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Host–pathogen gene expression profiles during infection of primary bovine mammary epithelial cells with *Escherichia coli* strains associated with acute or persistent bovine mastitis

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ABSTRACT

Escherichia coli intramammary infection (IMI) is often acute with local and systemic clinical manifestations that clear within 7 days. However, if not diagnosed early and treated, *E. coli* IMI could result in generalized systemic reaction and death. Persistent *E. coli* IMI is characterized by mild clinical manifestations followed by acute episodes of clinical mastitis during lactation. Factors responsible for pathogenesis of *E. coli* IMI and variation in clinical manifestations are not known. There are studies indicating that the outcome of *E. coli* IMI is mainly determined by cow factors. However, recent research demonstrated that virulence attributes of *E. coli* strains have significant impact on the outcome of *E. coli* IMI. The aims of this study were; (a) to compare gene expression profiles of PBMEC cocultured with strains of *E. coli* associated with acute or persistent IMI and; (b) to identify genes of *E. coli* induced during bacterial interaction with PBMEC. Utilizing cDNA we analyzed gene expression patterns of PBMEC cocultured with strains of *E. coli* using non-treated PBMEC as negative control. We evaluated also expression patterns of virulence associated genes of *E. coli* after co-culture with PBMEC using qRT-PCR. Our results showed that infection by both strains induced increased expression of pro-inflammatory cytokines, chemokines and innate immune response and apoptosis related genes. Our qRT-PCR results showed significant up-regulation of *ler*, *eae*, *flic* and *iutA* genes mainly in the strains of *E. coli* associated with persistent IMI. The pathogenesis and clinical severity of *E. coli* IMI may be determined by combined effects of host–pathogen factors.

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1. Introduction

Mastitis is the most economically imposing disease facing dairy producers in the world costing an estimated $2 billion annually (National Mastitis Council, 1996) in the United States alone. Current mastitis control programs which are based on milking time hygiene, antibiotic therapy, and culling of persistently infected cows led to considerable progress in reducing contagious mastitis pathogens such as *Streptococcus agalactiae* and *Staphylococcus aureus* worldwide. However, these same procedures have had a marginal effect on environmental pathogens such as *Escherichia coli* because of their ability to survive in the environment of dairy cows. Thus, environmental mastitis pathogens have become a major problem in well-managed dairy farms that have reduced the prevalence of contagious mastitis pathogens (Hogan et al., 1989). Usually, *E. coli* infect the mammary glands during the dry period progressing to clinical disease during the periparturient period, The infection is usually cleared within 7 days (Todhunter et al., 1991) or in some cases if not diagnosed early and treated, *E. coli* IMI could result in generalized systemic reaction leading to the death of the infected animal (Hogan et al., 1989). Persistent *E. coli* IMI is characterized by mild clinical manifestations that lasts for about one week, followed by subsequent episodes of

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clinical mastitis during lactation (Buitenhuis et al., 2011; Dogan et al., 2006; Dopfer et al., 2001). Rates of new IMI caused by *E. coli* are greater during the two weeks after drying off and the two weeks prior to calving than during lactation (Smith et al., 1985). Several studies have shown an increase in the importance of *E. coli* mastitis among all mastitis pathogens. A study from Wisconsin reported an increased prevalence of *E. coli* IMI from 17.7% in 1994 to 24.9% in 2001 of the total mastitis pathogens isolated (Makovec and Ruegg, 2003). In the United Kingdom, *E. coli* was also shown to be the most important pathogen in well managed dairies (Green et al., 2005). The parity number, season of the year, and lactation status affect susceptibility to clinical mastitis caused by *E. coli* (Todhunter et al., 1991). Results from some studies during the last decade, indicated that the severity of clinical manifestations of *E. coli* mastitis is mainly determined by cow factors rather than by virulence factors of *E. coli* (Burvenich et al., 2003). On the other hand, recent studies demonstrated that virulence attributes of *E. coli* strains may explain the outcome of *E. coli* IMI (Almeida et al., 2011; Bradley and Green, 2001; Dogan et al., 2006; Dopfer et al., 1999, 2001; Gonen et al., 2007; Makovec and Ruegg, 2003; Passey et al., 2008). We hypothesize that the pathogenesis and outcome of *E. coli* IMI is mainly determined by the combined effects of host–pathogen factors. Therefore, in order to define the interplay of host cells and *E. coli* factors during the initial steps in the host-cell/pathogen interaction we compare gene expression profiles of PBMEC cocultured with strains associated with acute and persistent *E. coli* IMI by DNA microarray and identify genes of these two strains expressed in these interactions by qRT-PCR. The knowledge of these host–pathogen factors responsible for pathogenesis of *E. coli* IMI and severity of clinical manifestations may enable us to design effective control measures against bovine *E. coli* mastitis.

2. Materials and methods

2.1. Experimental protocol

The design used includes 3 experimental groups: cocultures of primary bovine mammary epithelial cells (PBMEC) with acute and persistent *E. coli* strains (group 1 and 2, respectively), and PBMEC cultured alone (group 3) used as a negative control. Each group was incubated for 60 min and experiments were conducted in duplicate and run three independent times. From each of these conditions, total RNA of PBMEC and *E. coli* strains were isolated and analyzed by cDNA microarrays using GeneChip® Bovine Genome Array from Affymetrix and by qRT-PCR, respectively.

2.2. Primary bovine mammary epithelial cells growth conditions

PBMEC were grown in T75 tissue culture flasks (Corning Inc. Corning, NY, USA) in cell growth media (CGM) as described (Calvinho and Oliver, 1998; Almeida et al., 1996). PBMEC were incubated at 37 °C in 5% CO_2/95% air until 90–100% of confluence was achieved.

2.3. Bacterial strains and growth conditions

Escherichia coli strains ECA-727 and ECC-1470 isolated originally from acute mastitis and persistent mastitis (Dogan et al., 2006), respectively, were grown on Luria Bertani agar (LBA, Becton Dickinson Company, Sparks, MD, USA) overnight, and subcultured to mid-log phase at 37 °C in Luria Bertani broth (LBB, Becton Dickinson Company). After incubation, the bacterial suspension was washed three times in PBS (pH 7.4) and resuspended in DMEM at the density of $\sim 10^7$ colony forming units/ml (CFU/ml). The criteria sued for the selection of these strain was the knowledge, based on previous publications, that these strains induced transient and persistent mastitis and they presented different adherence and internalization rates when co-cultured with BMEC (Dogan et al., 2006; Dopfer et al., 2001). In addition to that, these strains were used extensively in our lab for experimentally induced *E. coli* IMI (Oliver et al., 2010) and to conduct studies focused on adherence to, internalization into, and trafficking of these strains in bovine mammary epithelial cells (Almeida et al., 2011).

2.4. Coculture assays

Bovine primary mammary epithelial cell monolayers in T75 (75 cm^2) tissue culture flasks were co-cultured with *E. coli* strains at 37 °C in 5% CO_2/95% air at a multiplicity of infection (M.O.I.) of 10 or left untreated as negative control.

2.5. Total RNA isolation procedures

Total eukaryotic RNA was isolated from *E. coli* – PBMEC cocultures using Mag MAX™ 96 total RNA isolation kit; total RNA from *E. coli* strains was isolated from the same *E. coli* PBMEC coculture using RiboPure™-Bacterial RNA purification kit (Ambion Inc., Austin, TX, USA) following manufacturer's instructions. To purify total RNA from PBMEC and *E. coli* separately from the same co-culture, mixed total RNA was first purified by RiboPure™-Bacterial kit (Ambion). Then DNA was removed using turbo DNAse (Ambion) and total RNA of *E. coli* was further purified using MICROBEnrich™ kit (Ambion). The first purification step with RiboPure™ bacterial kit isolate total RNA of both PBMEC and *E. coli*. The second purification step with MICROBExpress™ kit (Ambion) removes total RNA of *E. coli* using oligo magnetic beads. In this second purification step total RNA of PBMEC remain bound to oligo magnetic beads through their poly A tails, whereas total RNA of *E. coli* was eluted with elution buffer. Finally, the total RNA of PBMEC was eluted from oligo magnetic beads using TE buffer (pH 8.0) at 95 °C. The purity and integrity of RNA samples after purification was checked by Experion RNA HighSens Analysis Kit (Bio-Rad).

2.6. cDNA microarrays

Microarrays were conducted following Affymetrix protocol. Briefly, 500 ng of total RNA samples were labeled using the Target Amp One-Round Biotin aRNA kit from Epicentre Biotechnologies. A total of 8 μg of resultant cRNA