c. *When possible keep dilutions on ice*

Part II – *Bactericidal assay*

1. Isolate neutrophils according to standard protocol
2. Resuspend cells to $1x10^7$/ml in assay media
3. Warm neutrophils at this stage * add last to plate*
4. Make dilutions of opsonized *Klebsiella* as follows for the standard curve:
    a. 0% reduction – no dilution
    b. 30% reduction – 150 µl RPMI + 5% FBS + 1% L-g + 350 µl *Klebsiella*
    c. 60% reduction – 300 µl RPMI + 5% FBS + 1% L-g + 200 µl *Klebsiella*
    d. 90% reduction – 450 µl RPMI + 5% FBS + 1% L-g + 50 µl *Klebsiella*
5. In a 96-well flat bottom plate, add:
    a. Cell control wells: 50 µl cells + 50 µl media
    b. Test wells: 50 µl cells + 50 µl undiluted *Klebsiella*
    c. Standard curve wells: 50 µl *Klebsiella* (various dilutions) + 50µl media
    d. Blank wells: 100 µl media
    e. Note: Set everything up in quadruplicate
6. Incubate at 37°C for 1 hour in a plastic dish with a moist pad in the bottom (to prevent uneven drafts)
7. To each well, add 50 µl of 0.2 % sterile Saponin and mix thoroughly with multichannel pipettor (will lyse PMN)
8. Add 50 µl sterile MTT (2 mg/ml) to all wells and mix thoroughly. Wrap plate in aluminum foil
9. Incubate on shaker for 15 min and in incubator at 37°C for 10-15 min, or until color change is seen

10. Add 100 µl extraction buffer to each well and incubate 15mintues at 37°C
11. Read on ELISA plate reader at 595 nm and blank on the appropriate well
12. Calculate the percentage of bacteria killed by neutrophils using the following equation:

$$1 - \frac{(\text{OD sample}) - (\text{OD } 90\% \text{ dilution}) * 90\%}{(\text{OD } 0\ \% \text{ dilution}) - (\text{OD } 90\% \text{ dilution})}$$

***Klebsiella* dilutions (CFU)**

Purpose: To identify CFU/ml in *Klebsiella* cultures

Reagents:
LB broth
PBS

Procedure:

1. Inoculate 25 ml LB using one colony of Bacteria from Isolation plate
2. Incubate overnight (~12 hrs) at 37°C on shaker
3. Transfer culture to 50 ml centrifuge tube
4. Centrifuge 4000 rpm, 15 min, 4°C
5. Removed sup with vacuum
6. Resuspend pellet in 25 ml PBS, centrifuge and repeat
7. Resuspend pellet in 20 ml PBS
8. From culture make dilutions:

tube label	Dilution	ml of culture	ml of media
A	10	0.5	4.5
B	100	0.5	4.5
C	10^3	0.5	4.5
D	10^4	0.5	4.5

9. Continue making dilutions in a 96 well plate:
10. See SOP 2.5
11. Add 100 µl of bacterial cultures from <u>TUBE B</u> to Row A (1:2 dilution), mix

12. Plate appropriate dilutions (10^4 to 10^7) by dropping 3 x 25 μl drops onto blood plate.
13. Incubate blood plate overnight at 37°C.
14. Count number of colonies in 3 drops

    (colony count/3) x 40 x final dilution = CFU/ml

Reduction of *Klebsiella* Capsule Production

Purpose: To determine the extent of bacterial killing by bovine neutrophils of *Klebsiella* with suppressed capsule production.

Reagents:
LB broth
PBS
Bismuth Subsalicylate in propylene glycol (100mM with 400 mM NaOH @ pH 12) prepared fresh daily

Procedure:

1. Streak stock culture of *Klebsiella* onto a tryptic soy agar plate and incubate overnight at 37°C.
2. Using a sterile swab, inoculate approximately 10 CFU from single colony on TSA plate into both
3. 25ml LB broth with equal amount of propylene glycol as BSS (control)
4. 25 ml LB broth with correct dilution of BSS (test)
5. Incubate 37°C for 14 to 18 hrs on shaker.
6. Transfer culture to 50 ml centrifuge tube
7. Centrifuge 4000 rpm, 15 min, 4°C
8. Removed sup with vacuum
9. Resuspend pellet in 25 ml PBS, centrifuge and repeat.
10. Resuspend pellet in 20 ml PBS.
11. From culture make dilutions:

tube label	Dilution	ml of culture	ml of media
a	10	0.5	4.5
b	100	0.5	4.5
c	10^3	0.5	4.5
d	10^4	0.5	4.5

12. Continue making dilutions in a 96 well plate:
    a. See *Klebsiella* dilutions (CFU) protocol
13. Add 100 µl of bacterial cultures from TUBE B to Row A (1:2 dilution), mix
14. Plate appropriate dilutions (10^4 to 10^7) by dropping 3 x 25 µl drops onto blood plate.
15. Incubate blood plate overnight at 37°C.
16. Count number of colonies in 3 drops:

    (Colony count/3) x 40 x final dilution = CFU/ml

Appendix B: SAS Code

```
DM 'clear log'; DM 'clear output';

options ls=100;


data original; set work.ajn_a_t;

*proc contents;
*proc print;

data edited; set original;
mediatype=media_type;
if number=1 or number =15 or number=16 or number=17 then delete;
*if killpct > 100 then killpct=100;
*if killpct < -150 then killpct=.;
drop media_type;

ods rtf;
ods graphics on;
*proc print;
Proc means;

proc sort; by mediatype straintype;
proc univariate plot normal; by mediatype straintype;
  var killpct;

proc sort; by farm;
proc freq; by farm;
  tables mediatype*straintype;
/*
Title 'No restrictions on bacteria kill percent';

proc mixed data=edited;
  class mediatype straintype farm number cow;
  model bacteriak = mediatype|straintype|farm
                    capsule*mediatype capsule*straintype
capsule*mediatype*straintype
                    dim cfu cow cow*mediatype cow*straintype
cow*mediatype*straintype
                    / solution residual outpred=predictobs1;

  lsmeans mediatype straintype;
  lsmeans mediatype*straintype / slice=straintype;
  lsmeans mediatype*farm straintype*farm mediatype*straintype*farm/
slice=farm;
  lsmeans mediatype*cow straintype*cow mediatype*straintype*cow/ slice=cow;

  data outliers1; set predictobs1;
    if abs(studentresid)<-2 or abs(studentresid)>2;
proc print data=outliers1;
run;
```

```
`*/
Title 'Restrictions on bacteria kill (-20 <= kill <= 100%)';


Data restricted; set edited;
  if bacteriak < -20 then delete;
  if bacteriak > 100 then delete;

proc mixed data=restricted;

class mediatype straintype number cow farm;
  model bacteriak = mediatype straintype farm capsule cow
                         mediatype*farm mediatype*capsule straintype*cow
                    / solution residual outpred=predictobs2;

  lsmeans mediatype straintype farm mediatype*farm
straintype*cow /pdiff tdiff  adj= tukey;




  data outliers2; set predictobs2;
    if abs(studentresid)<-2 or abs(studentresid)>2;
proc print data=outliers2;

run;

  ods graphics off;
  ods rtf close;

*/
```

Appendix C: SAS Output

Variable	Label	N	Mean	Std Dev	Minimum	Maximum
Number	Number	68	9.2794118	2.9717355	5.0000000	14.0000000
Isolate	Isolate	53	472.5849057	246.8365571	199.0000000	822.0000000
runnum	runnum	68	2.6911765	1.4888376	1.0000000	6.0000000
bacteriaK	bacteriaK	68	33.7319342	30.3813130	-18.0180000	97.4200000
cfu	cfu	68	14897696.07	6791851.04	5330000.00	40000000.00
n___strain	n = strain	68	7.1470588	1.4788530	4.0000000	9.0000000
n_strain_media	n=strain/media	68	3.9117647	1.4008025	1.0000000	6.0000000
cow	cow	68	4165.41	19.6660441	4136.00	4184.00
DIM	DIM	68	175.9558824	72.3237815	78.0000000	306.0000000
capsule	capsule	68	2765.92	1369.14	674.3333333	5133.67

The Mixed Procedure

Model Information

Data Set	WORK.RESTRICTED
Dependent Variable	bacteriaK
Covariance Structure	Diagonal
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Residual

Dimensions

Covariance Parameters	1
Columns in X	19
Columns in Z	0
Subjects	1
Max Obs Per Subject	68

Number of Observations

Number of Observations Read	68
Number of Observations Used	68
Number of Observations Not Used	0

Covariance Parameter Estimates

Cov Parm	Estimate	Standard Error	Z Value	Pr > Z	Alpha	Lower	Upper
Residual	394.84	73.9595	5.34	<.0001	0.05	282.19	591.84

Fit Statistics

-2 Res Log Likelihood	543.9
AIC (smaller is better)	545.9
AICC (smaller is better)	546.0
BIC (smaller is better)	547.9

Solution for Fixed Effects

Effect	mediatype	strain Type	farm	cow	Estimate	Standard Error	DF	t Value	Pr > |t|
Intercept					9.9784	9.0724	57	1.10	0.2760
mediatype	LB				23.6035	6.6618	57	3.54	0.0008
mediatype	SM				0	.	.	.	.

The Mixed Procedure

Solution for Fixed Effects

<table>
<tr><th>Effect</th><th>mediatype</th><th>strain Type</th><th>farm</th><th>cow</th><th>Estimate</th><th>Standard Error</th><th>DF</th><th>t Value</th><th>Pr > |t|</th></tr>
<tr><td>strainType</td><td></td><td>m</td><td></td><td></td><td>9.0343</td><td>7.9492</td><td>57</td><td>1.14</td><td>0.2605</td></tr>
<tr><td>strainType</td><td></td><td>u</td><td></td><td></td><td>0</td><td>.</td><td>.</td><td>.</td><td>.</td></tr>
<tr><td>farm</td><td></td><td></td><td>a</td><td></td><td>18.1128</td><td>8.5346</td><td>57</td><td>2.12</td><td>0.0382</td></tr>
<tr><td>farm</td><td></td><td></td><td>c</td><td></td><td>9.3657</td><td>8.5140</td><td>57</td><td>1.10</td><td>0.2759</td></tr>
<tr><td>farm</td><td></td><td></td><td>m</td><td></td><td>-19.7046</td><td>7.7157</td><td>57</td><td>-2.55</td><td>0.0134</td></tr>
<tr><td>farm</td><td></td><td></td><td>y</td><td></td><td>0</td><td>.</td><td>.</td><td>.</td><td>.</td></tr>
<tr><td>cow</td><td></td><td></td><td></td><td>4136</td><td>27.5391</td><td>8.7939</td><td>57</td><td>3.13</td><td>0.0027</td></tr>
<tr><td>cow</td><td></td><td></td><td></td><td>4168</td><td>-25.6857</td><td>9.9487</td><td>57</td><td>-2.58</td><td>0.0124</td></tr>
<tr><td>cow</td><td></td><td></td><td></td><td>4184</td><td>0</td><td>.</td><td>.</td><td>.</td><td>.</td></tr>
<tr><td>strainType*cow</td><td></td><td>m</td><td></td><td>4136</td><td>-34.3692</td><td>12.3294</td><td>57</td><td>-2.79</td><td>0.0072</td></tr>
<tr><td>strainType*cow</td><td></td><td>m</td><td></td><td>4168</td><td>18.7118</td><td>13.0372</td><td>57</td><td>1.44</td><td>0.1567</td></tr>
<tr><td>strainType*cow</td><td></td><td>m</td><td></td><td>4184</td><td>0</td><td>.</td><td>.</td><td>.</td><td>.</td></tr>
<tr><td>strainType*cow</td><td></td><td>u</td><td></td><td>4136</td><td>0</td><td>.</td><td>.</td><td>.</td><td>.</td></tr>
<tr><td>strainType*cow</td><td></td><td>u</td><td></td><td>4168</td><td>0</td><td>.</td><td>.</td><td>.</td><td>.</td></tr>
<tr><td>strainType*cow</td><td></td><td>u</td><td></td><td>4184</td><td>0</td><td>.</td><td>.</td><td>.</td><td>.</td></tr>
<tr><td>capsule</td><td></td><td></td><td></td><td></td><td>0.002625</td><td>0.002465</td><td>57</td><td>1.06</td><td>0.2914</td></tr>
</table>

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
mediatype	1	57	12.55	0.0008
strainType	1	57	0.52	0.4743
farm	3	57	10.54	<.0001
cow	2	57	7.07	0.0018
strainType*cow	2	57	7.86	0.0010
capsule	1	57	1.13	0.2914

Least Squares Means

<table>
<tr><th>Effect</th><th>mediatype</th><th>strain Type</th><th>farm</th><th>cow</th><th>Estimate</th><th>Standard Error</th><th>DF</th><th>t Value</th><th>Pr > |t|</th></tr>
<tr><td>mediatype</td><td>LB</td><td></td><td></td><td></td><td>45.3115</td><td>3.7148</td><td>57</td><td>12.20</td><td><.0001</td></tr>
</table>

Least Squares Means

Effect	mediatype	strain Type	farm	cow	Alpha	Lower	Upper
mediatype	LB				0.05	37.8727	52.7503

The Mixed Procedure

Least Squares Means

Effect	mediatype	strain Type	farm	cow	Estimate	Standard Error	DF	t Value	Pr > |t|
mediatype	SM				21.7080	4.7420	57	4.58	<.0001
strainType		m			35.4174	3.7875	57	9.35	<.0001
strainType		u			31.6022	3.7123	57	8.51	<.0001
farm			a		49.6791	5.7969	57	8.57	<.0001
farm			c		40.9320	5.8436	57	7.00	<.0001
farm			m		11.8617	4.8221	57	2.46	0.0170
farm			y		31.5663	5.6996	57	5.54	<.0001
strainType*cow		m		4136	33.1886	7.8276	57	4.24	<.0001
strainType*cow		m		4168	33.0448	5.4370	57	6.08	<.0001
strainType*cow		m		4184	40.0187	6.5510	57	6.11	<.0001
strainType*cow		u		4136	58.5235	6.8374	57	8.56	<.0001
strainType*cow		u		4168	5.2986	7.9888	57	0.66	0.5098
strainType*cow		u		4184	30.9844	5.3505	57	5.79	<.0001

Least Squares Means

Effect	mediatype	strain Type	farm	cow	Alpha	Lower	Upper
mediatype	SM				0.05	12.2123	31.2038
strainType		m			0.05	27.8330	43.0018
strainType		u			0.05	24.1684	39.0359
farm			a		0.05	38.0710	61.2871
farm			c		0.05	29.2304	52.6337
farm			m		0.05	2.2057	21.5177
farm			y		0.05	20.1530	42.9796
strainType*cow		m		4136	0.05	17.5141	48.8631
strainType*cow		m		4168	0.05	22.1574	43.9322
strainType*cow		m		4184	0.05	26.9006	53.1368
strainType*cow		u		4136	0.05	44.8320	72.2151
strainType*cow		u		4168	0.05	-10.6986	21.2959
strainType*cow		u		4184	0.05	20.2701	41.6986

Differences of Least Squares Means

Effect	mediatype	strain Type	farm	cow	_mediatype	strain Type	farm	cow
mediatype	LB				SM			
strainType		m				u		
farm			a				c	
farm			a				m	
farm			a				y	
farm			c				m	
farm			c				y	

The Mixed Procedure

Differences of Least Squares Means

Effect	mediatype	strain Type	farm	cow	_mediatype	strain Type	farm	cow
farm			m				y	
strainType*cow		m		4136		m		4168
strainType*cow		m		4136		m		4184
strainType*cow		m		4136		u		4136
strainType*cow		m		4136		u		4168
strainType*cow		m		4136		u		4184
strainType*cow		m		4168		m		4184
strainType*cow		m		4168		u		4136
strainType*cow		m		4168		u		4168
strainType*cow		m		4168		u		4184
strainType*cow		m		4184		u		4136
strainType*cow		m		4184		u		4168
strainType*cow		m		4184		u		4184
strainType*cow		u		4136		u		4168
strainType*cow		u		4136		u		4184
strainType*cow		u		4168		u		4184

Differences of Least Squares Means

Effect	mediatype	strain Type	farm	cow	_mediatype	strain Type	farm	Estimate	Standard Error
mediatype	LB				SM			23.6035	6.6618
strainType		m				u		3.8152	5.2971
farm			a				c	8.7471	8.1744
farm			a				m	37.8174	7.1447
farm			a				y	18.1128	8.5346
farm			c				m	29.0704	7.6456
farm			c				y	9.3657	8.5140
farm			m				y	-19.7046	7.7157
strainType*cow		m		4136		m		0.1438	9.2565
strainType*cow		m		4136		m		-6.8301	10.5160
strainType*cow		m		4136		u		-25.3349	9.6472
strainType*cow		m		4136		u		27.8900	11.3745
strainType*cow		m		4136		u		2.2042	9.7152
strainType*cow		m		4168		m		-6.9739	8.7100
strainType*cow		m		4168		u		-25.4787	8.6449
strainType*cow		m		4168		u		27.7461	9.9406
strainType*cow		m		4168		u		2.0604	7.6175
strainType*cow		m		4184		u		-18.5048	9.8883
strainType*cow		m		4184		u		34.7201	10.4332
strainType*cow		m		4184		u		9.0343	7.9492
strainType*cow		u		4136		u		53.2249	10.8285
strainType*cow		u		4136		u		27.5391	8.7939
strainType*cow		u		4168		u		-25.6857	9.9487

The Mixed Procedure

Differences of Least Squares Means

Effect	mediatype	strain Type	farm	cow	_mediatype	strain Type	farm	DF	t Value
mediatype	LB				SM			57	3.54
strainType		m				u		57	0.72
farm			a				c	57	1.07
farm			a				m	57	5.29
farm			a				y	57	2.12
farm			c				m	57	3.80
farm			c				y	57	1.10
farm			m				y	57	-2.55
strainType*cow		m		4136		m		57	0.02
strainType*cow		m		4136		m		57	-0.65
strainType*cow		m		4136		u		57	-2.63
strainType*cow		m		4136		u		57	2.45
strainType*cow		m		4136		u		57	0.23
strainType*cow		m		4168		m		57	-0.80
strainType*cow		m		4168		u		57	-2.95
strainType*cow		m		4168		u		57	2.79
strainType*cow		m		4168		u		57	0.27
strainType*cow		m		4184		u		57	-1.87
strainType*cow		m		4184		u		57	3.33
strainType*cow		m		4184		u		57	1.14
strainType*cow		u		4136		u		57	4.92
strainType*cow		u		4136		u		57	3.13
strainType*cow		u		4168		u		57	-2.58

Differences of Least Squares Means

Effect	mediatype	strain Type	farm	cow	_mediatype	strain Type	farm	Pr > |t|
mediatype	LB				SM			0.0008
strainType		m				u		0.4743
farm			a				c	0.2891
farm			a				m	<.0001
farm			a				y	0.0382
farm			c				m	0.0004
farm			c				y	0.2759
farm			m				y	0.0134
strainType*cow		m		4136		m		0.9877
strainType*cow		m		4136		m		0.5186
strainType*cow		m		4136		u		0.0111
strainType*cow		m		4136		u		0.0173
strainType*cow		m		4136		u		0.8213
strainType*cow		m		4168		m		0.4266
strainType*cow		m		4168		u		0.0046
strainType*cow		m		4168		u		0.0071
strainType*cow		m		4168		u		0.7878

The Mixed Procedure

Differences of Least Squares Means

Effect	mediatype	strain Type	farm	cow	_mediatype	strain Type	farm	Pr > |t|
strainType*cow		m		4184		u		0.0664
strainType*cow		m		4184		u		0.0015
strainType*cow		m		4184		u		0.2605
strainType*cow		u		4136		u		<.0001
strainType*cow		u		4136		u		0.0027
strainType*cow		u		4168		u		0.0124

Differences of Least Squares Means

Effect	mediatype	strain Type	farm	cow	_mediatype	strain Type	farm	Adjustment
mediatype	LB				SM			Tukey-Kramer
strainType		m				u		Tukey-Kramer
farm			a				c	Tukey-Kramer
farm			a				m	Tukey-Kramer
farm			a				y	Tukey-Kramer
farm			c				m	Tukey-Kramer
farm			c				y	Tukey-Kramer
farm			m				y	Tukey-Kramer
strainType*cow		m		4136		m		Tukey-Kramer
strainType*cow		m		4136		m		Tukey-Kramer
strainType*cow		m		4136		u		Tukey-Kramer
strainType*cow		m		4136		u		Tukey-Kramer
strainType*cow		m		4136		u		Tukey-Kramer
strainType*cow		m		4168		m		Tukey-Kramer
strainType*cow		m		4168		u		Tukey-Kramer
strainType*cow		m		4168		u		Tukey-Kramer
strainType*cow		m		4168		u		Tukey-Kramer
strainType*cow		m		4184		u		Tukey-Kramer
strainType*cow		m		4184		u		Tukey-Kramer
strainType*cow		m		4184		u		Tukey-Kramer
strainType*cow		u		4136		u		Tukey-Kramer
strainType*cow		u		4136		u		Tukey-Kramer
strainType*cow		u		4168		u		Tukey-Kramer

Differences of Least Squares Means

Effect	mediatype	strain Type	farm	cow	_mediatype	strain Type	farm	Adj P	Alpha
mediatype	LB				SM			0.0008	0.05
strainType		m				u		0.4743	0.05
farm			a				c	0.7090	0.05
farm			a				m	<.0001	0.05
farm			a				y	0.1584	0.05

The Mixed Procedure

Differences of Least Squares Means

Effect	mediatype	strain Type	farm	cow	_mediatype	strain Type	farm	Adj P	Alpha
farm			c				m	0.0019	0.05
farm			c				y	0.6910	0.05
farm			m				y	0.0623	0.05
strainType*cow		m		4136		m		1.0000	0.05
strainType*cow		m		4136		m		0.9866	0.05
strainType*cow		m		4136		u		0.1075	0.05
strainType*cow		m		4136		u		0.1562	0.05
strainType*cow		m		4136		u		0.9999	0.05
strainType*cow		m		4168		m		0.9662	0.05
strainType*cow		m		4168		u		0.0502	0.05
strainType*cow		m		4168		u		0.0735	0.05
strainType*cow		m		4168		u		0.9998	0.05
strainType*cow		m		4184		u		0.4299	0.05
strainType*cow		m		4184		u		0.0183	0.05
strainType*cow		m		4184		u		0.8640	0.05
strainType*cow		u		4136		u		0.0001	0.05
strainType*cow		u		4136		u		0.0312	0.05
strainType*cow		u		4168		u		0.1186	0.05

Differences of Least Squares Means

Effect	mediatype	strain Type	farm	cow	_mediatype	strain Type	farm	Lower	Upper
mediatype	LB				SM			10.2635	36.9434
strainType		m				u		-6.7920	14.4224
farm			a				c	-7.6219	25.1160
farm			a				m	23.5105	52.1244
farm			a				y	1.0225	35.2031
farm			c				m	13.7603	44.3804
farm			c				y	-7.6833	26.4147
farm			m				y	-35.1551	-4.2542
strainType*cow		m		4136		m		-18.3921	18.6797
strainType*cow		m		4136		m		-27.8879	14.2278
strainType*cow		m		4136		u		-44.6532	-6.0166
strainType*cow		m		4136		u		5.1128	50.6671
strainType*cow		m		4136		u		-17.2501	21.6585
strainType*cow		m		4168		m		-24.4154	10.4676
strainType*cow		m		4168		u		-42.7898	-8.1677
strainType*cow		m		4168		u		7.8405	47.6518
strainType*cow		m		4168		u		-13.1934	17.3142
strainType*cow		m		4184		u		-38.3058	1.2961
strainType*cow		m		4184		u		13.8280	55.6121
strainType*cow		m		4184		u		-6.8837	24.9523
strainType*cow		u		4136		u		31.5412	74.9086
strainType*cow		u		4136		u		9.9297	45.1485

The Mixed Procedure

Differences of Least Squares Means

Effect	mediatype	strain Type	farm	cow	_mediatype	strain Type	farm	Lower	Upper
strainType*cow		u		4168		u		-45.6076	-5.7639

Differences of Least Squares Means

Effect	mediatype	strain Type	farm	cow	_mediatype	strain Type	farm	Adj Lower	Adj Upper
mediatype	LB				SM			10.2635	36.9434
strainType		m				u		-6.7920	14.4224
farm			a				c	-12.8862	30.3804
farm			a				m	18.9092	56.7256
farm			a				y	-4.4739	40.6995
farm			c				m	8.8365	49.3043
farm			c				y	-13.1664	31.8978
farm			m				y	-40.1240	0.7147
strainType*cow		m		4136		m		-27.1524	27.4400
strainType*cow		m		4136		m		-37.8401	24.1799
strainType*cow		m		4136		u		-53.7832	3.1134
strainType*cow		m		4136		u		-5.6519	61.4318
strainType*cow		m		4136		u		-26.4444	30.8528
strainType*cow		m		4168		m		-32.6585	18.7107
strainType*cow		m		4168		u		-50.9712	0.01375
strainType*cow		m		4168		u		-1.5671	57.0594
strainType*cow		m		4168		u		-20.4025	24.5233
strainType*cow		m		4184		u		-47.6640	10.6543
strainType*cow		m		4184		u		3.9542	65.4859
strainType*cow		m		4184		u		-14.4067	32.4753
strainType*cow		u		4136		u		21.2932	85.1566
strainType*cow		u		4136		u		1.6073	53.4709
strainType*cow		u		4168		u		-55.0229	3.6514

The UNIVARIATE Procedure
Variable: Resid (Residual)

Moments

N	68	Sum Weights	68
Mean	0	Sum Observations	0
Std Deviation	18.3277083	Variance	335.90489
Skewness	-0.1009487	Kurtosis	-0.0333378
Uncorrected SS	22505.6276	Corrected SS	22505.6276
Coeff Variation	.	Std Error Mean	2.22256109

Basic Statistical Measures

Location		Variability	
Mean	0.00000	Std Deviation	18.32771
Median	-0.00524	Variance	335.90489
Mode	.	Range	83.56304
		Interquartile Range	23.11294

Tests for Location: Mu0=0

Test	-Statistic-		-----p Value------	
Student's t	t	0	Pr > |t|	1.0000
Sign	M	0	Pr >= |M|	1.0000
Signed Rank	S	10	Pr >= |S|	0.9518

Tests for Normality

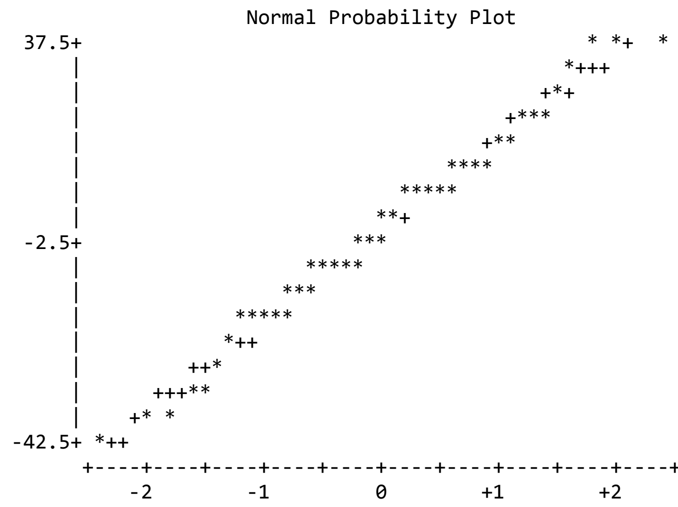
Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.98947	Pr < W	0.8395
Kolmogorov-Smirnov	D	0.062991	Pr > D	>0.1500
Cramer-von Mises	W-Sq	0.027483	Pr > W-Sq	>0.2500
Anderson-Darling	A-Sq	0.195548	Pr > A-Sq	>0.2500

Quantiles (Definition 5)

Quantile	Estimate
100% Max	39.48390502
99%	39.48390502
95%	33.41298971
90%	22.06385919
75% Q3	11.59754881
50% Median	-0.00523559
25% Q1	-11.51538740

```
              The UNIVARIATE Procedure
            Variable:  Resid  (Residual)

              Quantiles (Definition 5)

             Quantile          Estimate

             10%           -20.98600387
             5%            -33.16609498
             1%            -44.07913700
             0% Min        -44.07913700


                Extreme Observations

     ------Lowest-----        -----Highest-----

        Value      Obs           Value      Obs

     -44.0791       33         28.7439       17
     -39.9592       61         33.4130       38
     -35.4993       39         35.6195       49
     -33.1661       12         38.3999        5
     -31.0729       53         39.4839       13


  Stem Leaf                     #             Boxplot
     3 689                      3                |
     3 3                        1                |
     2 9                        1                |
     2 00224                    5                |
     1 589                      3                |
     1 0012234                  7             +-----+
     0 55666677799             11             |     |
     0 123                      3             |  +  |
    -0 444111                   6             *-----*
    -0 887777665                9             |     |
    -1 44210                    5             +-----+
    -1 98765                    5                |
    -2 100                      3                |
    -2 9                        1                |
    -3 31                       2                |
    -3 5                        1                |
    -4 40                       2                |
       ----+----+----+----+
   Multiply Stem.Leaf by 10**+1
```

```
Restrictions on bacteria kill (-20 <= kill <= 100%)                        12
                                              14:20 Tuesday, December 15, 2009

              The UNIVARIATE Procedure
            Variable:  Resid  (Residual)
```



Plot of Resid*Pred. Legend: A = 1 obs, B = 2 obs, etc.