

# Experimental Intramammary Infection with a Strain of *Escherichia coli* Isolated from a Cow with Persistent *E. coli* Mastitis

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## ABSTRACT

Transient *E. coli* intramammary infections (IMI) are usually associated with rapid onset of clinical signs including mammary gland swelling and abnormal milk with rapid clearance of bacteria from milk. Conversely, reports have described strains of *E. coli* showing very different clinical trends. Persistent *E. coli* IMI are associated with mild clinical symptoms that disappear shortly after the onset of infection, possibly flaring-up intermittently during lactation. In the present study, we evaluated a strain of *E. coli* isolated from a cow with persistent mastitis to determine if the experimental infection model mimics naturally occurring persistent *E. coli* IMI. Uninfected mammary quarters of 7 Holstein heifers were infused within 10 days of calving with 50 colony-forming units of a persistent *E. coli* strain. Six of 7 heifers developed mild clinical mastitis with elevated rectal temperatures within 9 to 36 h after infusion. The challenge strain was isolated intermittently in milk from all infected mammary quarters during the first two weeks after infusion and 3 animals continued to shed *E. coli* periodically during the sampling period. One animal shed *E. coli* intermittently in milk for 172 d after challenge and developed clinical mastitis four times during this period. The isolated strain had an identical pulsed-field gel electrophoresis profile as the *E. coli* strain used to infuse mammary glands. The experimental IMI model described here mimics very closely naturally occurring persistent *E. coli* IMI, thus providing an excellent *in vivo* model to better understand pathogenesis and to facilitate development of control strategies for this important mastitis pathogen.

**Keywords:** *Escherichia coli*; Intramammary Experimental Infection; Persistent Mastitis; Dairy Cows

## 1. Introduction

Current mastitis control programs devised in the 1960's are based on hygiene including teat disinfection, antibiotic therapy, and culling of persistently infected cows. Acceptance and application of these measures has led to considerable progress in controlling contagious mastitis pathogens. However, these same procedures are less effective against environmental pathogens such as *E. coli* because of the low susceptibility of *E. coli* to common mastitis treatments [1], lack of efficacy of teat disinfection for the prevention of new *E. coli* intramammary infections (IMI), and low efficacy of vaccination programs [2]. Therefore, it is not surprising that *E. coli* mastitis has become a major problem in many well-managed dairy farms that have successfully controlled contagious patho-

gens.

Transient *E. coli* mastitis is associated with rapid onset of clinical symptoms including mammary gland swelling and abnormal milk with rapid elimination of bacteria from milk. In some cases, severe systemic involvement occurs and when not properly diagnosed and treated, it could result in the death of the affected animal. Reports have also described strains of *E. coli* showing very different clinical trends. For example, there are reports on *E. coli* strains associated with persistent IMI which often times start with mild clinical symptoms that disappear soon after the onset of infection only to flare-up again during lactation, usually resulting in mild clinical mastitis [3-5]. *Escherichia coli* associated with persistent mastitis have been isolated in milk for long periods in spite of a high number of somatic cells in milk [3]. Published data suggests that adhesion to and internalization into

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mammary epithelial cells and subsequent intracellular survival might be important virulence attributes of strains of *E. coli* associated with persistent mastitis [3,5]. It was shown that internalization of persistent *E. coli* strains occurred by an endocytic mechanism that avoids bacterial uptake into acidified lysosomal compartments allowing bacteria to remain in the endosome evading host immune responses [6]. Studies conducted in our lab showed that transient *E. coli* strains internalize into bovine mammary epithelial cells preferentially exploiting receptor-mediated endocytosis (clathrin mediated), whereas persistent strains of *E. coli* appear to exploit caveolae-mediated endocytosis (CME). Exploitation of CME would allow persistent strains to circumvent host cell intracellular bactericidal/bacteriostatic mechanisms such as endosome acidification and endosome-lysosome fusion, which is consistent with characteristics of persistent IMI that they cause under natural conditions [7]. Experimental infection models have been an important tool for studying the pathogenesis of infections, management of infectious diseases, and development and evaluation of therapeutic and prevention strategies for disease control. In the present study, we evaluated a strain of *E. coli* isolated from a cow with persistent mastitis to determine if experimental intramammary infection mimics naturally occurring persistent *E. coli* mastitis in dairy cows.

## 2. Materials and Methods

### 2.1. Bacterial Strain and Growth Conditions

The *E. coli* strain ECC-1470 [3,7] isolated originally in milk from of a cow with persistent mastitis was used. The challenge inoculum was a frozen stock that was thawed, grown in Luria Bertani broth (Becton Dickinson Company, Sparks, MD, USA) overnight at 37°C and diluted serially to a concentration of approximately 50 colony-forming units (CFU) in 5 ml of sterile phosphate buffered saline (PBS, pH 7.4).

### 2.2. Experimental Animals

Seven healthy Holstein heifers free of mastitis and any other infectious disease were used. For the experimental infection protocol, animals were grouped together, milked last and included in normal herd practices.

### 2.3. Experimental Infection Protocol

One uninfected mammary gland of 7 Holstein heifers was infused with *E. coli* strain ECC-1470. Following disinfection of the teat end with ethanol, a bacterial suspension with a concentration of approximately 50 CFU in 5 ml of sterile PBS was infused into the teat cistern using sterile disposable syringes fitted with sterile dis-

posable teat canulas. Heifers were challenged within 10 days of calving; one heifer was challenged 2 d before calving, one at calving, two at 1 d after calving, one at 2 d after calving, one at 6 d after calving, and one at 8 d after calving. Post-calving infusions with the bacterial challenge suspension were administered within 20 min after milking. The protocol used for experimental infection of heifers was reviewed and approved by The University of Tennessee Institutional Animal Care and Use Committee.

### 2.4. Animal Inspection and Clinical Evaluation

Heifers and mammary glands were monitored extensively during the first week after experimental challenge. Animal observations including appetite, restlessness, mobility, and responsiveness were evaluated. Rectal temperatures were taken using a digital thermometer and recorded. Milk samples for microbiological evaluation and enumeration of somatic cell counts (SCC) were obtained twice daily for one week after challenge and twice weekly thereafter for a minimum of 56 days, or longer if *E. coli* was still isolated from milk. Pulsed-field gel electrophoresis (PFGE) patterns of *E. coli* isolated at intervals following challenge were evaluated. Milk and mammary condition were evaluated using the following scoring system: **Milk Score:** 1 = normal milk, 2 = flakes, 3 = small slugs, 4 = large slugs/clots, 5 = stringy/watery. **Mammary Gland Score:** 1 = normal; the udder was pliable when totally milked out. Heat, pain, redness, and/or swelling were not detectable; the animal exhibited no signs of discomfort; 2 = slight swelling; the udder was less pliable with some firmness as if not totally milked out. Additional milking or stripping did not return the gland to normal. Redness, heat and pain were generally not detectable and animals generally did not exhibit signs of discomfort; 3 = moderate swelling; the udder was definitely firm, reddened and warm to the touch. The udder did not return to normal size when milked out. The animal generally exhibited signs of discomfort (irritable, performs a stepping motion with feet and/or kicks) during prepping and milking procedures; 4 = severe swelling; the udder was very hard, red, hot and noticeably larger than other mammary quarters before milking with little or no change in size following milking. The animal was extremely uncomfortable and very irritable.

### 2.5. Milk Sample Collection and Processing for Bacteriological Evaluation

Milk samples were collected aseptically from challenged mammary glands for bacteriological evaluation and SCC every 3 h during the first 12 h (CH + 12 h), every 6 h from CH + 12 h to CH + 24 h, and every 12 h until CH + 168 h. At each sampling point, teats were washed thoroughly, dried with individual disposable paper towels, and teat

ends were cleaned with swabs containing 70% isopropyl alcohol. Milk samples for microbiological analysis were collected into sterile screw-cap tubes and kept at  $-20^{\circ}\text{C}$  until evaluation. Samples for SCC were collected into snap cap 50 ml tubes containing milk preservative pills and kept at  $4^{\circ}\text{C}$  until processing. Bacteriological evaluation of milk samples was conducted at the Tennessee Milk Quality Laboratory. Samples (10 and 100  $\mu\text{l}$ ) were plated onto blood and MacConkey agar, and one ml was placed on a Petrifilm Coliform Count Plate (3M Company, St. Paul, MN, USA). Somatic cell counts were conducted at the Tennessee Dairy Herd Improvement Association laboratory following standard procedures.

## 2.6. Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) patterns of *E. coli* isolated at intervals following challenge were analyzed as described [8]. Genomic DNA was digested with *Xba*I (Sigma Chemical Co. St. Louis, MO, USA) followed by PFGE. Gel DNA band patterns were analyzed using Molecular Analyst software version 1.6 (Bio-Rad Laboratories, Hercules, CA, USA) to determine strain relatedness. Band position tolerance of 2.5% was used for comparison of DNA patterns.

## 3. Results

### 3.1. Clinical Findings

Six of 7 heifers became infected following intramammary challenge. These heifers developed mild cases of clinical mastitis characterized by abnormal milk for a few days after challenge and slight to moderate swelling of the challenged mammary gland for up to a week after challenge. Initial clinical manifestations as evidenced by the presence of flakes and small slugs in milk and slight swelling and redness of the infused mammary gland were noticed at 18 h after inoculation reaching a peak at approximately CH + 72 h. At this inspection point, inoculated mammary glands showed swelling, firmness, redness, and were warm to the touch. Milk from inoculated mammary glands had moderate to large slugs. After 72 h, clinical symptoms declined, becoming moderate with slight swelling of the infused mammary gland and presence of small slugs in milk. Elevated rectal temperatures were detected approximately at CH + 9 h, reaching a peak approximately at CH + 12 h and declining to normal values 96 h after challenge (Figure 1).

### 3.2. Microbiological Findings

*Escherichia coli* were isolated in milk from all infected mammary glands frequently during the first week after challenge (Figure 2). Three animals continued to shed *E. coli* intermittently during the two-month sampling period.

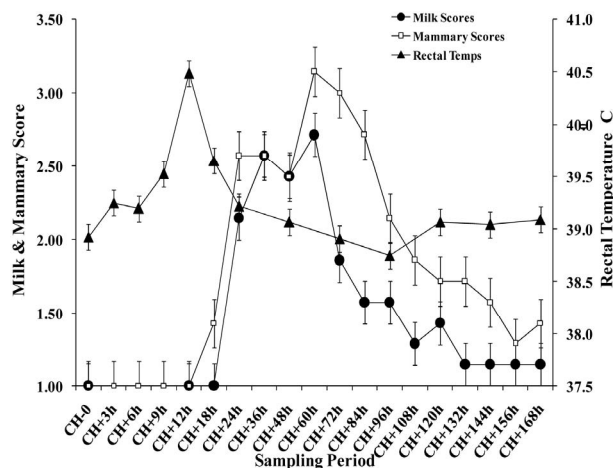


Figure 1. Milk scores, mammary scores, and rectal temperatures of heifers following intramammary challenge (CH) with a strain of *E. coli* (ECC-1470) isolated from a cow with persistent mastitis.

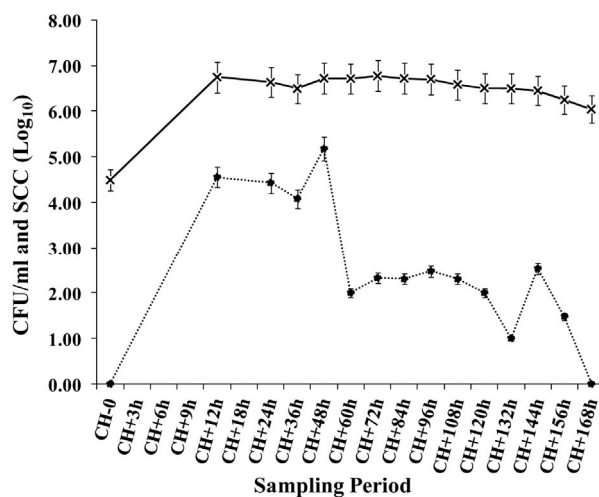


Figure 2. Milk somatic cell counts (\*;  $\log_{10}$  CFU/ml) and colony forming units per ml (X;  $\log_{10}$  SCC/ml) following intramammary challenge (CH) with a strain of *E. coli* (ECC-1470) isolated from a cow with persistent mastitis.

One animal shed *E. coli* in milk intermittently for 172 d after challenge (Figure 3), and developed clinical mastitis 4 times from which the challenge strain was isolated, as evaluated by PFGE typing (Figure 4). From all the infected animals (positive isolation of the challenge strain in milk), two animals cleared the infection within one week while the remaining animals cleared *E. coli* from milk between 11 and 172 days after infusion. For all infected animals, the range of the infection was 168 days, with a median of 40 days.

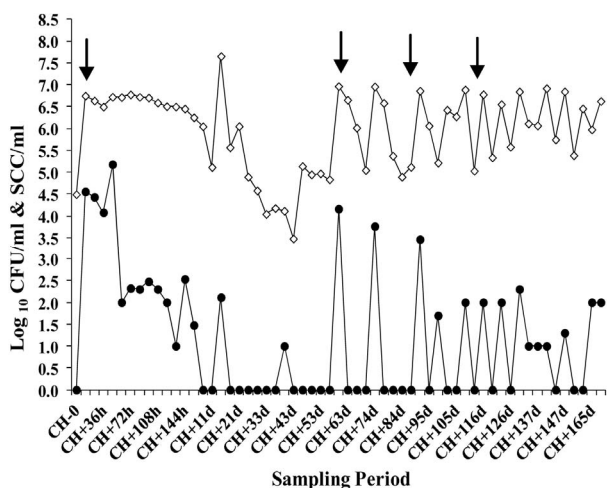
### 3.3. Somatic Cell Counts

In milk from all challenged mammary glands, SCC increased very rapidly during the first 9 h after inoculation

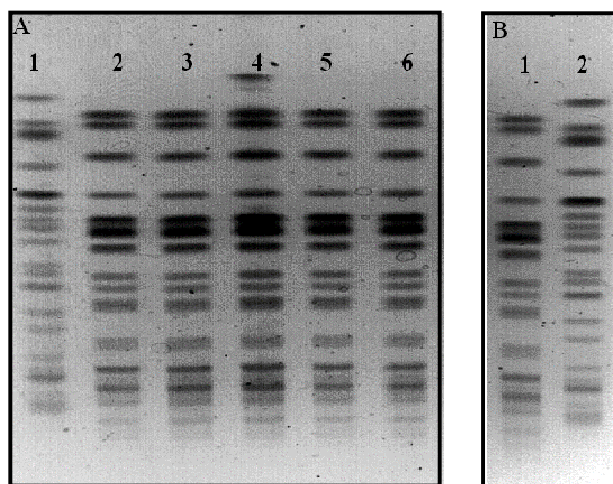
reaching values of  $\sim 7.0 \log_{10}$  at CH + 12 h. These SCC values were maintained throughout the entire sampling period and appeared to be unaffected by fluctuations in CFU/ml of *E. coli* in milk (Figures 2 and 3).

#### 4. Discussion

Several studies have shown the increasing importance of *E. coli* IMI and indicated that these IMI have become a major problem in many well-managed dairy farms since



**Figure 3.** Milk somatic cell counts per ml ( $\diamond$ ;  $\log_{10}$  SCC/ml) and colony forming units per ml ( $\bullet$ ;  $\log_{10}$  CFU/ml) of cow 7489 for 172 d following intramammary challenge (CH) with a strain of *E. coli* (ECC-1470) isolated from a cow with persistent mastitis. Clinical mastitis (arrows) was observed at 1, 60, 91 and 116 days after challenge.



**Figure 4.** PFGE patterns of *Escherichia coli* isolated from cow 7489. Panel A; lane 1: PFGE control G5244, lane 2: *E. coli* strain isolated from IMI at CH + 56 - 63, lane 3: from IMI at CH + 91, from IMI at CH + 116, and from milk of the infused mammary gland at CH+172. Panel B; lane 1: challenge strain (*E. coli* ECC-1470), lane 2: PFGE control G5244.

they cause significant milk loss and frequent culling and/or death of infected cows [9-12].

A study from Wisconsin reported an increased prevalence of *E. coli* from 17.7% to 24.9% of the total mastitis pathogens isolated [13].

In the United Kingdom, *E. coli* was also shown to be the most important pathogen in well-managed dairies [14]. Collectively, these studies demonstrate that *E. coli* is an increasingly important cause of mastitis in dairy cows.

Typically, *E. coli* mastitis is associated with acute onset of clinical signs including mammary gland swelling and abnormal milk with rapid elimination of bacteria from milk. In some cases, severe systemic involvement occurs and when not properly diagnosed and treated, it could result in death of the animal. However, an increasing number of reports have described strains of *E. coli* showing chronic and persistent IMI [3-5]. These persistent IMI usually start with mild clinical symptoms that disappear soon after the onset of infection only to flare-up again during lactation, generally resulting in mild clinical mastitis.

In this investigation using a strain of *E. coli* isolated from a cow with persistent mastitis, we were able to induce IMI with positive isolation of the challenge strain of *E. coli* in milk and clinical manifestations resembling persistent mastitis from which the strain was isolated initially [3,4]. Results from this investigation also showed that the strain used for infusing mammary glands was repeatedly isolated during the sampling period and into lactation. One animal shed *E. coli* in milk intermittently for 172 d after challenge (Figures 3 and 4) and developed clinical mastitis four times (Figure 3) which was caused by the challenge strain as evaluated by PFGE typing (Figure 4).

It is well accepted that clinical manifestations of any infectious disease result from the interplay between host defenses and virulence of the infectious agent. Results of the present study showed that experimental infection of heifer mammary glands during the periparturient period with a strain of *E. coli* isolated originally from a cow with persistent IMI caused mild clinical mastitis initially. In some animals, the challenge strain persisted for long periods of time in spite of a high number of somatic cells in milk similar to what was observed in cows with naturally occurring persistent *E. coli* mastitis [3,4]. Thus, based on experimental and natural infection data, *E. coli* strains associated with persistent IMI appear to behave quite differently from strains of *E. coli* that cause transient acute mastitis. Acute *E. coli* mastitis is usually associated with rapid onset of clinical signs of mastitis including mammary gland swelling and abnormal milk with rapid clearance of bacteria from milk. Persistent *E. coli* mastitis, on the other hand, results in mild clinical

mastitis and the strain persists in milk for very long periods in spite of a high number of somatic cells in milk. Previous results showed that persistent *E. coli* strains possess enhanced adherence and internalization capabilities as compared with acute *E. coli* strains [3,4]. Furthermore, work from our lab and other laboratories demonstrated that persistent strains of *E. coli* were capable of circumventing intracellular bactericidal mechanisms such as endosome acidification and endosome-lysosome fusion thus allowing intracellular persistence of these strains [6,7]. Such avoidance mechanisms are very likely the cellular basis to explain persistent IMI caused by these *E. coli* strains. From a practical standpoint, these results indicate that *E. coli* strains with these characteristics are able to persist for long periods in the affected mammary glands thus creating reservoirs of persistent *E. coli* strains in the herd. Such reservoirs are very difficult to control since these strains are resistant to host surveillance systems, and specialized in gaining access to microenvironments where host defenses or antimicrobials present in milk are not effective.

In conclusion, we have developed an experimental *E. coli* IMI model that mimics clinical symptoms observed in natural persistent *E. coli* mastitis. Strains of *E. coli* associated with persistent IMI appear to behave quite differently from strains of *E. coli* causing transient mastitis. The *in vivo* experimental infection model developed in the present study could be a useful and valuable tool to better understand pathogenesis and to facilitate development of control strategies for this important mastitis pathogen.

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