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Phylogenetic group, virulence factors and antimicrobial resistance of *Escherichia coli* associated with bovine mastitis

Yongxia Liu[1], Gang Liu[1], Wenjun Liu, Yong Liu, Tariq Ali, Wei Chen, Jinhua Yin, Bo Han*

College of Veterinary Medicine, China Agricultural University, Yuan Ming Yuan West Road No. 2, Haidian District, Beijing 100193, PR China

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Abstract

Escherichia coli is an important pathogen involved in the etiology of bovine mastitis. A total of 70 *E. coli* isolates recovered from clinical and subclinical mastitis samples were characterized with respect to their phylogenic group, virulence factors and antimicrobial susceptibility. Based on the presence of the specific genes *chuA*, *yjaA* and *TspE4.C2*, these isolates were found to belong to three different groups: group A(25), group B1(41) and group D(4). Twenty-five (35.7%) isolates harbored at least one virulence gene, and the most prevalent virulence genes were *f17A*, *irp2*, *astA*, *iucD* and *colV*. The *irp2*-coding gene was more often detected in group A than in group B1 isolates; in contrast, *colV* was identified more often in group B1 isolates. The majority of isolates (87.1%) were resistant to at least one antimicrobial compound. Forty-seven isolates (67.1%) were resistant to streptomycin, and those from group B1 were more resistant to streptomycin than isolates from group A. The latter feature was supported by the distribution of streptomycin resistance genes observed in group B1 compared to group A.

Keywords: *Escherichia coli*; Phylogenetic group; Virulence factors; Antimicrobial resistance; Bovine mastitis

1. Introduction

The dairy cow is becoming a major economically important animal throughout the world, and *Escherichia coli* is one of the important pathogens associated with bovine mastitis. Mastitis caused by *E. coli* is normally associated with severe clinical signs [16]. *E. coli* were mainly isolated from cases with a high somatic cell count (SCC) in well managed dairy farms [6,11]. The organism is generally considered an environmental pathogen that enters the udder via the teat canal and causes tissue damage to the mammary glands [36].

E. coli can be classified into pathogenic and non-pathogenic groups; pathogenic strains cause a variety of diseases in different animals, and these bacteria could be further divided into different types based on their associated pathogenic mechanisms [4]. Based on the well recognized phylogenetic grouping protocol, *E. coli* can be categorized into groups A, B1, B2 and D depending on the presence of *chuA*, *yjaA* and *TspE4.C2* genes [7,8]. Most extra-intestinal strains belong to phylo-group B2 and, to a lesser extent, to group D, while commensals belong to group A [19]. However, the correlation between *E. coli* phylogeny and intramammary infection is not yet well established [5,10].

The pathogenicity of *E. coli* was based on their virulence factors, and their combinations are present in mastitis isolates [4]. Adherence to mammary epithelial cells is an important first step for *E. coli* invasion of the mammary gland. Adhesin factors, including F17-, P-, S-fimbriae, afimbrial adhesins and intimins all play an important role in bovine *E. coli* mastitis [18,26]. Beyond that, *E. coli* can also produce other virulence factors that could improve their iron uptake ability, a feature that would contribute to providing greater bacterial resistance to host immunological defenses [37]. In addition, virulence factors may be linked to phylogeny groups and antimicrobial resistance traits [15].

* Corresponding author. Tel.: +86 10 62733801; fax: +86 10 62737865.
E-mail address: hanbo@cau.edu.cn (B. Han).
[1] These authors contributed equally to this work.

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The antimicrobial resistance developed by these pathogens is one of the main reasons for low cure rates in mastitis [3,17]. Streptomycin is a broad-spectrum antibiotic that is widely used on dairy farms, and information about the presence of antimicrobial resistance genes in *E. coli* associated with mastitis is limited [28,30,31].

The aim of the present study was to better understand the etiology of *E. coli* isolates associated with mastitis. All *E. coli* isolates were characterized and compared in terms of their phylogenetic group, virulence factors and antimicrobial resistance.

2. Materials and methods

2.1. Collection of samples and isolation of E. coli

Six-hundred and sixty-three mastitis milk samples were collected from 6 major dairy farms located in Beijing and the surrounding area during the period from September 2012 to October 2013. Clinical mastitis was determined by visual inspection and presentation of mammary glands, along with changes in milk morphology, while subclinical mastitis was identified using the California Mastitis Test [34]. Following the latter protocol, the teat was disinfected with 70% ethanol and the three initial streams were removed, quarter milk samples were aseptically collected in sterile tubes, cooled with freezer packs and transported to the laboratory [16]. Milk samples (50 μl) were cultured on trypticase soya agar (TSA; Sigma, Shanghai, China) supplemented with 5% defibrinated sheep blood. Presumptive isolated *E. coli* colonies were picked and purified. Primary identification of the *E. coli* isolates was done based on colony morphology, Gram stain and growth on MacConkey agar and eosin methylene blue (EMB) agar (Sigma, Beijing, China) [5]. All suspected isolates were later confirmed as *E. coli* by sequencing of 16S rDNA [14]. Confirmed *E. coli* isolates were stored in Luria–Bertani (LB) broth (Invitrogen, Beijing, China) with 25% glycerol at −80 °C.

2.2. Preparation of DNA templates

Bacterial isolates were grown overnight on TSA at 37 °C; one isolated colony was picked and suspended in 100 μl sterile distilled water and lysed by boiling for 15 min, followed by freezing and subsequently centrifuged at 14,000 rpm for 15 min to pellet the cell debris. The supernatant was collected and used as a template for amplification reaction. The DNA concentration of the supernatants was measured using a Nanodrop ND-1000 spectrophotometer (Thermoscientific, Wilmington, DE) and adjusted to be approximately 100 ng/μl.

2.3. Determination of phylogenetic group

E. coli isolates could be classified into four phylogenetic groups using a multiplex polymerase chain reaction (PCR) assay [7]. Different isolates were assigned to the corresponding groups based on three genetic markers, namely *chuA*, *yjaA* and *TspE4.C2*. Multiplex PCR reaction volumes consisted of 0.4 pmol of each primer (BGI, Beijing, China), 10 μl of 2× PCR Master Mix (Qiagen), 2 μl of DNA template (100 ng/μl) and 6 μl DEPC water. The procedure of amplification was as follows: initial denaturation at 95 °C for 15 min; 30 cycles of 5 s denaturation at 95 °C, 10 s annealing at 59 °C, 30 s extension at 72 °C; and final extension for 5 min at 72 °C. Amplicons were separated by electrophoreses in 2% (wt/vol) agarose gel stained with ethidium bromide (0.5 μg/ml) and visualized under ultraviolet illuminator gel documentation system (Syngene, NIFSAT).

2.4. Detection of virulence factor genes

Virulence genes for study were chosen based on those previously identified in extra-intestinal pathogenic *E. coli* and enterohemorrhagic *E. coli* (EHEC) [33]. The virulence-associated genes assayed by PCR were: *f17A*, *papC*, *sfaD*, *saa*, *afa8E*, *eaeA*, *iss*, *cnf2*, *colV*, *vat*, *tsh*, *iucD*, *irp2*, *ehxA*, *astA*, *stx1* and *stx2* (Bertin et al., 1996; Jansen, 2001; Paton et al., 1998; Lalioui et al., 1999; Horne et al., 2000; Maurer et al., 1998; Yamamoto et al., 1996 [13,25,29,35]). As previously described, amplicons were analyzed by gel electrophoresis.

2.5. Detection of antimicrobial susceptibility and associated resistance genes

Seventy *E. coli* isolates were tested by the Kirby–Bauer disc diffusion method in Mueller–Hinton agar (Sigma, Shanghai, China) according to the guidelines of the Clinical and Laboratory Standards Institute [9]. Susceptibilities to ampicillin, cefalotin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfafurazole and tetracycline were tested; *E. coli* ATCC™25922 was used as quality control. To further characterize the resistance mechanisms associated with streptomycin, the following resistance genes were selected for study by PCR: *strA*, *strB*, *aadA*, *ant* (*3″*), *aph*(*3″*), *aph*(*6*)*-1d*, *aph*(*6*)*-1c*, *ant*(*6*) [31].

2.6. Statistical analysis

Chi-square tests were used to compare phylogenetic groups A and B1 for distribution of the virulence and antimicrobial resistance of the isolates. The statistical significance level was set at 0.05. All analyses were performed using Statgraphics version 5.1.

3. Results

3.1. Phylogenetic grouping of E. coli isolates

Seventy *E. coli* isolates were cultured from 663 mastitis samples. Among them, 41 (58.6%) belonged to phylo-group B1, 25 (35.7%) to group A and 4 (5.7%) to group D. None of the isolates was found to belong to group B2.