Escherichia coli Isolates' Serotypes, Genotypes, and Virulence Genes and Clinical Coliform Mastitis Severity

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ABSTRACT

Dairy cattle with clinical mastitis caused by *Escherichia coli* exhibit a wide range of disease severity, from mild, with only local inflammatory changes of the mammary gland, to severe, with significant systemic derangement. The present study was designed to examine the relationship between serotype and virulence genes of *E. coli* mastitis isolates, different levels of systemic disease severity, and farm from which the *E. coli* strain was obtained. One hundred twenty-three *E. coli* milk isolates were obtained from cows with clinical mastitis of varying systemic disease severity from 6 different farms. No predominant serotype was identified by farm or by systemic disease severity; however, the most frequent serotype, O158:NM (n = 3), was isolated from cows in the moderate severity group. Virulence genes evaluated were identified infrequently and were not associated with systemic disease severity. Evaluation of genetic similarity showed no clustering assigned by farm or mastitis severity based on systemic disease signs. We concluded that a high degree of genotypic variability is characteristic of *E. coli* strains causing clinical mastitis within and between different farms and systemic severity groups, and that specific cow factors probably play a more important role in determining systemic disease severity.

Key words: coliform mastitis, virulence, severity

INTRODUCTION

Dairy cattle with acute coliform mastitis, caused primarily by *Escherichia coli,* exhibit a wide range of systemic disease severity, from mild, with only local inflammatory changes of the mammary gland, to severe, with significant systemic signs including rumen stasis, dehydration, shock, and even death (Wenz et al., 2001a). Studies have shown that up to 23% of clinical coliform mastitis presents with acute systemic disease signs (Smith et al., 1985; Gonzalez et al., 1990). Previously, we demonstrated that bacteremia occurs in a significant proportion of cows with severe systemic disease signs (Wenz et al., 2001b). Often genotypically similar *E. coli* strains were isolated from both the milk and blood of affected cows, suggesting that virulence factors associated with septicemia may play a role.

Disease severity is determined by interactions between the host, the environment, and the infectious agent. Virulence factors of the bacterial strain can assist in colonization, multiplication, and survival in the face of host selective pressures. Virulence varies not only among different species, but also among strains of the same species. Numerous studies have been conducted to identify virulence factors of *E. coli* isolated from cows with clinical mastitis (Barrow and Hill, 1989; Kaipainen et al., 2002). These studies have typically found that a variety of virulence genes were present in mastitis strains; however, the majority did not possess any of the virulence genes evaluated. The only virulence characteristic consistently associated with *E. coli* isolated from cows with clinical mastitis was serum resistance (Hill, 1994). However, none of the studies designed to identify virulence factors of *E. coli* mastitis strains have evaluated clinical disease severity. In our previous work, we showed important differences in clinicopathologic changes, the number of bacteria in secretions from the affected mammary gland, and outcomes of an acute coliform mastitis episode in cows grouped based on systemic disease severity (Wenz et al., 2001a,b).

The *eaeA* gene encodes for the intimin protein, which is associated with attaching and effacing lesions and bacterial adherence to epithelial cells (Jerse et al., 1990). The cytotoxic necrotizing factor (**CNF**) toxins (*CNF1* and *CNF2* genes) are associated with damage to vascular endothelial cells and thrombotic microangiopathy. The *cs31a* gene was found in strains of *E. coli* obtained from cases of diarrhea and septicemia (Bertin et al., 1998; Bertin et al., 2000). The presence of any one of these factors could logically be associated with varying levels of clinical disease through their potential for causing tissue damage or mediating bacteremia as-

sociated with acute coliform mastitis showing severe systemic disease signs. However, there is little information about the association of strain possession of virulence genes, serotype, and genotype of strains and variation in clinical disease severity or source farm.

The present study was designed to examine the relationship between different *E. coli* mastitis isolates (presence of virulence genes, serotype, and genotype), different levels of systemic disease severity, and farm from which the *E. coli* isolate was obtained.

MATERIALS AND METHODS

Animals

Escherichia coli isolates were obtained from 123 cows with acute coliform mastitis that were housed at 6 dairies near Fort Collins, Colorado, between July 1997 and January 1999. All cows were milked 3 times daily and were housed in dry-lot pens or free stalls. The average monthly bulk tank SCC of cooperating dairies, reported by the processor, was less than 300,000 cells/mL during the sampling period. All cows were vaccinated with a bacterin containing the J-5 strain of *E. coli*.

Clinical Disease Severity Classification

Cows with clinical coliform mastitis were classified as having mild, moderate, or severe disease on the basis of rectal temperature, hydration status, rumen contraction rate, and attitude at time 0, as described previously (Wenz et al., 2001a).

Bacterial Isolates

Secretions from individual mammary glands with clinical mastitis were collected in sterile vials following teat end disinfection with 70% ethanol and removal of the first 3 to 4 streams of milk. One hundred twenty-three *E. coli* milk isolates were obtained from single colonies of growth from MacConkey agar. The isolates were identified as *E. coli* based on colony morphology and color, Gram stain, and API 20E (bioMérieux, Durham, NC). Colonies subcultured on blood agar were frozen on horse red blood cell-coated sterile glass beads and stored at −70°C.

Serotype and Virulence Gene Determination

Serotype and virulence gene determinations were performed at the Pennsylvania State University *E. coli* Reference Center. Serum agglutination assay was used to screen isolates for the presence of 187 O antigens and 52 H antigens. The isolates were also examined for the presence of genes that code for intimin (*eaeA* gene),

CNF (*CNF1* and *CNF2* genes), and the *cs31a* gene using 4 separate PCR assays (DebRoy and Maddox, 2001).

Genotyping

Polymerase chain reaction using Enterobacterial Repetitive Intergenic Consensus (**ERIC**) primers was used to identify strains (Lipman et al., 1995a). Briefly, 2 primers with the sequences CATTAGGGGTCCTCG AATGTA and AAGTAAGTGACTGGGGTGAGCG were used to amplify repetitive sequences contained in the chromosomal DNA of *E. coli* isolates. The amplicons were electrophoresed on 2% agarose gel, stained with ethidium bromide to visualize the DNA fragment size banding pattern, viewed with a UV light transilluminator, and photographed for further analysis.

Analysis of ERIC Data

The ERIC banding patterns of 115 *E. coli* mastitis isolates were available for evaluation. The banding information was coded as a matrix of 1 (band present) and 0 (band absent), and bins that did not contain any bands were removed. A pairwise distance matrix was generated using RAPDPLOT (available by anonymous file transfer protocol from lamar.colostate.edu/pub/ wbc4) based on the measure $1 - M$, where [M (similarity) $= N_{ab}/N_T \times N_{ab}$] and M is the total number of matches in individuals a and b, and N_T is the total number of bands scored. A value of $M = 1$ indicates the 2 individuals have identical banding patterns, and a value of $M = 0$ indicates they have no bands in common. Cluster analysis using the unweighted pair group method with arithmetic averages was performed on the values of $1 - M$ in RAPDPLOT using the method of Nei and Li (1979), modified for bootstrapping by setting N_T to 1 when no ERIC bands were present in either of the isolates being compared.

Statistics

The percentage of isolates possessing virulence genes was compared between severity groups by using χ^2 tests, except that Fisher exact tests were used when the expected count in >25% of the categories was <5 (PROC FREQ, SAS v. 9.01; SAS Institute Inc., Cary, NC). Continuous variables (DIM and lactation number) were compared among severity groups by ANOVA (PROC GLM, SAS v. 9.01; SAS Institute Inc.).

RESULTS

Fifty-seven, 38, and 28 cows were classified as mild, moderate, and severe, respectively, based on systemic

Table 1. Frequency of O and H types identified from 72 *Escherichia coli* milk isolates from cows with acute coliform mastitis of varying systemic disease severity

Frequency	O-type	H-type
1	3, 18, 19, 21, 25, 37, 48, 53, 65, 78, 82, 86, 129, 135, 140, 141, 37/158, X13w, X18	8, 10, 17, 39, 46, 52, 12w/21w, 15w, 4/32
2	16, 36, 169, M,[1] X7	2, 12, 48, 12/21, 12w, M
3	2, 15, 113, 158	—
4	20	—
5	8, 146	—
18	—	NM[2]
22	NT[3]	—
35	—	NT

[1]M = Motile.

[2]NM = Nonmotile.

[3]NT = Not typable.

disease signs. Serotype data were available for 72 of 123 milk *E. coli* isolates. O-type 8 and 146 were the most common, shared by 5 isolates each. The most frequent H-type after not typable (**NT**, n = 35) was nonmotile (**NM**, n = 18; Table 1). No predominant serotype was identified by farm or by systemic disease severity. Isolates of the most frequent serotype O158:HNM (n = 3) were isolated from cows in the moderate severity group from 3 different farms.

Of the 123 milk isolates, 15 (10.7%) had a single virulence gene detected by PCR. The most common gene identified was *CNF2* (n = 12; Table 2). No combinations of genes were detected in any of the isolates, and there was no association between severity of systemic disease signs and presence of virulence genes evaluated (Table 2).

Genotype data were available for 115 of the 123 milk *E. coli* isolates. A dendrogram was created based on an analysis by the unweighted pair group method with arithmetic averages of presence–absence data for bands obtained with ERIC primers of the *E. coli* isolates. The cluster analysis showed no association between genotype and disease severity or farm from which the isolate was obtained. A consensus-tree bootstrap analysis showed that only 18% of the branches were supported ≥50%.

DISCUSSION

The results of this study are consistent with previous reports suggesting that *E. coli* strains causing acute coliform mastitis in dairy cattle do not possess specific virulence factors that contribute to clinical disease (Barrow and Hill, 1989; Kaipainen et al., 2002; Bean et al., 2004). Unlike previous studies, the current study investigated the associations among possession of isolate virulence genes and clinical mastitis systemic disease severity and farm of origin. No association was identified between severity of systemic disease signs and the presence of toxin genes evaluated or strain type characterized by serologic and genotypic methods. Through classification of cows and inclusion of a wide range of mastitis severity, we hoped to increase the probability of identifying strains possessing virulence factors or specific genotypes associated with systemic disease severity. Bean et al. (2004) evaluated the "health status" of cows from which isolates were obtained to study virulence genes. However, their health status determination was not clearly defined nor were their data presented.

In the present study, we did not evaluate LPS levels in the secretion from affected quarters. Lipopolysaccharide, also known as endotoxin, is a structural compo-

Table 2. Distribution of *E. coli* mastitis isolates with detected virulence factor genes by systemic disease severity of cows with acute coliform mastitis

Severity	n[1]	eae No.[2]	eae Percent[3]	cs31a No.	cs31a Percent	CNF1 No.	CNF1 Percent	CNF2 No.	CNF2 Percent	All No.	All Percent
Mild	57	0	0.0	0	0.0	1	1.8	7	12.3	8	14.0
Moderate	38	0	0.0	1	2.6	0	0.0	3	7.9	4	10.5
Severe	28	0	0.0	0	0.0	1	3.6	2	7.1	3	10.7

[1]n = Number of isolates in each severity group.

[2]No. = Number of isolates within a severity group possessing a virulence gene.

[3]Percent = Percentage of isolates within a severity group possessing a virulence gene.

nent of the outer membrane of all gram-negative bacteria. Endotoxin is a potent stimulator of the immune system of animals, and clinical disease occurs either when an excessive amount is liberated or the host responds in an overly exuberant manner. Most of the clinical disease signs associated with acute coliform mastitis are attributed to the induction of endogenous inflammatory mediators, in particular the cytokine tumor necrosis factor-α, by endotoxin (Hirvonen et al., 1999; Hoeben et al., 2000). The pathophysiologic responses to experimental intramammary or i.v. endotoxin infusion are dose dependent, and experimental coliform infections suggest that the maximal number of bacteria attained in the gland determines clinical disease severity (Lohuis et al., 1988). Bacterial numbers in the gland are, in turn, likely determined by the innate immunity of the cow, specifically the neutrophil function (Burvenich et al., 2003).

In the present study, we chose to evaluate the presence of *cs31a* because of its association with septicemic bovine *E. coli* isolates as well as our recent finding that over 40% of cows with severe acute coliform mastitis were bacteremic (Girardeau et al., 1988; Wenz et al., 2001b). A study by Lipman et al. (1995b), indicating that 11 of 20 *E. coli* mastitis strains possessed genetic sequences coding for F17 fimbriae proteins, suggests that the presence of *F17* genes may be more relevant. Nevertheless, bovine *E. coli* strains from diarrheic calves, where adhesion is important to the pathogenesis of disease, frequently appear to produce both F17 and cs31a adhesins (Bertin et al., 2000). In the current study, of the 123 isolates obtained from cattle exhibiting a wide range of systemic disease severity, only 1 isolate possessed the *cs31a* gene. These results are consistent with electron microscopy and cell culture data indicating that attachment to mammary epithelium is not necessary in the pathogenesis of acute coliform mastitis (Opdebeeck et al., 1988). Similarly, the *eae* gene (important for adherence to intestinal epithelia) was present in only a single isolate.

The *CNF2* gene was most commonly identified; however, it was still uncommon (12 of 128 isolates), and there was no difference in gene presence based on severity of systemic disease signs.

Serum resistance was not evaluated because numerous other studies have clearly identified it as important for causing acute coliform mastitis and have identified serum resistance in 64 to 100% of isolates (Barrow and Hill, 1989; Nemeth et al., 1991; Fang and Pyörälä, 1996). The *TraT* gene was associated with serum resistance in human and avian *E. coli* isolates (Moll et al., 1980; Vandekerchove et al., 2005). However, Nemeth et al. (1991) found no significant relationship between the *TraT* gene and serum resistance in 95 *E. coli* bovine mastitis isolates.

The low bootstrap values obtained from the cluster analysis indicate that the *E. coli* isolates were not clearly definable into related groups based on the ERIC primer PCR product banding pattern obtained. This may reflect significant diversity of the isolates or the inability of ERIC primer PCR products to define the real genetic relatedness of the isolates. The former interpretation is consistent with the commonly held view of *E. coli* as an opportunistic pathogen. Furthermore, unlike bacteremic and enteropathogenic strains, the *E. coli* isolates examined exhibited a high number of different serotypes.

Burvenich et al. (2003) concluded, in a comprehensive review of physiological studies from the previous 10 yr, that severity of *E. coli* mastitis is mainly determined by cow factors rather than bacterial pathogenicity. The results of the present study, which failed to identify an association between serotype, genotype, or virulence gene possession and clinical disease severity, are consistent with that conclusion.

CONCLUSIONS

Escherichia coli strains causing clinical coliform mastitis are opportunistic pathogens of diverse strain types. There appears to be no association between strain serotype, genotype, or the presence of specific virulence genes and clinical disease severity. We concluded that a high degree of genotypic variability is characteristic of *E. coli* strains causing clinical mastitis within and between different farms and clinical severity groups, and that specific cow factors probably play a more important role in determining clinical disease severity.

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