Susceptibility of *Escherichia coli* isolated from Intramammary Infections to Phagocytosis by Bovine Neutrophils

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**ABSTRACT**

Thirteen *Escherichia coli* isolated from naturally occurring IMI were tested for susceptibility to phagocytosis by bovine blood neutrophils. Isolates were opsonized in pooled serum collected from nine healthy lactating cows. Bacteria isolated from IMI first diagnosed within 3 d after calving were more resistant to phagocytosis than were isolates from IMI originating during either the first half of the dry period or later during lactation. Duration of the IMI was negatively correlated with both phagocytic index and percentage of neutrophils phagocytizing within bacterial isolates from IMI originating at calving and during lactation. Phagocytosis was independent of duration of IMI within isolates from IMI originating during the first half of the dry period. Susceptibility to in vitro phagocytosis by neutrophils was not related to O antigen serotype, encapsulation, or growth in dry cow secretion.

(Key words: *Escherichia coli*, phagocytosis)

**INTRODUCTION**

Rates of *Escherichia coli* IMI are not constant among the different stages of lactation. These differences in rate of IMI can be explained partially by changes in the ability of the gland to defend against bacterial growth. The lowest rate of *E. coli* IMI occurs during the first half of the dry period (14). The increased resistance of the involuted gland to *E. coli* IMI was related directly to high concentrations of the iron-binding protein lactoferrin in mammary secretion (12). The rate of *E. coli* IMI is highest at calving and during early lactation (14). Hill (5) reported that the major factor affecting the severity of *E. coli* mastitis during early lactation was the ability of the cow to mobilize neutrophils into the gland for phagocytosis and intracellular kill of bacteria.

*Escherichia coli* isolated from naturally occurring IMI differ greatly in the phenotypic expression of virulence factors, such as ability to grow in dry cow secretion (12), serum susceptibility (7), hemagglutination and hemolysis production (8), and presence of capsule (1). The proportion of *E. coli* isolated from bovine IMI that express various virulence factors may depend on the stage of lactation at which the IMI originated. For example, coliform bacteria isolated from IMI originating during the early dry period were better able to overcome the antibacterial activity of lactoferrin in dry cow secretion than were isolates from IMI originating during lactation (12). The ability of *E. coli* to evade phagocytosis by neutrophils and to cause mastitis during lactation appears to be related to cell surface O antigens and capsule formation (6). Data are limited on the relationship between the ability of *E. coli* to evade...
phagocytosis and the stage of lactation at which the IMI originated. The purpose of this study was to compare the in vitro phagocytosis of *E. coli* isolated from IMI originating during the early dry period, at calving, and during lactation.

**MATERIALS AND METHODS**

**Origin of Bacterial Isolates**

Thirteen *E. coli* isolates were tested. Bacteria were isolated from naturally occurring bovine IMI in the Ohio Agricultural Research and Development Center dairy herd. Five isolates were from IMI originating during the first 30 d of the dry period, 4 isolates were from IMI first diagnosed within 3 d after calving, and 4 isolates were from IMI first detected between lactation d 3 and drying off. Criteria for diagnosis and determination of origin and duration of IMI were previously described (14). Species were identified by the API 20E system (Analytab Products, Plainview, NY).

**Bacterial Cultures**

Isolates were stored on trypticase soy agar (BBL Microbiological Systems, Cockeysville, MD) slants at room temperature (26°C) prior to testing. Bacteria were subcultured on MacConkey agar (Difco Laboratories, Detroit, MI), and a portion of one colony was inoculated into 12 ml of trypticase soy broth (BBL Microbiological Systems) for incubation at 37°C for 18 h at 200 rpm on a gyratory shaker. A total of .1 ml of the 18-h culture was inoculated into 12 ml of fresh trypticase soy broth (BBL Microbiological Systems) for incubation at 37°C for 18 h at 200 rpm on a gyratory shaker. A total of .1 ml of the 18-h culture was inoculated into 12 ml of fresh trypticase soy broth (BBL Microbiological Systems) for incubation at 37°C for 18 h at 200 rpm on a gyratory shaker. A total of .1 ml of the 18-h culture was inoculated into 12 ml of fresh trypticase soy broth (BBL Microbiological Systems) for incubation at 37°C for 18 h at 200 rpm on a gyratory shaker. A total of .1 ml of the 18-h culture was inoculated into 12 ml of fresh trypticase soy broth (BBL Microbiological Systems) for incubation at 37°C for 18 h at 200 rpm on a gyratory shaker. A total of .1 ml of the 18-h culture was inoculated into 12 ml of fresh trypticase soy broth (BBL Microbiological Systems) for incubation at 37°C for 18 h at 200 rpm on a gyratory shaker. A total of .1 ml of the 18-h culture was inoculated into 12 ml of fresh trypticase soy broth (BBL Microbiological Systems) for incubation at 37°C for 18 h at 200 rpm on a gyratory shaker.

**Preparation of Blood Neutrophils**

Blood samples from four healthy midlactation cows were pooled, and neutrophils were isolated. Cows were bled according to a schedule whereby four cows from a group of six were bled each day that assays were conducted, and no cow was bled more than 4 consecutive d. Blood samples were collected as described by Carlson and Kaneko (2). The final cell preparations were washed twice in HBSS. Viable cells were determined by trypan blue exclusion and counted with a hemocytometer. A portion of each cell preparation was stained (Diff-Quik<sup>®</sup>; AHS del Caribe, Inc., Aguadilla, Puerto Rico) for differential counts. Pooled cell preparations averaged (X ± SD) 89.2 ± 8% neutrophils, 10.3 ± 8% monocytes, and 96.9% ± 1% viability. Cell concentrations were adjusted to 40 × 10<sup>6</sup> viable neutrophils/ml of HBSS.

**Neutrophil Assay**

Phagocytosis and intracellular kill of bacteria by neutrophils were measured by modifications of the fluorochrome assay described by Goldner et al. (4). Suspensions of neutrophils and opsonized bacteria were added to incubation tubes in a ratio of 1:3 (neutrophils: bacterial colony-forming units) and incubated at 100 rpm for 90 min. Bacterial numbers were confirmed by removing a portion of assay suspension prior to incubation, serially diluting bacteria, and plating bacteria on trypticase soy agar. Five isolates were assayed per day, and assays were in duplicate. Each isolate was assayed on 2 separate d during a 4-wk period. Assays were conducted blind; laboratory personnel had no prior knowledge of origin of IMI for isolates tested. Data were expressed as percentage of neutrophils phagocytizing, phagocytic index (live plus dead intracellular bacteria divided by phagocytizing neutrophil), and percentage of intracellular kill (dead intracellular bacteria divided by live plus dead intracellular bacteria).

**Growth in Dry Cow Secretion**

*Escherichia coli* isolates were tested earlier for growth in a pooled source of dry cow secretion (12). Briefly, dry cow secretions from nine cows at 21 d into the dry period...
were pooled and centrifuged at 48,000 × g for 60 min, the fat layer was removed, and supernatant was filter-sterilized (0.45 μm; Millipore Corp., Bedford, MA). In vitro growth consisted of duplicate cultures containing 250 μl of dry cow secretion inoculated with 10 μl of bacteria (10⁴ cfu). Cultures were incubated aerobically at 37°C for 18 h. Bacterial counts were determined by removing a portion of culture, serially diluting bacteria, and plating bacteria on MacConkey agar (12).

**O Antigen Grouping**

Presence of O antigens on each isolate was determined by The Pennsylvania State University *Escherichia coli* Reference Center (University Park, PA). Isolates were cultured on veal infusion yeast extract agar for 18 h at 37°C. Isolates were then suspended in 0.06% phenol saline and heated at 100°C for 2 h. Bacteria were tested for O antigens by an agglutination system (3, 15).

**Capsule Staining**

Cellular capsules were detected by the India ink (Faber-Castell Co., Newark, NJ) staining procedure. Bacteria were cultivated overnight in trypticase soy broth supplemented with 6% dextrose and 10% homologous serum. Ten microliters of culture and 10 μl of India ink were mixed, smeared on a microscope slide, and air dried. Bacteria were counterstained with gentian violet (Harleco Stains, Gibbstown, NJ). Isolates with a clear zone of greater than 2 μm were considered to be encapsulated.

**Statistical Analysis**

Differences among bacteria in neutrophil assays were tested by least squares means analysis of covariance (10). Ratio of neutrophil:bacteria in each neutrophil assay was a significant (P < .05) effect on phagocytic index and was included as a covariate to adjust for neutrophil assay variability unrelated to treatment. The correlations among growth in dry cow secretion, duration of IMI from which bacteria were isolated, and phagocytic parameters were quantified using linear regression (11).

**RESULTS**

Bacteria isolated from IMI present at calving had lower phagocytic index (P < .05; Figure 1) than did isolates from IMI occurring either during the dry period or lactation. Although the mean percentage of neutrophils phagocytizing isolates from IMI at calving was approximately 20% lower than that for isolates from IMI at lactation and during the dry period, differences among stage of lactation groups were not significant (P > .05; Figure 2). Intracellular kill also did not differ among stage of lactation groups (P > .05; Figure 3). Duration of IMI from which isolates were obtained and growth of isolates in dry cow secretion are in Table 1. Two phagocytic parameters were negatively correlated (P < .05) with duration of IMI for isolates from IMI at calving and lactation. Correlation coefficients for the relationships between duration of IMI with phagocytic index and percentage of neutrophils phagocytizing isolates were −.66 and −.61, respectively. Duration of IMI was not related to phagocytosis within isolates from IMI during the dry period. Relationships among phagocytic parameters and growth of bacteria in dry cow secretion were not significant (P > .05). Twelve of the *E. coli* isolates were grouped into nine O antigen types (Table 2). The one isolate that was not typeable was also the only encapsulated isolate. Encapsulation and O antigen types were not related to phagocytic measures.
**DISCUSSION**

*Escherichia coli* isolated from IMI at calving were more resistant to phagocytosis than isolates from IMI occurring during lactation or the dry period. The difference among isolates was due to both a reduced number of bacteria per phagocytizing neutrophil and a trend for a lower percentage of neutrophils phagocytizing isolates from IMI at calving. These findings contrast those of Hill et al. (6), in which each of the *E. coli* isolates tested were phagocytosed and killed by bovine neutrophils in presence of 10% pooled bovine serum. Phagocytosis of isolates in the previous study (6) differed when the amount of serum used for opsonization was diluted to less than 10% and differences were attributed to bacterial cell surface components. The contrasts between the current trial and the previous trial (6) were possibly due to differences in opsonin concentrations in sera and assay procedures to measure phagocytosis. Nevertheless, results of both trials revealed differences in susceptibility of *E. coli* isolated from IMI to in vitro phagocytosis.

Isolates from IMI at calving were more likely to evade engulfment than were isolates from IMI originating during either the dry period or lactation. The resistance of *E. coli* strains isolated from bovine IMI to phagocytosis was attributed to specific O antigen serotypes and encapsulation (6). In the current study, nine different serotypes were represented by the isolates with little overlap of serotype among origin of IMI. Evidence did not suggest that phagocytic measurements and O antigen serotype were related. Only one (7.7%) isolate was encapsulated, which is

**TABLE 1.** Mean duration of IMI and growth in dry cow secretion for *Escherichia coli* isolated from IMI originating at calving (C), during lactation (L), and during the first half of the dry period (DP).

<table>
<thead>
<tr>
<th>Origin of IMI</th>
<th>Variable</th>
<th>C (n = 4)</th>
<th>L (n = 4)</th>
<th>DP (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Duration of IMI¹</td>
<td>X SE</td>
<td>X SE</td>
<td>X SE</td>
</tr>
<tr>
<td></td>
<td>Growth in dry cow secretion²</td>
<td>1.27 .28</td>
<td>52 .35</td>
<td>1.56 .41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.40 .70</td>
<td>3.15 1.25</td>
<td>4.40 2.01</td>
</tr>
</tbody>
</table>

¹Duration of IMI expressed as days $\log_{10}$.

²Growth in dry cow secretion expressed as colony-forming units $\log_{10}$ per milliliter.
TABLE 2. Distribution of O antigen serotypes by origin of IMI for *Escherichia coli* isolated from IMI originating at calving (C), during lactation (L), and during the first half of the dry period (DP).

<table>
<thead>
<tr>
<th>O Antigen group</th>
<th>Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>(no.)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>169</td>
</tr>
<tr>
<td>9</td>
<td>727</td>
</tr>
<tr>
<td>16</td>
<td>767</td>
</tr>
<tr>
<td>17</td>
<td>584</td>
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<tr>
<td>18</td>
<td>778</td>
</tr>
<tr>
<td>74</td>
<td>636</td>
</tr>
<tr>
<td>460</td>
<td>446</td>
</tr>
<tr>
<td>471</td>
<td>460</td>
</tr>
<tr>
<td>80</td>
<td>414</td>
</tr>
<tr>
<td>91</td>
<td>28</td>
</tr>
<tr>
<td>101</td>
<td>83</td>
</tr>
<tr>
<td>NT1</td>
<td>470</td>
</tr>
</tbody>
</table>

1 NT = Not typeable.

Comparable with the 2% encapsulated *E. coli* reported earlier (1). Interestingly, the single encapsulated isolate in the present study was not O antigen typeable. Encapsulation was probably not related to the inability to serotype this strain because the bacteria were heated for an additional 1 h at 120°C to expose O antigens masked by heat-resistant capsular antigen (3, 15). Barrow and Hill (1) reported that 75% of *E. coli* isolated from bovine IMI had clear zones (less than 2 μm) surrounding the cells. A population of isolates with such a narrow zone of capsule was not evident in the current study. Although the encapsulated isolate was resistant to phagocytosis, several of the unencapsulated isolates were equally resistant. Apparently, bacterial factors other than O antigen serotype or capsule may allow certain isolates to evade phagocytosis. These factors may be associated more frequently with isolates that cause IMI at calving than those causing IMI during the first half of the dry period or lactation.

The concentration and relative importance of different mammary gland antibacterial systems change with lactational status. The primary factors that determine the severity of *E. coli* mastitis during lactation are the speed at which neutrophils are mobilized into the gland and the opsonic capacity of the milk (5). Duration of IMI originating at calving and IMI originating during lactation from which isolates were obtained was correlated with the ability of isolates to avoid phagocytosis in the current study. Bacteria that were more resistant to in vitro phagocytosis were isolated from IMI of longer duration during lactation. These correlations suggest that factors enabling isolates to evade in vitro phagocytosis are also associated with evading host defenses in vivo during lactation. Another example of in vitro measurements correlating with in vivo IMI parameters was the growth of isolates obtained from IMI originating during the dry period in a standard dry cow secretion. The increased resistance of the involuted gland to *E. coli* IMI was related directly to the high concentration of the iron-binding protein lactoferrin (12). Isolates from dry period IMI overcame the antibacterial properties of lactoferrin more effectively than did isolates from IMI at calving or lactation (12, 13). In contrast, the correlation was not significant between phagocytosis and duration of IMI within isolates from IMI originating during the dry period. *Escherichia coli* isolates may realize little advantage in establishing IMI by evading phagocytosis during the dry period, because phagocytic function diminishes as involution advances (9). Results of the current and previous trials support the theory that the proportion of *E. coli* isolated from bovine IMI that express various virulence factors depend on the stage of lactation in which the IMI originated.

REFERENCES

PHAGOCYTOSIS OF ESCHERICHIA COLI


