J. Dairy Sci. 101:1–9
https://doi.org/10.3168/jds.2017-13159

The phylogenetic group, antimicrobial susceptibility, and virulence genes of *Escherichia coli* from clinical bovine mastitis

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ABSTRACT

Bovine mastitis is still a central problem on dairy farms despite control programs, and *Escherichia coli* is a crucial pathogen during the development of bovine mastitis. The virulence genes, antimicrobial susceptibility, and mortality of mice infected with different *E. coli* isolates from bovine mastitis were determined in this study. According to the presence of the specific genes *chuA*, *yjaA*, and *TspE4.C2*, these isolates mainly belonged to 2 different groups: group A (47/79) and group B1 (22/79). The *ompC* gene was detected in all the isolates, followed by *fimH* (89.9%), *ECs3703* (88.6%), and *ompF* (73.4%), whereas most of the virulence genes were not detected in these isolates. The results of the antimicrobial susceptibility tests indicated that the isolates were susceptible to the fluoroquinolones and aminoglycosides. An inverse relationship was shown between the expression level of *ompF* and antimicrobial resistance; additionally, the isolates that were nonsusceptible to at least 4 classes of antimicrobial agents showed a lower mortality to mice in comparison with the susceptible isolates. This study indicated that antibiotic resistance had emerged in *E. coli* from bovine mastitis in this area, and appropriate measures should be taken to avoid potential threats to humans and other animals.

Key words: *Escherichia coli*, bovine mastitis, antimicrobial susceptibility, virulence factor, *ompF* expression

INTRODUCTION

Mastitis in dairy cows is one of the most frequent diseases and causes economic loss worldwide due to treatment costs, loss of milk yields, lower milk value, and an increased risk of culling (Seegers et al., 2003; Huijps et al., 2008; Hogeveen et al., 2011). Several bacteria are involved in mastitis in dairy cows, with *Escherichia coli* as the most common pathogen isolated from mastitis in dairy cows. Bovine mastitis caused by *E. coli* is often characterized by a short-lived IMI and is usually spontaneously eliminated (Burvenich et al., 2003). However, IMI caused by *E. coli* can be severe, with clinical signs and significant tissue damage to the mammary gland (Wenz et al., 2001).

Although *E. coli* can be classified as pathogenic or nonpathogenic, within both of these groups there can be 4 phylogenetic groups, including groups A, B1, B2, and D, which can have different prevalences (Clermont et al., 2000, 2013). A previous study indicated that phylogroup B2 was predominant among the extra-intestinal strains (Johnson and Stell, 2000), but the majority of *E. coli* isolated from dairy cows with mastitis belonged to phylo-group A (Suojala et al., 2011). In addition, virulence genes are essential for the pathogenicity of bacteria, and various virulence genes have been found in *E. coli* from mastitis in dairy cows, including genes encoding hemagglutinin, aerobactin, P-fimbria, enterohemolysin, intimin, and autoagglutinating adhesion proteins (Bekal et al., 2003; Ewers et al., 2007; Johnson et al., 2008). This may explain why *E. coli* is able to colonize many different host tissues and causes various diseases in many animals. The phylogenetic grouping and distribution of virulence genes in *E. coli* from dairy cows with mastitis in Liaoning, China, still needed to be determined.

Antimicrobial agents are important in curing and preventing bovine clinical mastitis (Bengtsson et al., 2009). Unfortunately, *E. coli* has the potential to become nonsusceptible to almost all antimicrobial agents, and the wide use of antibiotics in curing bovine mastitis (Metzger and Hogan, 2013) and in feed additives in animals (Jost et al., 2003) has set a selective pressure driving the spread of resistance through conjugative plasmids or pathogenicity islands (Boerlin and White, 2006), with nonsusceptible *E. coli* isolates from mastitis

Received May 19, 2017.
Accepted August 21, 2017.
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in dairy cows being reported repeatedly. For example, reports indicated that 20 to 33% of isolates from bovine mastitis were shown to be nonsusceptible to at least one antimicrobial agent (Suojala et al., 2011; Fairbrother et al., 2015), whereas 87.1% of the 70 *E. coli* isolates from bovine mastitis was nonsusceptible to streptomycin, kanamycin, and ampicillin in Beijing, China (Liu et al., 2014). The downregulation of outer membrane proteins is considered to be involved in resistance to many kinds of antimicrobial agents, and the reduction in outer membrane proteins (OmpF and OmpC) leads to a decrease in the accumulation of antimicrobial agents in the cell, and hence a decrease in antibiotic susceptibility (Huguet et al., 2013).

The evolution of bacteria has been reshaped because susceptible pathogenic populations are decreasing, whereas nonsusceptible populations are increasing through the wide use of antibiotics. A fitness cost is often incurred in nonsusceptible populations, as the genetic burden is a disadvantage for bacteria to grow in an antibiotic-free environment (Beceiro et al., 2013). Previously reported data indicate that *E. coli* that is nonsusceptible to tigecycline showed significantly reduced virulence in a mouse model (Linkevicius et al., 2013); similarly, the nonsusceptible isolates were less competitive in comparison with the susceptible isolates when they were cultured together (Phan and Ferenci, 2013). Therefore, it is of high importance to evaluate the fitness cost of antibiotic resistance in *E. coli*.

The objectives of the present work were as follows: (1) to determine the phylogeny groups and the distribution of virulence genes, (2) to investigate antimicrobial susceptibility, (3) to measure the expression levels of *ompF*, and (4) to observe the mortality of mice infected with the *E. coli* isolates from dairy cows with mastitis in Liaoning, China.

MATERIALS AND METHODS

Sample Collection

The *E. coli* strains involved in this study were from 7 dairy farms, which were meant to represent the dairy farms located in Liaoning, China. Briefly, the herds were conveniently selected according to location, housing type, and bulk tank SCC, with SCC >250,000 deemed as a criterion that mastitis may be occurring in the cows (Pilla et al., 2013). Duplicate quarter milk samples were acquired from each cow with the symptoms of clinical mastitis, including swelling of the udder, tenderness to touch, fever, and depression, and were examined by veterinarians (Oliveira and Ruegg, 2014) between January 2013 and April 2013 using aseptic technique. Approximately 5 mL of milk was collected from each quarter of the udder by trained workers immediately after the teats were cleaned using towels and disinfected with 2% iodine tincture and 75% ethanol. The samples were frozen at the farm and sent to our laboratory within 6 h.

E. coli Isolates

Escherichia coli isolates were obtained as outlined according to previous reports with minor modifications (Suojala et al., 2011). Milk samples were examined microbiologically by plating 10 μL of sample on MacConkey agar plate (Aobox, Beijing, China), and the plate was incubated 24 h at 37°C. Isolates were also further identified by colony morphology and Gram stain, and identification was confirmed by the API 20E test (bioMerieux, Marcy l'Etoile, France).

Antimicrobial Susceptibility Tests

Susceptibility to antimicrobial agents was determined by the broth microdilution method as described by the Clinical Laboratory Standards Institute guidelines (CLSI, 2013). Mueller-Hinton broth [MH(B), Aobox] was selected as the medium for susceptibility testing. The concentration of bacteria was adjusted to approximately 5×10^5 to 10^6 cfu/mL. Trays were maintained in a normal atmosphere incubator for 24 h at 37°C, and *E. coli* ATCC 25922 was used as a reference strain.

The 21 antimicrobial agents tested in this study were as follows: tetracycline, doxycycline, oxytetracycline, ampicillin, amoxicillin, cefazolin, ceftiofur, streptomycin, gentamicin, amikacin, chloramphenicol, thiamphenicol, florfenicol, colistin, sulfamonomethoxine, sulfamethoxazole, enrofloxacin, ciprofloxacin, ofloxacin, maquindox, and azithromycin. The antimicrobial agents were purchased from the China Institute of Veterinary Drugs Control.

DNA Extraction

A single colony from a fresh bacterial culture from Muller-Hinton agar [MH(A), Aobox] was picked and inoculated into 3 mL of sterile MH(B) at 37°C for 24 h in an incubator. The DNA extraction was carried out by using a bacterial DNA extraction kit (Takara, Dalian, China) according to the manufacturer's protocol. All DNA preparations were stored at −20°C until subsequent use.

Phylogenetic Grouping

The *E. coli* isolates were phylogenetically grouped into the A, B1, B2, or D groups using the triplex PCR