Curli Production and ERIC-PCR Fingerprinting of *Escherichia coli* from Clinical Cases of Bovine Mastitis

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(ABSTRACT)

Mastitis caused by *Escherichia coli* is among the most common and severe forms of environmental mastitis. Currently, the severity of *E. coli* mastitis is thought to be more related to cow factors than bacterial virulence. Some strains of *E. coli*, however, may be adapted to colonizing mammary tissue, increasing clinical severity, and impairing recovery. Curli are adhesive surface structures produced by some *E. coli* and *Salmonella* strains that bind a number of host proteins and have recently been found to play a role in the pathogenesis of bacterial sepsis. Sixty-one *E. coli* isolates from 36 clinical cases of bovine mastitis were characterized using ERIC-PCR and screened for their ability to produce curli by binding Congo-red dye. The effect of curli production on case recovery, based on a return to milk production, was investigated for a subset of 43 isolates from 20 quarters of 19 cows. Fifty-eight of the 61 isolates were clustered into two clonal groups at 52% genetic similarity. Thirty-five of all 61 isolates (57%) were curli-positive. Twenty-three isolates from 13 cows clustered in clonal group I, of which 5 cases (38%) were curli-positive; 35 isolates from 22 cows were clustered in clonal group II, 15 of which were curli-positive cases (68%). No association was found between genetic similarity and phenotypic curli expression of isolates from cows with clinical *E. coli* mastitis cases ($p=0.16$). Phenotypic curli expression in isolates did not affect the recovery of cows' milk yield to pre-mastitis production levels ($p=0.18$).

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Table of Contents

List of Tables

List of Figures

Introduction

Mastitis is an economically important disease on United States dairy farms and is caused by contagious and environmental bacteria. Coliform mastitis, a type of environmental mastitis, is most often caused by *Escherichia coli* and commonly occurs within the first 30 days of lactation. Mild cases are usually self-limiting and cure spontaneously, while moderate to severe cases can induce fever, inflammation of the udder, milk clots, watery secretions, decreased milk production, and, occasionally, sepsis and death. In general, *E. coli* associated with coliform mastitis does not posses any specific virulence factors, with the exception of serum resistance. Currently, it is believed that the severity of coliform mastitis is more related to physiologic factors in the cow, rather than bacterial virulence, and *E. coli* is viewed as an opportunistic disease agent of the mammary gland. However, some strains may be adapted to infecting and colonizing mammary tissue.

Curli are thin, coiled protein fibers produced by some strains of *E. coli*. The expression of curli is strictly regulated through a combination of environmental and genetic factors and is preferentially expressed during starvation, stationary phase growth, and at low temperatures (<30ºC). The genes for curli production have been shown to be ubiquitous in *E. coli*; however, not all strains are capable of producing curli, even under ideal environmental conditions. Deficiencies in curli production are often caused by small alterations in the genes responsible for curli formation and regulation and are related to host, bacterial pathogenicity, and source. Curli are capable of binding to a number of host proteins, triggering inflammatory mediators, contributing to sepsis related

clinical signs, and play a role in the formation of biofilms. Curli is a potential virulence factor in mastitis due to its ability to adhere to mammary epithelium and stimulate inflammatory pathways.

Review of Literature

Bovine Mastitis

Mastitis is the inflammation of the mammary gland and is among the most common diseases in dairy cows. This inflammation can be a reaction to a traumatic injury, chemical irritation, but, most often, infections caused by bacteria. Cows with mastitis may exhibit clinical signs associated with inflammation, bacterial toxemia, and occasionally septic shock. Mastitis results in increased costs for the producer from veterinary expenses, production loss, and increased labor.

Bacteria associated with mastitis in cows are divided into contagious and environmental species. Contagious mastitis pathogens, such as *Streptococcus agalactiae* and *Staphylococcus aureus,* are transmitted from cow to cow and usually result in chronic or subclinical mastitis cases. Environmental mastitis bacteria are present in great numbers in the cows' surroundings, such as bedding, manure, and mud. A particular group of environmental mastitis organisms are the coliforms, which include *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter aerogenes*. Coliform mastitis cases are usually short in duration; however, the majority of cows infected with coliforms will develop clinical signs as opposed to subclinical or asymptomatic infections (Smith et al., 1985). The incidence of mastitis associated with contagious pathogens has decreased over the past 40 years, while the impact and importance of environmental mastitis cases has increased (Smith et al., 1985; Philpot and Nickerson, 2000).

Cows with coliform mastitis may display mild, moderate, or severe clinical signs. Affected mammary quarters may be swollen, firm, or hot with straw-colored to watery

secretions, with or without clots. In more severe cases, systemic signs may develop, including, but not limited to, depression, pyrexia, anorexia, rumen stasis, tachycardia, tachypnea, diarrhea, and dehydration. Septic shock may develop, decreasing blood pressure and body temperature, and possibly leading to recumbency and death. The majority of acute cases of *E. coli* mastitis occur during the first 30 days in lactation. Cows with mild signs often recover spontaneously within seven days. Cows with a more chronic course will not continuously display clinical signs, but instead, have periodic, short-lived flare ups (Smith et al., 1985; Philpot and Nickerson, 2000).

Escherichia coli exhibit a diverse range of host interactions and can exist as both an ubiquitous, commensal organism or as a severe pathogen. *Escherichia coli* possess a wide variety of virulence factors that contribute to its ability to cause a broad spectrum of diseases. Lipopolysaccharide (LPS), an endotoxin, is produced by all Gram-negative organisms as part of its outer membrane and is considered an important component in the pathogenesis of *E. coli* mastitis. Intramammary infusion of LPS induces similar clinical signs as those observed in *E. coli* mastitis (Paape et al., 1974; Diez-Fraile et al., 2002). The growth and lysis of *E. coli* within the mammary tissue releases LPS locally, where the endotoxin triggers several complex inflammatory events, including increased vascular permeability, the migration of neutrophils and other leukocytes into the gland, formation of nitric oxide (NO), and the release of interleukin 1 (IL-1) from mammary epithelial cells. Less commonly, LPS may diffuse from the mammary gland and enter the general circulation leading to systemic effects such as toxemia, shock, and death (Lohuis et al., 1988; Boudjellab et al., 2000; Boulanger et al., 2001; Burvenich et al., 2003).

Highly adapted *E. coli* strains often express specific virulence traits and thus, develop a set of stable pathotypes. Enteropathogenic *E. coli* (EPEC) possess and express specific sets of genes that allow them to adhere to enterocytes in the small bowel and cause cytoskeletal alterations, destroying the underlying microstructure and causing severe diarrhea in the host (Kaper et al., 2004). *Escherichia coli* associated with bovine mastitis are not considered to belong to a specific pathogenic group and do not appear to possess any consistent virulence factors, with the exception of serum resistance, which is expressed in 64-100% of *E. coli* isolated from clinical mastitis cases (Sanchez-Carlo et al., 1984a, b; Barrow and Hill, 1989; Nameth et al., 1994; Kaipainen et al., 2002). Lactating cows challenged intramammarily with serum resistant organisms developed clinical mastitis, whereas those challenged with serum sensitive organisms did not, and only serum resistant organisms could be recovered from infected quarters (Barrow and Hill, 1989).

Attachment of *E. coli* to mammary epithelium has not been observed in vivo in experimental intramammary challenges studied by electron microscopy (Frost et al., 1980; Bramley, 1991) and does not appear to be required for the pathogenesis of coliform mastitis (Frost, 1975). However, partial or temporary adherence in vivo cannot be ruled out (Burvenich et al., 2004). It has also been demonstrated that *E. coli* can adhere to and invade MAC-T mammary epithelial cells in vitro. It may also be possible that invading *E. coli* can reside within host cells and serve as a reservoir for future or chronic intramammary infections (Dopfer et al., 2000, 2001).

Escherichia coli isolates from bovine mastitis cases have been examined on a genetic level. Fingerprinting using molecular based methods has been performed on mastitis isolates in several studies. Lipman et al. (1994) reported a variety of genotypes obtained from 30 diverse *E. coli* mastitis cases with no special genotype associated with disease characteristics or farm location. Recurrent episodes of mastitis in the same cow often yielded the same genotype, suggesting that some strains may persist within the mammary gland (Lam et al., 1996; Dopfer et al., 1999). These persistent and recurrent strains can also spread to other quarters in the same cow, though this occurs rarely (Dopfer et al., 1999; Bradley and Green, 2001). Based on the biochemical and virulence traits, *E. coli* associated with bovine mastitis behave as opportunistic, environmental pathogens and are not represented by a specific pathogenic or genetic population. However, it is still possible that certain *E. coli* strains are better suited to residing in the mammary gland, initiating more severe, recurrent, or persistent clinical episodes.

The severity of *E. coli* mastitis is more dependant on cow-related factors than bacterial pathogenicity. Parturition, early lactation, low somatic cell count (SCC), or a negative energy balance impair the cow's defenses against invading bacteria making the cow more susceptible to intramammary infections and more likely to develop severe mastitis. Cows that become infected in mid to late lactation usually develop very mild clinical signs and recover spontaneously (Hill, 1991; Burvenich et al., 2003).

Neutrophils are a primary defense against bacterial invaders, and their function is vital for eliminating *E. coli* and other infectious organisms from the mammary gland. After infection, host chemotactic factors and LPS attract neutrophils into the mammary

tissue where they sequester and kill invading bacteria. The speed and efficacy at which the neutrophils respond to bacterial invaders determines the severity of the clinical episode (Hill, 1991; Burvenich et al., 2003). Following parturition and the onset of lactation, the immune system is less able to react appropriately to bacterial challenges. Though the exact mechanisms are not clear, a combination of metabolic and hormonal influences may temporarily suppress the immune system in the periparturient period. Additionally, the altered nutritional and energy demands that occur in the periparturient cow during the last trimester and early lactation increase fat metabolism, leading to a buildup of ketone metabolites (ketosis), which also negatively impact the microbicidal properties of circulating neutrophils and increase the cow's susceptibility to mastitis (Suriyasathaporn et al., 2000). This temporary and transient immunosuppression increases the cow's susceptibility to opportunistic organisms and increases the likelihood for environmental bacteria to invade the udder and cause mastitis (Guidry et al., 1975; Hoeben, 1999; Suriyasathaporn et al., 2000; Diez-Fraile et al., 2003).

Despite the common clinical incidence of coliform mastitis, treatment is not well defined. Treatment protocols vary depending on the severity of the clinical signs, but they often consist of a combination of supportive care, anti-inflammatory agents, frequent milkout, and antimicrobials. Most mild infections are self-limiting and will cure spontaneously, regardless of any treatment. Supportive care in the form of fluid therapy, electrolyte supplementation, and anti-inflammatory drugs are considered beneficial in moderate to severe cases. Frequent milkout is a common practice; though it has recently been documented that frequent milkout does not affect the outcome in moderate to severe

cases of mastitis when compared to non-frequent milkout cow controls (Leininger et al., 2003). The use of antimicrobial drugs in cases of coliform mastitis is very controversial. Though often implemented in severe cases, antibiotics have little value in controlling coliform mastitis or the environmental reservoir of bacteria (Smith et al., 1985; 1993).

Vaccination against mastitis agents are among the more recent managerial approaches to reducing the incidence of coliform mastitis. A vaccine based on the mutated, rough-coated *E. coli* strain, J5, produces antibodies to the common core antigens of Gram-negative bacteria and provides cross-protection to a variety of coliforms (Tyler et al., 1992). A field trial conducted by Gonzalez et al. (1989) found that the J5 vaccine was protective against natural challenge by Gram-negative bacteria and reduced the incidence of clinical mastitis in the first three months of lactation. The J5 vaccine did not prevent mastitis from developing in cows experimentally challenged intramammarily with *E. coli;* however, vaccinated cows experienced less severe clinical signs (Hogan et al., 1992). Though the vaccine has proven to be effective and beneficial in reducing the incidence and clinical severity of coliform mastitis, vaccination should not be a substitute for proper nutrition, management, and hygiene on the dairy farm.

The most commonly practiced method to identify the mastitis causing organism is through bacteriologic culture of a milk sample. Numerous methods exist for the diagnosis of Gram-negative pathogens (Hogan et al., 1999). The protocol used by the Production Management Medicine Department at the Virginia-Maryland Regional College of Veterinary Medicine typically obtains a result within 24 to 36 hours. After an initial milk culture on Columbia Blood Agar, colonies are tested with KOH to

differentiate Gram-negative from Gram-positive organisms. A small amount of the bacterial colony is mixed with a few drops of 3% KOH on a glass slide. A positive reaction will cause the KOH to become thick and stringy and is caused by Gram-negative organisms. An isolated colony is then plated onto MacConkey (MAC) and eosin methylene blue (EMB) agar for further differentiation. *Escherichia coli* and *Klebsiella* spp. will usually both produce dry to mucoid, pink colonies on MAC, a positive result. *Escherichia coli* will produce a green, metallic sheen on EMB (positive), whereas *Klebsiella* will typically lack this sheen (negative) (MacFadden, 1985; Hogan et al., 1999; Leininger et al., 2003).

Molecular Bacterial Typing

Disease outbreaks are often caused by a single strain of infectious agent or originate from a specific source. Identification and classification of the disease agent is a crucial part of epidemiologic investigation. *Escherichia coli* is usually a normal, commensal inhabitant of the animal gastrointestinal tract, but, depending on the strain of bacteria, *E. coli* can also exist as an important disease causing organism. Several molecular techniques have been developed to identify and classify bacteria beyond the species level in order to help distinguish non-pathogenic bacteria from their disease-causing counterparts, as well as classify groups of closely related strains.

Pulse field gel electrophoresis (PFGE) is considered the "gold standard" of molecular, bacterial typing. Plugs of bacteria are combined with molten agarose gel and digested with detergents, lysing enzymes, and specific restriction endonucleases. The

plug is placed through a specialized gel electrophoresis in which the polarity of the current is periodically altered, resulting in the separation of several, large DNA fragments. The presence and size of these fragments are documented and used to identify the particular bacterial strain (Olive and Bean, 1999). Pulse field gel electrophoresis is highly discriminatory and is often superior to other subtyping procedures, but it is also very expensive and time consuming to perform (Olive and Bean., 1999; Jonas et al., 2002; Hahm et al., 2003). While PFGE can identify a particular bacterial strain with a high degree of discrimination, its ability to infer the degree of genetic relatedness between strains is limited (Davis et al., 2003). As a result, PCR based typing methods have become a popular, reliable, and faster alternative to PFGE in the identification of bacterial strains and evaluating the genetic relationships they share. Two common PCR-based typing methods include the use of random amplified polymorphic DNA (RAPD) and repetitive-element (rep) PCR.

Random amplified polymorphic DNA-PCR, also called arbitrarily primed (AP) PCR, utilizes short, nine to ten base pair primers that target random sequences in the genomic DNA (Welsh and McClelland, 1990; Williams et al., 1990). The random primers anneal to the DNA at low temperatures and amplify random segments of the bacterial genome, resulting in numerous and variably sized PCR products. The presence, absence, and/or number of these arbitrary primer binding sites and their resulting PCR products vary for each bacterial subtype, with similar sites more common among closely related strains. Under ideal conditions, gel electrophoresis of the PCR products creates a unique banding pattern for each bacterial strain (Welsh and McClelland, 1990; Williams

et al., 1990; Olive and Bean, 1999). In addition to its simplicity and speed, the primary advantage of RAPD-PCR is that one does not need to know of or target specific genetic loci. As a result, RAPD methods have been used to quickly type and analyze a broad range of organisms including animals (Smith et al., 1996), fungi (Lehmann et al., 1992; Meyer et al., 1993), protozoa (Tibayrenc et al., 1993), and a large variety of bacterial species (Cave et al., 1994; Niederhauser et al., 1994; Lin et al., 1996; Lipman et al., 1996).

Though simple in design and theory, RAPD-PCR does have its shortcomings. Without targeting a specific gene or sequence in the genome, variable and sometimes conflicting results can be obtained with different primers. Furthermore, imperfect hybridization between the DNA and primer can lead to unstable PCR products that can vary between reactions. It also appears that RAPD-PCR is very sensitive to inter-experimental variations in temperature, reagents, lab conditions, and equipment, lending itself to questionable reproducibility and making RAPD-PCR difficult to standardize (Gao et al., 1993; Meunier and Grimont, 1993; Penner et al., 1993; Olive and Bean, 1999).

Repetitive element (rep) PCR, unlike RAPD, targets well-defined elements within the DNA that repeat themselves frequently throughout the genome. These repetitive elements are common among prokaryotes and are highly conserved in each generation. Selective primers can be used to bind and amplify these repetitive elements, forming a unique banding pattern upon electrophoresis for each species and strain of bacteria (Versalovic et al., 1991). Repetitive element PCR of multiple colonies or repeated

isolates of the same strain will reveal the same fingerprint, demonstrating that the fingerprint is stable over time (Woods et al., 1992). Several repetitive elements have been identified, with repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) sequences being two of the more frequently targeted sequences (Versalovic et al., 1991; Lupski and Weinstock, 1992).

Repetitive extragenic palindromic (REP) sequences, a type of rep-PCR, are occasionally referred to as palindromic units (PU) and are a 38 nucleotide consensus sequence that forms a palindrome (Glison et al., 1984). Initially studied in *E. coli* and *Salmonella typhimurium* (Higgins et al., 1982; Glison et al., 1984), these REP sequences are organized into clusters and can occur at up to 1000 locations. By nature, REP sequences are capable of folding on themselves and forming stable stem-loop structures with a variable five base pair (bp) loop in the center (Stern et al., 1984). This ability has caused many to examine the role of REP sequences in DNA regulation (Yang and Ames, 1988; Gilson et al., 1990), but, currently, REP sequences are thought of as a form of 'selfish' DNA (Higgins et al., 1988; Lupski and Weinstock, 1992).

Enterobacterial repetitive intergenic consensus (ERIC) sequences are another type of highly conserved rep element. Also known as intergenic repeat units (IRU), ERIC elements are about 126 bp long and located in extragenic (noncoding) regions of the genome (Sharples and Lloyd, 1990; Hulton et al., 1991). Compared with REP sequences, ERICs are a distinctly different group of repeated elements and are not related to REPs, though they both may have similar functions in the bacterial genome (Hulton, et al., 1991). It can be debated which primer set, REP or ERIC, will produce a more stable

fingerprint with repeated reactions. Lipman et al. (1994) obtained more reproducible PCR fingerprints using ERIC primers compared to those obtained with REP primers; they suspected this was attributed to the fact that ERIC primers are longer than those used for REP sequences. However, Wong and Lin (2001) produced more stable fingerprints using REP sequences rather than ERICs.

Repetitive element PCR based techniques are relatively fast, easy, and inexpensive methods of typing and relating bacteria. Single or multiple sets of primers can be used to obtain a variety of stable, complex results in order to differentiate closely related strains (Olive and Bean 1999). Fingerprints obtained with rep elements, such as ERIC and REP sequences, are also more robust, stable, and reproducible than those created with RAPDs, producing less day-to-day variation (Liu et al., 1995; de la Puente-Redondo et al., 2000). Repetitive element PCR with REP and ERIC primers has been successfully performed on numerous bacterial organisms including *E. coli* (Lam et al. 1996; Dopfer et al. 1999; de Moura et al. 2001), *Enterobacter* spp. (Georghiou et al., 1995; Zaher and Cimolai, 1997), *S. aureus* (Lipman et al., 1996), and *Salmonella* spp. (Bennasar et al., 2000).

Gel electrophoresis of both rep and RAPD-PCR products will yield a banding pattern that is unique to each bacterial strain. These products can be compared visually to determine the occurrence or persistence of a particular strain. However, when dealing with a large number of isolates, further examination beyond visual assessment is required. Computer assistance with pattern analysis becomes necessary to interpret the

numerous and complex fingerprints that arise from typing several isolates (Rademaker and Bruijn, 1997; Ooyen, 2001).

When two banding patterns are compared, their similarity can be expressed as a numerical value known as a similarity or proximity coefficient. This coefficient takes values ranging from 0, indicating no common trait, to +1, indicating the two strains are identical. Coefficients between 0 and +1 are proportional to the genetic similarity, with more similar banding patterns having higher coefficients. Alternatively, a dissimilarity index can be constructed, resulting in inverse values. By comparing each strain to every other, the resulting similarity values can be compiled into a similarity or resemblance matrix. A second analysis, known as clustering, is performed to extrapolate the matrix into a dendrogram, expressing the similarity coefficients in a visual form (Romesburg, 1990; Rademaker and Bruijn, 1997; Ooyen, 2001). Similarity coefficients of rep-PCR products are obtained by comparing one or more characteristics from each strain's gel electrophoresis pattern. The information obtained from each strain can be selected using band-based or curve-based characterization.

Band-based similarity coefficients are derived from binary variables that are based solely on the presence or absence of a band at a particular location or molecular weight. Two common band-based similarity measures used in PCR fingerprinting include Jaccard and Dice coefficients. The coefficient of Jaccard expresses the similarity between two tracks of bands and is calculated by the number of matching bands divided by the total number of bands in both tracks. Jaccard coefficients are expressed as:

$$S_{i,j} = \frac{n_{i,j}}{n_i + n_j - n_{i,j}}$$

where $S_{i,j}$ is the similarity coefficient between strains *i* and *j*; $n_{i,j}$ is the number of matching bands in strains *i* and *j*; n_i is the total number of bands in strain *i*; and n_j is the total number of bands in strain *j* (Ooyen, 2001).

Dice coefficients are calculated in a similar fashion as Jaccard's method, but place more emphasis on common bands. These are expressed as:

$$S_{i,j} = \frac{2n_{i,j}}{n_i + n_j}$$

where $S_{i,j}$ is the similarity coefficient between strains *i* and *j;* $n_{i,j}$ is the number of matching bands for strains *i* and *j*; n_i is the total number of bands in strain *i*; and n_j is the total number of bands in strain *j* (Ooyen, 2001).

Band-based coefficients rely on the selection of bands to include in the analysis. This can be performed manually by the researcher or by using computer software. The process of band assignment is often tedious, labor intensive, and subject to the viewer's interpretation. Additionally, variations in the staining and photographing of the gel can add temperamental alterations of the band's appearance. Multiple reviewers may have different interpretations of what should be included in a similarity analysis and faint bands may be overlooked or not included in the selection process. For these reasons, a binary, band-based selection system may not be appropriate for fingerprints as complex

as those obtained with rep-PCR (Rademaker and Bruijn, 1997; Ooyen, 2001; Meacham et al., 2003).

Curve-based similarity coefficients are based on the optical density (OD) of the bands, that is, the saturation of the pixels in a digital photograph of the gel. In this method, the banding pattern is converted into a transverse, linear graph of the band density running the length of the gel. The correlation coefficient of two strains is a direct comparison of the valleys and peaks in the graph, as well as the different ratios in peak height and width. The height of each band's peak, which corresponds to the intensity of the band, correlates to the quantity of the DNA in the band. As a result, the degree of band intensity can be quantified and factored into the comparison (van Belkum, 1995; Rademaker and Bruijn, 1997; Ooyen, 2001).

Pearson's or product moment correlation coefficients are another measure of similarity, but, unlike Dice or Jaccard's method, use a curve based algorithm. With regard to rep-PCR, the similarity between two strains is calculated as the correlation between the densitometric values or optical density of the band(s) expressed as:

$$S_{i,j} = \frac{\sum_{k=1}^{N}(C_{k,i} - \overline{C}_i)(C_{k,j} - \overline{C}_j)}{\sqrt{\sum_{k=1}^{N}(C_{k,i} - \overline{C}_i)^2 \sum_{k=1}^{N}(C_{k,j} - \overline{C}_j)^2}}$$

where $S_{i,j}$ is the similarity coefficient between strains *i* and *j*; $C_{k,i}$ is the densitometric value of strain *i* at location *k* (such as the molecular weight of the band); $C_{k,j}$ is the densitometric value of strain *j* at location *k;* $\overline{C}_i$ is the mean of all the densitometric values for strain *i;* and $\overline{C}_j$ is the mean of all the densitometric values for strain *j* (Ooyen, 2001).

Pearson's coefficient is a more stable measurement of similarity than band-based methods because whole densitometric curves are compared, omitting subjecting band scoring steps (Rademaker and Bruijn, 1997). Additionally, by applying Pearson's coefficient to a densitometric graph, artifactual differences between gels can be normalized and removed so that they do not alter the result (Ooyen, 2001).

Regardless of the method used, correlation analysis results in a similarity matrix of all the strains compared to each other. In theory, a correlation coefficient can range from -1 to +1. However, due to the type of data used (ODs for Pearson's, 0 or 1 for Jaccard or Dice), the result is not less than zero (Ooyen, 2001). The data within the similarity matrix is then clustered to form a visual representation of the genetic relatedness of the isolates in a dendrogram.

Cluster analysis sequentially converts the similarity data into more inclusive groupings, combining like strains into clusters based on their similarity coefficient. Two very similar strains are grouped together and then joined with another cluster to form a new, larger, and more inclusive cluster. This is repeated until all strains and associated clusters are tied completely together (Romesburg, 1990).

Several mathematical clustering methods exist, with the unweighted pair groups method analysis (UPGMA) being the most commonly used to cluster PCR band-based data (Romesburg, 1990). Also known as the average linkage clustering method, UPGMA groups two elements, such as the similarity coefficient of bacterial strains, together based on the arithmetic average of their similarity to each other and to groups already formed. The UPGMA states that if cluster *l* is formed by joining elements/clusters *i* and *j*, the

similarity of element/cluster *k* to cluster *l* is the average of the similarities between *k* and *i,* and *k* and *j*. Mathematically this is expressed as:

$$S_{k,l} = \frac{N_i S_{k,i} + N_j S_{k,j}}{N_i + N_j}$$

where $S_{k,l}$ is the similarity between elements/clusters *k* and *l*; $S_{k,i}$ is the similarity coefficient of elements/clusters *k* and *i;* $S_{k,j}$ is the similarity coefficient of elements/clusters *k* and *j;* N_i is the number of objects in cluster *i*; and N_j is the number of objects in cluster *j* (Ooyen, 2001). Simply put, when joining two objects or clusters together, the similarity of the new group to all other strains is the average similarity coefficient of each of the joined strains to the strains not in the cluster.

When comparing complex fingerprints of numerous strains, computer assisted analysis is essential. This is certainly the case when examining ERIC-PCR fingerprints and calculating their similarity coefficients from densitometric curves using Pearson's product moment coefficients. Several commercial computer software packages with a variety of features exist to assist in microbial fingerprint pattern analysis, including, but not limited to, the AMBIS system (Scanalytics, Waltham, MA), GelCompar II, Bionumerics (Applied Maths, Inc., Austin, TX), Multi-Analyst, and Molecular Analyst (Bio-Rad, Philadelphia, PA) (Vauterin and Vauterin, 1992; Rademaker and Bruijn, 1997; Johnson and O'Bryan, 2000). Digital images of the gel can be normalized to correct for inter and intra-gel variations. By comparing standard lane ladders, each lane can be adjusted to a standard size by elongating or shortening the lane. Background florescence can be removed so it is not factored into the similarity coefficient. Additionally, the

greatest advantage to computer assisted analysis lies in high speed and great accuracy (Rademaker and Bruijn, 1997).

Fingerprinting clinical isolates, such as those obtained from mastitis cases, using a PCR-based method can be used to gain more insight into the causative agent. The fingerprints can be compared to determine if severe clinical signs are associated with a particular genotype or a group of closely related strains. Once the fingerprints are compiled into a dendrogram, groups or clusters of isolates can be examined for common traits such as geographical location, expression of a virulence factor, and case outcome. Attributes can be analyzed to determine if they occur in a specific group or cluster, or if they are randomly distributed throughout the population. Should a particular bacterial group or strain be more likely to cause severe signs or posses a particular virulence trait, efforts to further explore, classify, and combat these strains can be focused to gain better insight into these organisms and their diseases.

Curli

Curli are a unique group of thin, coiled, fibrous structures found on the surface of some types of bacteria. Expression of curli is regulated through complex environmental and genetic interactions. Curli are capable of binding to a number of substrates including fibronectin, laminin, and Congo-red dye (Olsen et al., 1989) and also play a role in the auto-aggregation of bacteria and biofilm formation. Additionally, curli expression contributes to the pathogenesis of bacterial invasion, colonization, and sepsis (Vidal et al., 1998; Bian et al., 2001; Gophna et al., 2001; Ryu et al., 2004).

Curli were first described on *E. coli* isolates from cases of bovine mastitis (Olsen et al., 1989). Similar structures known as thin, aggregative fimbriae have also been observed on *Samonella enterica* serovar Enteritidis *(S. enteritidis)* strains which posses a strong genetic similarity and are homologous to curli in structure (Olsen et al., 1989; Collinson et al., 1992), function (Collinson et al., 1993; Sjobring et al., 1994), and regulation (Arnqvist et al., 1992; Römling et al., 1998a; Gerstel and Römling, 2003). Genes encoding curli organelles are referred to as *csg* (Hammar et al., 1995), whereas the genes encoding thin, aggregative fimbriae are called *agf* (Collinson et al., 1996). For convenience, thin, aggregative fimbriae in *Salmonella* species and curli in *E. coli* will be treated as identical structures in this paper.

Expression of curli fibers is influenced by several environmental factors. In general, curli are produced in low temperature (<30ºC) and low osmolarity conditions during stationary phase growth (Olsen et al., 1989; Olsen et al., 1993b; Maurer et al., 1998). Curli producing bacterial strains are typically observed by their ability to bind the dye Congo-red (Hammar et al., 1996) which provides a simple method to screen for bacteria capable of in vitro curli production. When incubated at 26º-28ºC for 48 hours on media containing Congo-red dye, curli producing colonies will usually display a red, dry, and rough morphotype (rdar) while curli deficient strains often appear smooth, white, and moist (saw). Examples of low nutrient and low salt media that will induce curli production include colony factor antigen (CFA) or YESCA agar (Hammar et al. 1995; 1996). Changes in the environment, such as increasing the NaCl content or the temperature to 37ºC, will cause some curli producing strains to revert to the white

phenotype (Olsen et al., 1993b; Römling et al., 1998b). This is not always the case as some bacterial strains are capable of expressing curli independent of temperature or other environmental factors (Romeling et al., 1998b; Bian et al., 2000; Uhlich et al., 2001).

The genes required for curli formation in *E. coli* are encoded within two divergently transcribed operons: *csgBAC* and *csgDEFG*, with a 521 base pair intergenic region between *csgD* and *csgB* (Hammar et al., 1995). The curli structural subunit, known as curlin or CsgA, is secreted extracellularly and polymerized on the surface by the nucleator protein CsgB (Hammar et al., 1996). In absence of the CsgB nucleator, CsgA will continue to be secreted in a soluble, less adhesive form (Bian and Normark; 1997). The outer membrane lipoprotein, CsgG, is required to maintain the stability of the CsgB and CsgA proteins during the curli assembly and may also transport curli proteins across the outer membrane. Without the CsgG protein, CsgB and CsgA will accumulate within the periplasmic space (Loferer et al., 1997). CsgD serves as an important transcriptional regulator of the *csgBAC* operon (Hammar et al., 1995; Römling et al., 1998a). The exact roles of the *csgC*, *csgE*, and *csgF* genes are not completely understood. CsgC is a possible production protein. Mutations in the *csgF* gene will halt curli formation but still allow CsgA to be secreted outside the cell. Disruption of the *csgE* region will not significantly impair curli formation; however, CsgE is required for binding of fibronectin and Congo-red dye (Stathopoulos et al., 2000; Hammar et al., 2005).

The genes required for the formation of curli fibers have been found in several other enterobacteria species. The two operons are practically ubiquitous in *E. coli* and

Salmonella spp. (Baumler et al., 1997; Maurer et al., 1998; Römling et al., 1998a). The *csg* genes have also been found in *Shigella* (Sakellaris et al., 2000), *Enterobacter, Citrobacter*, and *Klebsiella* spp. (Zogaj et al., 2003). However, the presence of the curli genes does not always lead to curli expression, as not all *E. coli* or *Salmonella* spp. are capable of curli expression, even under ideal environmental conditions. Additionally, deletions or insertions within the curli genes themselves commonly occur, deactivating expression (Maurer et al., 1997; Sakellaris et al., 2000; Zogaj et al., 2003).

Curli expression is variable among wild-type, laboratory, and pathogenic strains of bacteria. Curli expression can be as high as 90% among *S. typhimurium* and *S. enteritidis* isolates from a variety of human and animal sources under optimal conditions of low temperature, low salt, and stationary growth (Gerstel and Romeling, 2001). Strains with a more limited host range, such as *Salmonella typhi* and *Salmonella choleraesius*, do not express the fibers (Romeling et al., 2003). Furthermore, Romeling and coworkers (2003) reported that the curliated morphotype is serovar specific and correlated among disease causing strains. Curli production among *E. coli* strains is also variable, depending on the pathotype and source. Curli expression occurred in 100% of *E. coli* isolated from the colon of sudden infant death syndrome cases (Goldwater and Bettelheim, 2002), 55% of bovine mastitis isolates (Olsen et al., 1989), 38% of non-O157 Shiga-toxin producing *E. coli* (STEC) isolates, and is extremely rare among O157 strains (Cookson et al., 2002). Zogaj et al. (2003) found the curli related genes in *Enterobacter* and *Klebsiella* spp., but could not get these species to produce the curliated

phenotype. In many cases, curli forming ability is lost primarily through alterations in the promoter region gene, *csgD*, through sequence insertions or deletions.

CsgD is a transcriptional regulator belonging to the LuxR protein family and is considered the primary regulator of the *csgBAC* operon (Hammar et al., 1995; Chirwa and Harrington, 2003). An increase in *csgD* activity is associated with increased curli expression as well as increased invasion and virulence of bacteria (Uhlich et al., 2001; 2002). It was originally believed that *csgD* directly activated the *csgBAC* operon by binding to an upstream promoter, leading to increased CsgA and CsgB production. However, direct activation of *csgBA* by CsgD has not been proven. More recently, it has been proposed that CsgD (directly or indirectly) upregulates the activity of the serine hydroxymethyltransferase gene, *glyA*, increasing the cellular glycine metabolism to be used for the production of the glycine-rich curlin protein (Chirwa and Harrington, 2003). CsgD also acts as a promoter for the secretion of the cellulose exopolysaccaride, which contributes to the formation of the biofilms and the rdar morphotype in *Salmonella* spp. (Chirwa and Harrington, 2003; Zogaj et al., 2003).

The *csgD* gene is strongly regulated through environmental and genetic factors. Curli production as a reaction to environmental cues is primarily mediated through the up or down regulation of the *csgD* gene. Though trace amounts of CsgD may be present in exponentially growing cells (Prigent-Combaret et al., 2001), CsgD is primarily produced during curli favorable conditions (i.e., low osmolarity, low temperature, stationary growth), increasing curli expression (Hammar et al., 1995; Gerstel and Römling, 2003). During stationary phase growth, *csgD* transcription can increase up to 370-fold as

available nutrients are depleted (Gerstel and Römling, 2001). Interestingly, iron starvation will cause curli expression to become independent of temperature regulation (Römling et al., 1998b). Gerstel and Römling (2001) also noticed that curli and *csgD* expression were affected by oxygen tension, with maximal expression occurring under aerobic conditions on minimal agar and under microaerobic conditions with rich, but low salt, media. Supplementing bacterial media with salt reduces the expression of both *csgD* and curli fibers (Römling et al., 1998b; Prigent-Combaret et al., 2001).

The *csgD* gene and its affect on curli expression is also positively or negatively regulated by several other maintenance gene products that are influenced by environmental cues. These include the regulatory outer membrane protein R, OmpR, (Römling et al., 1998b), the stationary phase and stressed induced sigma factor, RpoS (σS) (Olsen et al., 1993b), Crl (Arnqvist et al., 1992), and the histone-like nucleoid structuring protein (H-NS) (Gerstel and Romeling, 2003; Gerstel et al., 2003). In short, the *csgD* promoter is highly regulated through a sophisticated network of proteins that react to environmental and genetic cues.

Curli's properties enhance the virulence of bacteria that can produce it. When expressed, curli are very adhesive structures and are capable of binding to a number of biological substrates including fibronectin, Congo-red dye (Olsen et al., 1989), laminin (Olsen et al., 1992), plaminogen (Sjobring et al., 1994), human contact phase proteins (Ben Nasr et al., 1996), and class I human major histocompatibility complex (MHC) (Olsen et al., 1998). Curli also facilitate bacterial auto-aggregation and contribute to biofilm formation on abiotic surfaces, such as glass or polystyrene (Vidal et al., 1998;

Prigent-Combaret et al., 2000; Brown et al., 2001; Cookson et al., 2002). Additionally, curliated bacteria are capable of invading and entering eukaryotic cells (Gophna, et al., 2001).

Clinical studies in vitro and in vivo suggest that curli producing bacteria are more pathogenic than non-curliated strains in their ability to adhere to, persist, and invade host cells. Curli increased the colonization and persistence of bacteria in avian colibacillosis (Ragione et al., 2000), while similar strains demonstrated reduced persistence once their curli production was inhibited (Ragione et al., 1999). Mice challenged with curli producing (red) *E. coli* variants displayed significantly shorter survival times than those challenged with curli deficient (white) variants (Uhlich et al., 2002). Curli producing *Salmonella* strains also achieved higher levels of growth and yolk invasion in chicken eggs when compared to their curli deficient counterparts (Cogan et al., 2004). Curli facilitated the internalization of *E. coli* by epithelial and HEp-2 cell lines, possibly mediated by fibronectin binding, where they may persist and evade detection by immune cells (Gophna et al., 2001; Gophna et al., 2002).

More recently, curli have been found to play a role in human sepsis. In vitro studies observed that curli are capable of binding human kininogen, resulting in the release of the vasoactive and proinflammatory peptide, bradykinin, and activating the contact-phase system (Nasr et al., 1996). Antibodies to the curlin protein, CsgA, were found in serum samples from convalescent human sepsis cases but not in healthy controls (Bian et al., 2000). Bian et al. (2000) also demonstrated that a significant number of *E. coli* (52%) cultured from human blood were capable of expressing curli at 37ºC in vitro.

Additionally, curli producing *E. coli* and a curli deficient mutant that secreted CsgA, triggered the release of several sepsis related cytokines (Bian et al., 2000). Curli components are also capable of directly inducing the NO progenitor, type 2 nitric oxide synthase, in vitro and in vivo, increasing the amount of NO in the blood leading to an increased risk of sepsis related deaths (Bian et al., 2001). These studies prove that curli's virulence extends beyond increased adhesion.

Curli were initially discovered on isolates from cases of bovine mastitis (Olsen et al., 1989); however, the possibility that curli may have a role in the pathogenesis of bovine mastitis has not been thoroughly explored. Todhunter et al. (1990) conducted a study to examine the clinical outcome of 12 cows that were vaccinated with curli-positive and curli-negative strains of *E. coli*. Each group of cows (n=6) was vaccinated on day 14 of lactation and then administered an intramammary infusion of a wild-type, curli-producing strain of *E. coli* on day 30 of lactation. All cows developed clinical mastitis that spontaneously resolved by day 10 post-challenge (Todhunter et al., 1990). This study did not use a control group of cows to examine differences in the clinical course of mastitis in non-vaccinated cows or cows infused with a curli-negative strain. Additionally, the study was conducted before it was known that curli interact with immunologic pathways. Currently, Todhunter et al. (1990) is the only published study examining the effect of curli in bovine mastitis.

Curli may contribute to the pathogenesis of *E. coli* mastitis through several mechanism. It has been shown that some bacterial strains can produce curli at increased temperatures, such as those encountered within the in vivo mammary environment. Curli

may increase the ability of bacteria to adhere to, colonize, and/or persist within the teat lumen and mammary tissue as well as evade the immune systems. As a protein, curli may also activate immunologic pathways, increasing inflammation, recovery time, and clinical severity.

Curli's strong affinity for binding fibronectin may serve as an adherence mechanisms in the udder and allow the bacteria to invade local cells. Fibronectin is a component of the basement membranes and is also present in myo-epithelial cells and fibroblasts. Fibronectin is not normally exposed on the apical side of healthy teat epithelium; however, epithelial lesions that occur as a result of *E. coli* mastitis expose the basement membrane (Frost et al., 1982). Lipopolysaccharides have also been shown to increase the fibronectin from surfaces of fibroblasts in vitro (Kubo et al., 1996). Exposed fibronectin may serve as a nidus to curli producing bacteria, promoting adherence. Fibronectin is also suspected to mediate the internalization of curliated *E. coli* into epithelial cells (Gophna et al., 2002). Should bacteria be able to reside inside mammary epithelial cells, they could persist in the gland and evade the immune system, leading to a longer clinical course, or serve as a reservoir for reinfection.

In addition to curli's adhesive and invasive abilities, both assembled curli and the curlin protein, CsgA, are capable of triggering inflammatory events and clinical signs associated with septic shock (Nasr et al., 1996; Bian et al., 2000). The presence of curli related proteins in mammary tissue, combined with the effects of LPS, can add to the inflammatory process resulting in more tissue damage and slowing the rate of recovery.

Furthermore, curli proteins can activate NO, causing a drop in blood pressure along with other sepsis related clinical signs, increasing systemic severity and case mortality.

Should curli play a role in mastitis pathogenesis and/or mastitis related septic events, then it could be beneficial to target curli in mastitis treatment and prevention plans. Possible strategies include vaccinations against curli proteins, therapeutics that suppress curli formation or bind curli fibers, and screening for and removing curli-positive cows.

Materials and Methods

Clinical Definition and Sampling

Bacterial isolates were obtained from milk samples from clinical mastitis incidents in dairy cows enrolled in a clinical mastitis study. Cows were eligible for enrollment if they suffered from clinical mastitis as defined by having abnormal milk (discoloration, flakes, clots, and/or wateriness) with or without the presence of additional signs of inflammation (redness, swelling, heat, and pain), plus any two of the following systemic conditions: abnormal temperature (<37.8°C or >39.5°C), abnormal heart rate (>98 beats per minute), abnormal attitude (depression or shivering), sharply decreased milk production (defined by 20% or greater drop in production from previous day), dehydration (determined by skin tenting of eyelids), rumen contractions <2 per minute, diarrhea, or recumbency. Diagnosis was made by the investigating veterinarian or his/her assistant. Cows with complicating injuries or other concurrent diseases were excluded from the study. Milk was collected on day zero (D0) and day five (D5) of the clinical mastitis event and shipped to a designated diagnostic laboratory for culture and identification using National Mastitis Council methods (Hogan et al., 1999).

Identification and Storage

All isolates were cultured on MacConkey (MAC) agar plates (Becton, Dickinson and Company, Cockeyville, MD) and on Levine's Eosin-Methylene Blue (EMB) agar (Difco, Detroit, MI) for 12 hours at 37°C. Isolates that produced dry, pink colonies on MAC and green, reflective colonies on EMB (positive reactions for lactose-fermenting

coliforms) were considered to be *E. coli.* Spot indole and oxidase tests (BBL, Cockeyville, MD) were performed on isolates with a weak or negative EMB test result with indole-positive and oxidase-negative results identifying *E. coli* isolates.

Isolates were cultured overnight in Brain-Heart-Infusion broth (Remel, Lenexa, KS) combined with 30% glycerol (v/v) and maintained at -20ºC for long term storage (years). Stock cultures for regular (daily) use were maintained on modified trypticase soy agar (TSA II) deeps (BBL, Cockeyville, MD) and maintained at 4ºC.

Bacterial Isolates

Three diagnostic laboratories sent a total of 55 isolates from 29 cows. Three isolates were determined to not be *E. coli* and excluded from the study. The Quality Milk Promotion Services Laboratory, Cornell University, Ithaca, NY, sent 36 *E. coli* isolates from 16 cows; the Colorado State University Veterinary Diagnostic Laboratory, Fort Collins, CO, sent 11 isolates from 6 cows; and the Kansas Veterinary Diagnostic Laboratory, Kansas State University, Manhattan, KS, sent 5 isolates from 5 cows. An additional 9 isolates from 9 cows were received from the Production Management Medicine Program, Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, VA, and included in this study. In total, 61 isolates from 36 different cows were included in this study.

Isolates were designated by a naming scheme based on the site origin (B, C, F, K, or V), cow number, and day cultured (D0 or D5). Thirty-three of 36 Cornell isolates were cultured from 14 cows from one farm, designated 'B', with the remaining 3 isolates

from 2 cows from other farms designated as 'C'. Colorado isolates, designated 'F', were all obtained from one farm. Kansas isolates were designated 'K' and Virginia isolates 'V'. Multiple isolates from the same cow were designated as a, b, c, or d. For example, isolate B1420D5d indicates an isolate received from Cornell University, from cow #1420 on farm B, cultured on day five of the mastitis episode. One cow (B1300) presented with one case of mastitis affecting two different quarters, yielding two isolates from each infected quarter. These isolates were differentiated by identifying left rear (LR) or right rear quarters (RR).

Individual Case Histories

Milking records were obtained from 6 cows from site F and 13 cows from site B from five days prior (day -5) to seven days after (day 7) the clinical mastitis episode. Cows were milked three times a day and the total milk obtained from each cow for three consecutive milkings in a 24 hour period constitutes the daily milk yield (DMY). Cows that were able to produce ≥75% of their initial, pre-mastitis (day -5) DMY by day 7 were considered to have recovered. Cows that did not regain at least 75% of their day -5 DMY were classified as non-recovered. Forty-three of the 61 isolates examined were isolated from these 19 cows.

Curli Phenotype

Curli production was determined by the binding of Congo-red dye (Hammar et al., 1995). Isolates were incubated at 26ºC for 48 hours on Congo-red indicator (CRI)

agar, which is YESCA agar supplemented with 20 mg/L Congo Red (Alfa Aesar, Ward Hill, MA) and 10 mg/L Coomassie Brilliant Blue G dye (Tokyo Kaes Kogyo, Tokyo, Japan). Each liter of YESCA agar contains 10 g Casamino Acids, 1 g Yeast Extract, and 20 g Bacto Agar (Becton, Dickinson and Company, Sparks, MD; Hammar, et al., 1995). Curli-positive colonies are red and curli-negative colonies are white. All isolates were directly recultured onto CRI agar and reincubated for 48 hours at 26ºC.

DNA Extraction and Quantification

Bacteria were grown in Luria-Bertani broth (Becton, Dickinson and Company, Sparks, MD) at 37ºC for 16 hours while being shaken at 200 rpm. DNA was extracted from 4 ml of culture using the Qiagen DNA Mini kit (Qiagen Inc., Valencia, CA) according to kit instructions.

DNA was quantified by measuring absorbance at λ=260 nm using a Shimadzu UV-1201 spectrometer (Shimadzu, Columbia, MD). DNA was then diluted to aliquots of 100 ng/µl with distilled, DNase/RNase free water (Gibco, Grand Island, NY) and stored at -20ºC.

csgA and *csgD* PCR

The genes for the curlin subunit protein, *csgA*, and the curli transcriptional regulator, *csgD*, were targeted and amplified in a PCR to determine their presence or absence in each isolate. Primers for *csgA* were selected as listed in Maurer et al. (1998). The *csgD* primers were manually selected from the *csgD* listing in Genbank (accession

number X90754; www.ncbi.nlm.nih.gov; National Institute of Health, Bethesda, MD; accessed 6/1/2004). Primer sequences are presented in Table 1.

Both the *csgA* and *csgD* PCRs were performed in a total volume of 50 µl with 300 ng DNA, 20 pmol of each primer, and 45 µl of Platinum PCR SuperMix High Fidelity (Invitrogen Life Technologies, Carlsbad, CA). The PCR amplification for *csgA* was as follows: denaturation at 94°C for 2 minutes (min), followed by 35 cycles of 30 seconds (sec) at 94°C, 30 sec at 54°C, and 30 sec at 68°C with a final extension of 10 min at 72°C. The PCR reaction for *csgD* was cycled with the following protocol: denaturation at 94°C for 2 min, followed by 35 cycles of 30 sec at 94°C, 30 sec at 46°C, and 30 sec at 68°C, with a final 10 min extension at 72°C. The reactions were performed in an Eppendorf Mastercycler Gradient thermocycler (Eppendorf AG, Hamburg, Germany).

ERIC-PCR

All strains were fingerprinted by ERIC-PCR using the ERIC2 primer sequence (Table 1; Meacham et al., 2003). Reactions were carried out in a final volume of 25 µl mixture consisting of: 100 ng DNA, 25 pmol of ERIC2 primer, 2 U of Platinum *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA), 0.4 m*M* of each dNTP, and 5 m*M* $MgCl_2$ in a 1x PCR buffer (provided with Platinum *Taq*). ERIC-PCR reactions were conducted simultaneously on all isolates in a 96-well PCR plate (Bio-Rad Laboratories, Hercules, CA) in an Eppendorf Mastercycler Gradient thermocycler with the following protocol: denaturation for 2 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 1 min at 57°C, 5 min at 72°C with a final extension step of 10 min at 72°C.

Gel Electrophoresis of PCR Products

csgA and *csgD* PCR products were electrophoresed in 1.0% agarose gel (Gibco, Grand Island, NY) buffered with sodium borate (SB) at 300 V for 10 minutes (Brody and Kern, 2004). Each gel contained a Low DNA Mass Ladder (Invitrogen Life Technologies, Carlsbad, CA) and was stained with 10 ng of ethidium bromide precast in the agarose. The expected length of *csgA* and *csgD* PCR products was 200 and 650 bp, respectively.

ERIC-PCR products were electrophoresed in a 1.5% agarose gel buffered with Tris-boro-EDTA (TBE) (Bio-Rad Laboratories, Hercules, CA) at 90 V for 2 hours and 45 minutes. Gels were stained for 40 minutes with a 1x solution of SYBR green (Molecular Probes, Inc., Eugene, OR) in a TBE buffer adjusted downward to pH=7.95, using 12 *M* HCl. Each gel contained 15 wells and was run with three lanes of 1 Kb Plus standard DNA ladder (Invitrogen Life Technologies, Carlsbad, CA).

ERIC-PCR gels were illuminated with UV fluorescence and photographed with a Kodak Imaging Station 440CF (Eastman Kodak Company, New Haven, CT) for a 2 minute exposure at f-2 through a #16, yellow, high-pass filter. Digital images were converted and exported to Tiff format using Kodak 1D Image Analysis Software v. 3.5.5.B (Eastman Kodak Company, New Haven, CT).

Statistical Analysis

Tiff images of ERIC-PCR results were standardized and analyzed using GelCompar II software (Applied Maths, Inc., Austin, TX). Similarity matrices were constructed using Pearson's correlation coefficient based on densitometric readings of the banding patterns. Isolates were clustered based on their similarity coefficients using unweighted pair group method analysis (UPGMA). One dendrogram was constructed using the data from all isolates. A second dendrogram was constructed by the same method using data from the 43 isolates from 19 cows for which milking records were available.

Isolates were examined for trends in clustering with regard to curli production on CRI agar and case recovery. Fisher's Exact tests were performed using Simple Interactive Statistical Analysis (SISA) online statistical analysis software (Uitenbroek, 1997; http://home.clara.net/sisa; accessed 1/1/2005).

Results

Phenotypic Curli Expression

Sixty-one *E. coli* isolates were cultured on CRI agar. Thirty-five isolates (57%) expressed the red phenotype (curli-positive) and 26 (43%) expressed the white phenotype (curli-negative). Neither variant, red nor white, altered its phenotype when recultured on CRI agar (Figure 1).

When more than one isolate was obtained from a cow, those isolates displayed the same curli phenotype, with one exception. Two isolates cultured on day 0 from cow B303 were curli-negative (B303D0a, B303D0b), while a third isolate obtained on day 5 (B303D5c) was curli-positive. Curli-positive *E. coli* isolates were cultured from 21 cows, and curli-negative strains were cultured from 15 cows (Table 2 and 4).

Presence of *csgA* and *csgD* Detected by PCR

The curlin subunit gene, *csgA*, and the curli transcriptional regulator gene, *csgD,* were both present in all phenotypically curli-positive and negative isolates (Figure 2).

ERIC-PCR Dendrogram

Genetic fingerprinting with the ERIC2 primer produced consistent and stable fingerprints (data not shown; Figures 3 and 4). The raw similarity matrix is presented in Appendix I.

At a threshold of 52% similarity, 58 bacterial isolates were clustered into two DNA clonal groups. Group I contained 23 isolates from 13 cows, and group II contained

35 isolates from 22 cows. Three isolates were genetically distinct from the isolates in the two groups: V3758D0a shared 49.9% genetic similarity with isolates in the two groups, while isolates B1290D5a and B1290D5b had only a 6.7% similarity with the two groups, but were 87.7% similar to each other (Figure 5).

A single *E. coli* isolate was obtained from 22 mastitis cases, while multiple *E. coli* isolates were obtained from 14 mastitis cases such that 2 'sibling' isolates were obtained from 7 cases, 3 isolates from 3 cases and 4 isolates from 4 cases. In 8 cases, sibling isolates possessed ≥90% similarity to each other and were clustered closely. In the remaining 7 cases, sibling isolates were <90% similar and were clustered on different or slightly more distant branches. In total, 28 of the 39 isolates clustered at ≥90% similarity to isolates within the same cow. B1167D5b and c were 95% similar to each other, but only 85% similar to B1167D0a. C1030D0a was 86% similar to C1030D5b, but more closely related to and clustered with B1090D0a and B1080D5a. Isolates F1300LRD0c and F1300LRD0d were only 83% similar to each other and placed in separate clusters. Additionally, F1300LR0c and F1300LRD0d had an average similarity of 69% to their other sibling isolates, F1300RRD0a and b. F12544D0a and F12544D0b were 81% similar and, as a result, clustered on different branches. B303D0a and b, two curli-negative isolates, were placed in group I and were 99% similar, whereas B303D5c, a curli-positive isolate, was placed in group II, which had an average similarity of 52% to group I. B1450D0a was only 60% similar to its sibling isolates (B1450D0b, B1450D5c, B1450D5d), which shared greater than 96% genetic similarity with each other.

A separate cluster analysis was performed and a dendrogram produced for the 43 isolates from 19 cows for which production records were available. This dendrogram also divided at a threshold of 52% similarity into two distinct groups (III and IV). Group III consisted of 18 isolates from 8 cows. Group IV was composed of 23 isolates from 11 cows. Isolates B1290D5a and B1290D5b were located outside of these two groups and possessed only 6.7% genetic similarity to these groups (Figure 6).

Curli Distribution by ERIC-PCR

Curli-positive isolates were present in both groups in both dendrograms (Figures 5 and 6). Group I had 8 curli-positive isolates from 5 cows and 15 curli-negative isolates from 8 cows. Group II contained 26 curli-positive *E. coli* isolates from 15 cows and 9 curli-negative isolates from 7 cows ($p=0.157$; Figure 5; Table 4).

Group III contained 5 curli-positive isolates from 2 cows and 13 curli-negative isolates from 6 cows, group IV had 19 curli-positive *E. coli* isolated from 9 cows and 4 curli-negative isolates from 2 cows ($p=0.024$; Figure 6; Table 4).

The p-values were calculated based on number of cow-cases in each group.

Recovery From Mastitis Events

The dairy records of 19 cows included milk yields on day -5, day 0, and day 7 related to mastitis detection. Eight cows (42%) regained ≥75% of their initial, pre-mastitis (day -5) daily milk yield by day 7. Of the remaining 11 cows (58%), 8 cows regained 36-69% of their pre-event daily milk production by day 7, 2 cows died within 24

hours of detection of severe clinical signs, and 1 cow died within seven days of clinical onset (Table 3).

When multiple isolates were recovered from a cow, they expressed all the same phenotype, except for isolates from cow B303 which yielded two curli-negative isolates on day zero, and one curli-positive isolate on day 5. Since a curli-positive *E. coli* was isolated, B303 was counted as a curli-positive case.

Curli-positive isolates were obtained from 3 of the 8 cows in the recovered group and from 8 of 11 cows in the non-recovered group ($p=0.18$; Tables 3 and 4). Group III contained 3 recovered cows and 5 non-recovered cows and group IV had 4 recovered cows and 7 non-recovered cows ($p=1.0$; Figure 6; Table 4).

Discussion

The overall incidence of curli expression in all 61 isolates was 57%. This is similar to the 55% incidence initially reported in the first report of curli fibers and the only published report of curli expression in *E. coli* from cases of bovine mastitis (Olsen et al., 1989). Additionally, Olsen et al. (1989) reported curli expression in 55% of bovine fecal isolates. None of the curli-negative or positive isolates changed its phenotype after successive culturing on CRI agar.

The rate of curli expression in other bacteria has been shown to be related to the bacteria's behavior, host, and environment. Curli expression has been reported as high as 90% in *S. typhimurium* and *S. enteritidis* strains, whereas it was absent in *Salmonella typhi* and *Salmonella choleraesuis* (Gerstel and Romeling, 2001; Romeling et al., 2003). Different *E. coli* strains have also been observed possessing varying rates of curli expression ranging from rare to 100%, depending on the associated host or disease (Cookson et al., 2002; Goldwater and Bettelheim, 2002). Mastitic *E. coli* are thought of as opportunistic, environmental bacteria, and the curli expression in bovine fecal and mastitic *E. coli* reported by Olsen et al. (1989) and in this study may indicate that the normal incidence of curli expression in non-pathogenic *E. coli* from bovine feces is in the range of 55-60%. These data also suggest that curli expression is a stable phenotype among the mastitic *E. coli* investigated, and even repeated culture among white (negative) variants did not cause them to express the red (positive) phenotype. However, it should be noted that curli expression was observed under ideal growth

conditions, and such stability may not be the case in the changing, in vivo environment of the udder.

All isolates possessed the genes for the curlin subunit protein, *csgA*, and the curli activator gene, *csgD*, regardless of phenotype expressed. Similar findings have been reported (Maurer et al., 1998; Zogaj et al., 2003), and our findings support the claim that both genes are normal background genes and ubiquitous in all *E. coli* strains. The presence of both these genes does not explain the difference among white and red variants. In this experiment, only the presence of the *csgA* and *csgD* gene was evaluated, and it is possible that, though present, either gene was not expressed or activated in white variants. Another gene required for curli expression may also be missing, altered by insertions or deletions, or not expressed. Additionally, since the PCR performed in this experiment only targeted the beginning and end of each gene, either gene could possess defects that could inactivate the normal function. The ubiquitous nature of the *csg* genes does not allow these genes to be used in screening for curliated *E. coli*. At this time, it appears that physical evaluation of the binding of Congo-red dye (or another substrate) is the simplest and fastest method to determine if an *E. coli* isolate is capable of producing curli.

Curli expression was present in 59% of the cow-cases, which is also similar to the occurrence reported on a per isolate basis and by Olsen et al. (1989). Interestingly, in the 15 cases in which more than one isolate was cultured from the same cow, with one exception, all isolates cultured from the same case displayed the same phenotype. The most likely reason for this observation is that additional isolates cultured from the same

case were either very closely related or identical clones. Another possibility is that curli producing bacteria may have a selective advantage within the mammary environment of some cows, increasing the likelihood of culturing a curli-positive strain. With the observation that curliated isolates did not change their phenotype when cultured on CRI agar, it is possible that the phenotype is also stable in vivo, and even isolates cultured five days apart retained their ability or inability to express curli.

The *E. coli* isolated from cow B303 were the only isolates to have a mixed curli phenotype. *Escherichia coli* isolated on day zero were curli-negative and the isolate cultured on day five was curli-positive. One possible explanation for this outcome is that the initial infecting population experienced a mutation or phase shift during the clinical episode that allowed it to activate the curli production. However, the genetic distance observed between B303D0a/b and B303D5c by ERIC-PCR supports the possibility that multiple, unrelated isolates were cultured from cow B303. Based on the raw similarity matrix data, the relatedness between B303D5c and its sibling isolates, B303D0a and B303D0b, is 30.1 and 32.1, respectively. Possible mechanisms for the isolation of multiple *E. coli* strains from B303 include co-infection of B303's mammary gland by two or more strains, a separate infection that occurred after D0, or contamination of the culture by an environmental *E. coli.*

Though no other group of isolates obtained from the same cow expressed both curli phenotypes, there were other incidences where genetically diverse isolates were cultured from the same cow. However, of the seven cases where all sibling isolates did not cluster at ≥90%, four of the cases clustered with their siblings at ≥80% similarity, and

the remaining isolates from three cows: B303, B1450, and F1300 clustered at < 70% similarity. Cow B1450 had three isolates that clustered closely together (≥96% similarity), but also contained isolate B1450D0a that was only about 60% similar to the others. As with cow B303, these data also suggest that cow B1450 was infected with at least two genetically diverse *E. coli* strains.

Cow F1300 was also a unique case in that it was the only case in this study that presented with mastitis in two quarters. Isolates from both quarter possessed a positive curli phenotype. Additionally, the isolates from the RR quarter were very similar (96%) to each other, but were only about 70% similar to the isolates cultured from the LR quarter. These data support that each quarter was simultaneously infected with different strains of *E. coli*. It is very possible that this is a clinical coincidence; however if cow F1300's mastitis was a result of a systemic condition (such as stress, hormonal or nutritional influence) that caused temporary immunosuppression, both strains could be opportunistic invaders.

Isolates that appeared to be identical clones, or were very similar, still had a slight amount of variation. This raises a query as to the similarity threshold at which similar isolates should be considered identical. In this experiment, a threshold of 90% was designated to determine if sibling isolates clustered close together. Sufficient random variation exists in the banding patterns of closely related isolates to add a small amount of dissimilarity. Intra- and inter-experimental variation is still a possible cause of error when calculating similarity. In this experiment, variations in ERIC-PCR reactions were minimized by running all PCR reactions simultaneously with the same reagents and

equipment. However, slight variations in staining, consistency of electrophoresis gels, DNA concentrations, and other random events that could not be controlled may play a role in introducing slight differences that impact the similarity score.

Fingerprinting with ERIC-PCR only examines a portion of the genetic data. The banding patterns that are produced are a result of binding numerous ERIC sequences through the bacterial genome and replicating the DNA between them. The greatest advantages of genotyping by ERIC-PCR lie in its accessibility, speed, relative ease of use, and general stability. Unfortunately, ERIC-PCR does not always offer a complete picture of genetic relatedness. Identical bands are based on their size, and not necessarily their genetic makeup. It is possible that two genetically diverse segments between two ERIC segments, can be counted as equal, provided that the ERIC targets are the same distance apart on the DNA. The presence of ERIC sequences throughout the bacterial genome is only one of many molecular traits that can be used to compare bacteria. Further detail could be obtained by combining ERIC-PCR data with additional rep-PCRs (such as REP elements) or the presence/absence of other genetic markers or virulence factors.

In the isolates investigated in this experiment, ERIC-PCR did not differentiate curliated *E. coli* from those unable to produce curli. Given the stability of the curli phenotype, curliated *E. coli* do appear to be different from their non-curliated strains. Possible mechanisms for why such differences may not have been detectable by ERIC-PCR include that the genetic capabilities required for curli expression may not be located between ERIC elements or significantly alter the length of DNA (such as with the

deletion of one base pair). Additionally, curli production in these isolates may be entirely regulated by RNA, which was not examined, or the diversity of ERIC sequences and locations within the genome may overshadow any differences that contribute to curli production.

In this study, curli expression did not affect the outcome of a cow's recovery, as measured by a return to milk production. However, the return to production does not always reflect the clinical severity of a mastitis case. Other than the mastitis case definitions, clinical signs of mastitis were not evaluated or associated with curli incidence. Cows with severe clinical signs may still have recovered by day 7, especially if aggressive, supportive care was implemented. This study focused on curli's local and immediate effects within the mammary gland. Conversely, curli's impact on mastitis may extend beyond milk production, such as increasing the severity of systemic clinical signs.

Curli have been reported to enhance bacterial virulence not only through enhanced adhesion, but also through the stimulation of inflammatory mediators, cytokines, and septic related clinical signs (Nasr et al., 1996; Bian et al., 2000). This study did not examine cows for evidence of curli outside the mammary gland, such as antibodies to the curlin subunit, CsgA. Curli have also been reported in cellular internalization of *E. coli*. Curli may play a role in chronic and recurrent mastitis by allowing some strains to reside in the mammary gland, serving as a reservoir for future infections. Possible areas of investigation to assess curli effect on bovine mastitis can include the effect of curli on local and systemic clinical signs exhibited by a cohort of

cows with mastitis, experimental mammary challenges with curliated and non-curliated *E. coli* strains, the presence of CsgA antibodies in the serum of cows with severe or septic mastitis, and the role of curli in chronic, recurring mammary infections.

Summary and Conclusions

Curli are thin fibers expressed in some *E. coli* and other bacteria, and are able to increase bacterial virulence through its ability to bind host proteins, form biofilms, and initiate inflammatory pathways. The incidence of curli expression among mastitic *E. coli* evaluated in this study was approximately 55-60% and was similar to the reported curli incidence in other bovine mastitis and fecal *E. coli*. Two genes that are use in curli formation and regulation, *csgA* and *csgD*, were present in all examined isolates, regardless of their ability to express curli. Isolates capable of producing curli and binding Congo-red dye did not selectively cluster when fingerprinted by ERIC-PCR. Additionally, isolates did not cluster together based on case outcome, recovered or non-recovered, when fingerprinted by ERIC-PCR.

Generally, the severity of a clinical *E. coli* mastitis case is more dependent on cow-factors than bacterial virulence. In this study, curli production in *E. coli* isolated from clinical cases of mastitis did not impact a cow's return to milk production.

Tables

Table 1 Primer sequences*

Primer	Target	Sequence (5'-3')
M464F[a]	*csgA*	ACTCTGACTTGACTATTACC
M465R[a]	*csgA*	AGATGCAGTCTGGTCAAC
csgDF	*csgD*	ATGTTTAATGAAGTCCATAGTATT
csgDR	*csgD*	TTATCGCCTGAGGTTATCGTTTGC
ERIC2[b]	ERIC	AAGTAAGTGACTGGGGTGAGCG

* Primers produced by Integrated DNA Technologies, Coralville, IA
a Obtained from Maurer et al., 1998
b Obtained from Meacham et al., 2003

Table 2 Isolate, site of origin, curli phenotype on CRI agar, and similarity group of all isolates

Isolate	Site	Curli	Similarity Group
B303D5c	B [a]	+	II
B303D0a	B	-	I/III
B303D0b	B	-	I/III
B528D0a	B	+	I/III
B528D0b	B	+	I/III
B541D5c	B	+	I/III
B541D0a	B	+	I/III
B541D0b	B	+	I/III
B1080D5a	B	+	II
B1083D5a	B	+	II/IV
B1090D0a	B	+	II
B1129D5a	B	+	II/IV
B1135D5c	B	+	II/IV
B1135D5d	B	+	II/IV
B1135D0a	B	+	II/IV
B1135D0b	B	+	II/IV
B1167D5b	B	-	I/III
B1167D5c	B	-	I/III
B1167D0a	B	-	I/III
B1290D5a	B	-	
B1290D5b	B	-	
B1343D0a	B	-	II/IV
B1343D0b	B	-	II/IV
B1420D5c	B	+	II/IV
B1420D5d	B	+	II/IV
B1420D0b	B	+	II/IV
B1420D0a	B	+	II/IV
B1450D0b	B	-	I/III
B1450D5c	B	-	I/III
B1450D5d	B	-	I/III
B1450D0a	B	-	I/III
B1525D0a	B	-	I/III
B1525D0b	B	-	I/III
C1030D5b	C [b]	+	II
C1030D0a	C	+	II
C1400D5a	C	-	I

Isolate	Site	Curli	Similarity Group
F506D0a	F [c]	+	II/IV
F506D0b	F	+	II/IV
F554D0a	F	+	II/IV
F1300RRD0a	F	+	II/IV
F1300LRD0d	F	+	II/IV
F1300RRD0b	F	+	II/IV
F1300LRD0c	F	+	II/IV
F5403D0a	F	-	I/III
F12364D0a	F	-	I/III
F12544D0a	F	-	II/IV
F12544D0b	F	-	II/IV
K2726D0a	K [d]	-	II
K3142D0a	K	+	II
K3208D0a	K	+	II
K3376D0a	K	+	II
K3440D0a	K	+	II
V000Doa	V [e]	+	I
V503D0a	V	-	I
V556D0a	V	+	I
V1138D0a	V	-	II
V1421D0a	V	-	II
V1429D0a	V	-	II
V3367D0a	V	+	I
V3543D0a	V	-	II
V3758D0a	V	+	

a B= Isolates obtained through Cornell University from farm B
b C= Isolates obtained through Cornell University from other sites
c F= Isolates obtained through Colorado State University
d K= Isolates obtained through Kansas State University
e V= Isolates obtained through Virginia-Maryland Regional College of Veterinary Medicine

Table 3 Milk production of cows based on milking records and isolate phenotype

Cow ID	Site	D-5 DMY[a]	D0 DMY[b]	D7 DMY[c]	Recovery[d]	Curli Phenotype	Associated Strains
1083	B	20.00	3.64	32.73	1.64	+	B1083D5a
506	F	19.09	26.09	29.23	1.53	+	F506D0a, F506D0b
12544	F	48.18	25.95	45.45	0.94	-	F12544D0a, F12544D0b
5403	F	50.00	36.00	45.32	0.91	-	F5403D0a
12364	F	34.55	22.14	31.32	0.91	-	F12364D0a
1420	B	54.55	6.82	46.36	0.85	+	B1420D0a, B1420D0b, B1420D5c, B1420D5d
1290	B	47.27	0.91	39.09	0.83	-	B1290D5a, B1290D5b
1525	B	37.73	21.36	29.55	0.78	-	B1525D0a, B1525D0b
1080	B	50.00	3.64	34.55	0.69	+	B1080D5a
1135	B	36.36	1.82	24.55	0.68	+	B1135D0a, B1135D0b, B1135D5c, B1135D5d
303	B	37.27	3.18	25.00	0.67	-/-/+[e]	B303D0a, B303D0b, B303D5c
1450	B	41.36	13.18	26.36	0.64	-	B1450D0a, B1450D0b, B4150D5c, B4150D5d
1167	B	39.09	7.73	25.00	0.64	-	B1167D0a, B1167D5b, B1167D5c
554	F	57.27	2.91	30.36	0.53	+	F554D0a
541	B	45.45	5.45	23.64	0.52	+	B541D0a, B541D0b, B541D5c
1300[f]	F	20.00	7.27	6.18	0.31	+	F1300RRD0a, F1300RRD0b, F1300LRD0c, F1300LRD0d
1129	B	36.82	10.00	D[g]	-	+	B1129D5a
528	B	45.91	1.82	D	-	+	B528D0a, B528D0b
1343	B	47.73	1.64	D	-	-	B1343D0a, B1343D0b

a 24 hour milk yield 5 days before clinical incident (kg)
b 24 hour milk yield at diagnosis (kg)
c 24 hour milk yield 7 days after diagnosis (kg)
d Ratio of D-5 DMY to D7 DMY
e B303D0a and b were curli – and isolate B303D5c was curli +
f Isolates obtained from cases of mastitis in different quarters. LR= Left Rear; RR= Right Rear
g D= Cows that died

Table 4 Composition of similarity groups

Similarity Group	Total Isolates	Curli + Isolates	Curli - Isolates	Total Cases[c]	Curli + Cases	Curli - Cases	Non-recovered Cases[c, d]
I	23	8	15	13	5	8	
II	35	26	9	22	15	7	
Total[I+IIa]	58	34	14	35	20	15	
III	18	5	13	8	2	6	5
IV	23	19	4	11	9	2	7
Total[III+IVb]	41	24	17	19	11	8	12

a Three isolates from two cases did not cluster within the two main groupings
b Two isolates from one case did not cluster within the two main groupings
c B303D0a and b were in group I/III and B303D5c was in group II/IV; each occurrence was counted
d Cows that did not regain 75% of D-5 daily milk yield by day 7

Figure 1 Curli-negative (top) and curli-positive (bottom) strains displaying the white and red phenotypes.

Figure 2 Gel electrophoresis of *csgA* and *csgD* PCR reactions. Lanes marked "M" are molecular weight standard ladders.

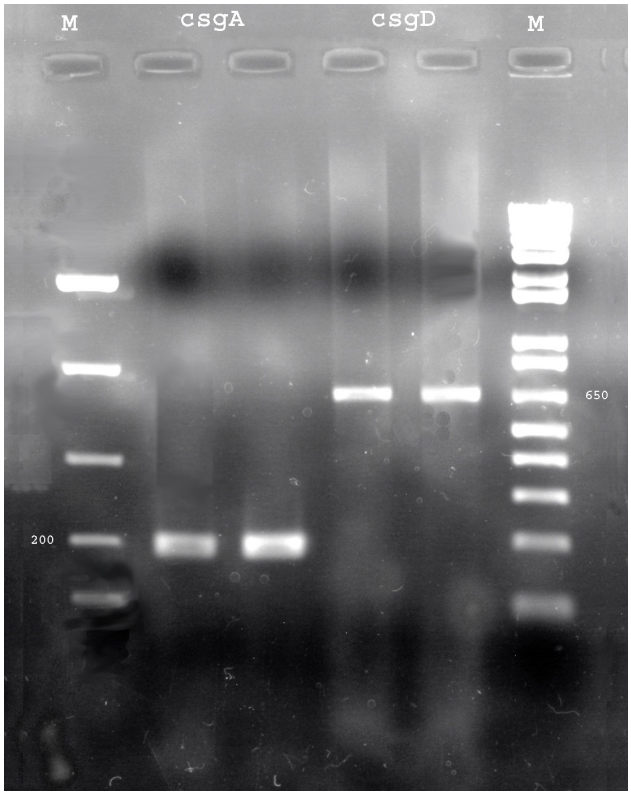


Figure 3 Gel electrophoresis of ERIC-PCR reaction. Lanes marked “M” are standard molecular weight marker ladders.

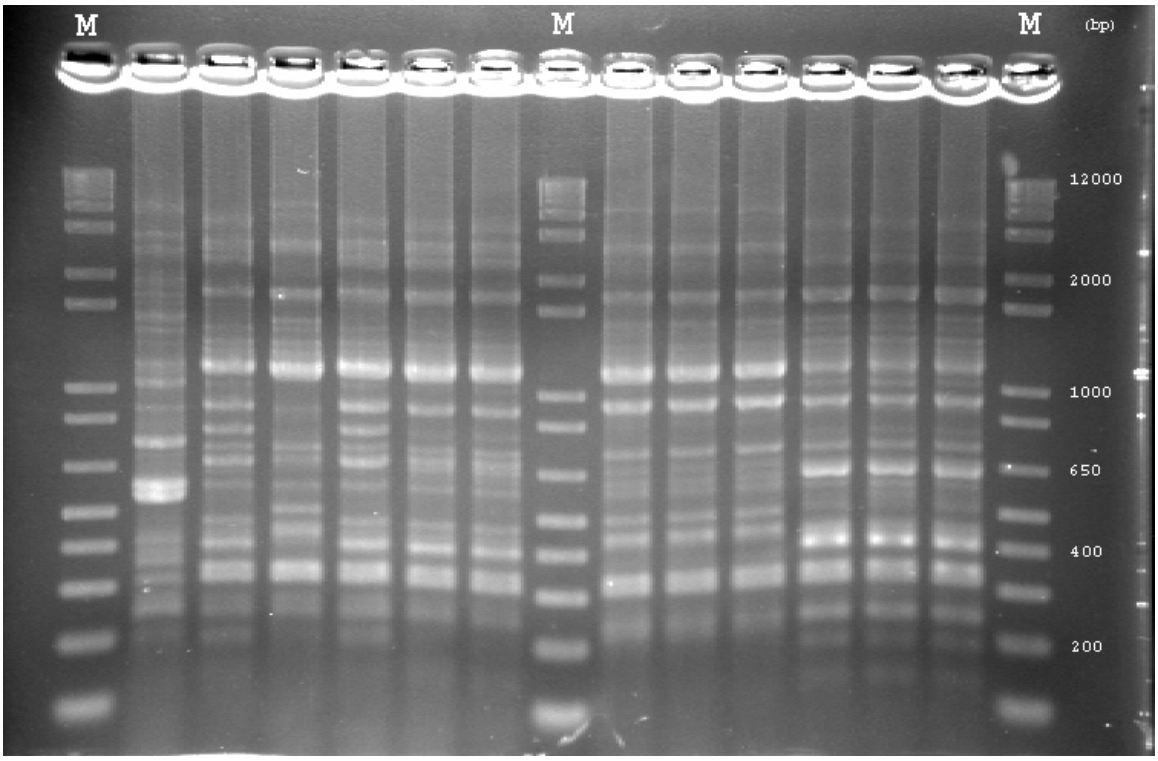


Figure 4 ERIC-PCR banding patterns and resulting dendrogram of all *E. coli* isolates. The horizontal images are the standardized ERIC-PCR banding patterns for each isolate. The numbers at each branch represent the clustered (average) similarity of the joining branches. Curli phenotype of each isolate is represented by + or –.

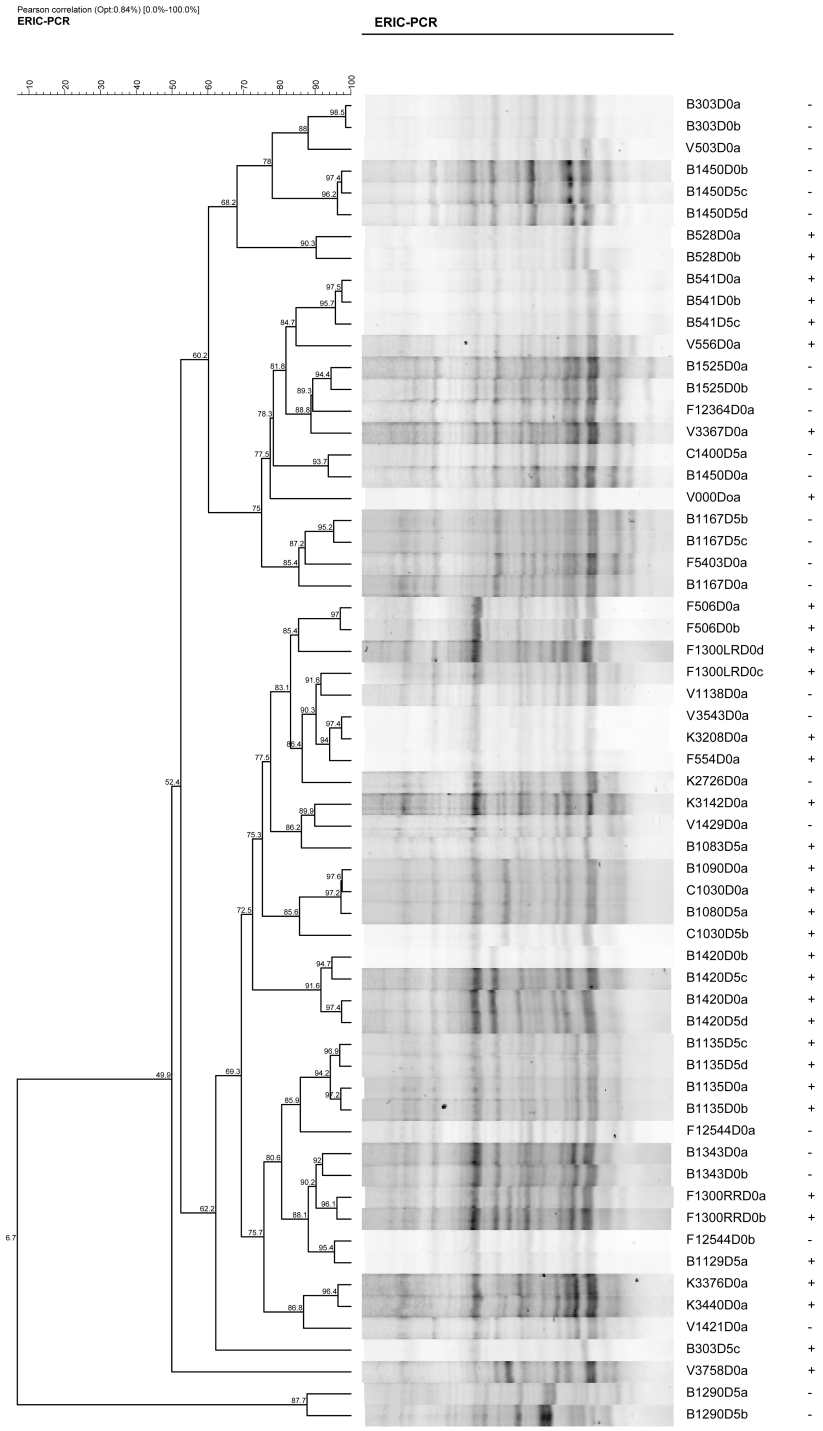


Figure 5 Dendrogram of all *E. coli* isolates with two distinct groups forming at 52% genetic similarity.

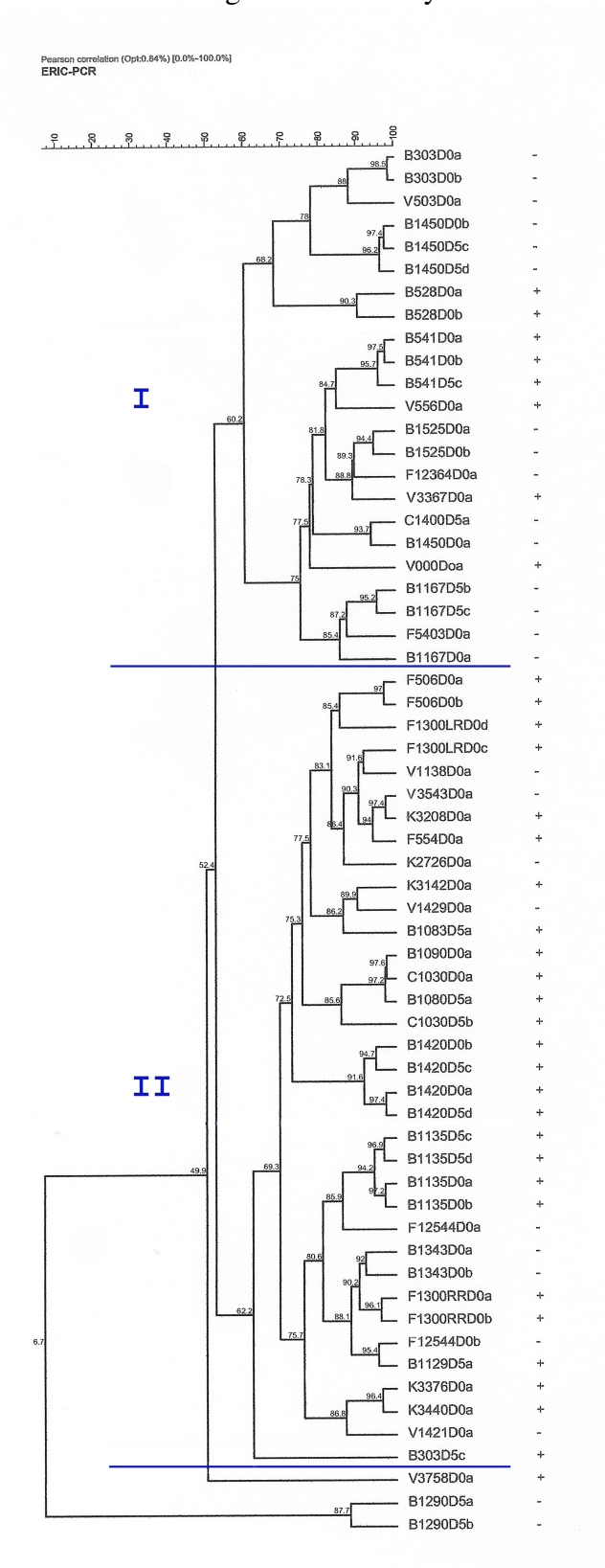
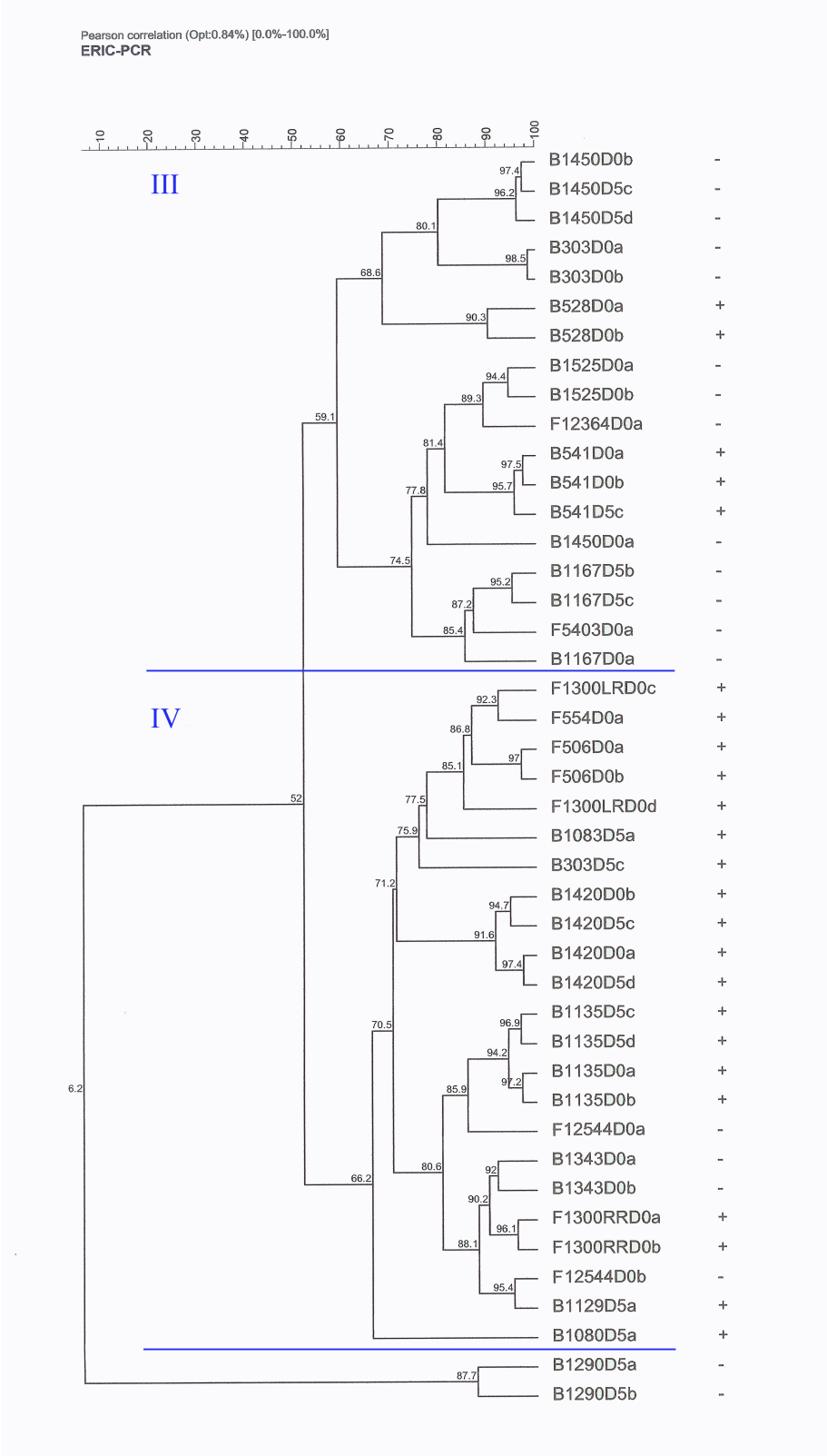


Figure 6 Dendrogram of 43 selected *E. coli* isolates from the mastitis cases of cows with production records with two distinct groups forming at 52% genetic similarity.



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Vita

John Dyer was born on October 11, 1977 in Decatur, Alabama. He attended Virginia Tech and graduated in 2000 with a B.S. in Animal and Poultry Science. In August of 2000, he began his medical education at the Virginia-Maryland Regional College of Veterinary Medicine. After his first year, John enrolled in the M.S./D.V.M. parallel program and would spend his summer performing research for the Department of Large Animal Clinical Sciences under Dr. François Elvinger. In 2004, he graduated with a Doctorate of Veterinary Medicine and spent the following summer finishing his research. John is the son of John and Patricia Dyer in Vienna, Virginia and was married to Lisa Brake in November, 2004. Currently, John lives with his wife in Richmond, Virginia and is a small animal practitioner for Fairfield Veterinary Hospital. In addition to camping and hiking, John also enjoys playing the cello and breeding poison dart frogs in his spare time.