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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.

3.2. Virulence factors

Among the 70 isolates, 25 contained at least one virulence gene. The *f17A* gene was identified in 11 isolates (15.7%), followed by *irp2* (10, 14.3%), *astA* (7, 10%), *iucD* (5, 7.1%) and *colV* (4, 5.7%), whereas *papC*, *sfaD*, *saa*, *eaeA*, *iss*, *ehxA*, *stx1* and *stx2* were not detected. Distribution of the virulence genes among various *E. coli* isolates varied, and most of the genes were detected in different combinations. Nine isolates from phylo-group A (36.0%) were positive for 5 different virulence gene combinations, while 16 isolates from group B1 (39.0%) were positive for 7 types of virulence combination (Table 1). As to the main virulence genes, *irp2* was detected more often among isolates of phylo-group A. In contrast, the *colV* gene was detected more often among isolates of phylo-group B1, and genes *f17A*, *astA* and *iucD* did not show a significant difference between them (Table 2).

3.3. Antimicrobial susceptibility testing

The susceptibility of all 70 *E. coli* isolates to a panel of ten antimicrobial compounds was tested. Most of the isolates were resistant to streptomycin (32.9%), kanamycin (37.1%) and ampicillin (47.1%). In comparison, most of the collections were susceptible to chloramphenicol (91.3%), ciprofloxacin (81.4%) and sulfafurazole (80.0%). Furthermore, group B1 showed a higher level of antimicrobial resistance than group A (Table 3).

Among the *E. coli* isolates tested, 47 (67.1%) were resistant to streptomycin. Group B1 isolates were more resistant to streptomycin than those of group A ($P < 0.05$). Among streptomycin resistance genes, *strA* (26.8%) was the most common genotype identified, followed by *strB* (24.4%), *ant (3″)* (9.8%), *aph(3″)* (7.3%), *aadA* (4.9%), *aph(6)-1c* (4.9%), *aph(6)-1d* (2.4%) and *ant(6)* (2.4%) in isolates of group B1. Nineteen of the 32 (59.4%) resistant isolates in group B1 contained at least one resistance gene. However, in group A, the proportions were *strA* (20.0%), *strB* (16.0%), *ant (3″)* (8.0%), *aph(6)-1c* (8.0%) and *aph(3″)* (4.0%). Genes *aadA*, *aph(6)-1d* and *ant(6)* were not found in group A. Six of the 25 (46.2%) resistant isolates of group A had at least one resistance gene (Table 4).

Table 1
Distribution and combination patterns of virulence genes detected in *E. coli* isolates associated with mastitis.

Virulence genes combinations				Number of isolates in various phylo-groups		
				Group A	Group B1	Total
f17A	*irp2*			1	2	3
f17A	*irp2*	*tsh*		1	0	1
f17A	*iucD*	*cnf2*		1	0	1
f17A	*astA*			0	1	1
irp2	*iucD*	*colV*	*Afa8E*	0	1	1
irp2	*astA*			0	1	1
iucD	*colV*			0	3	3
f17A				0	5	5
irp2				4	0	4
astA				2	3	5
Total				9	16	25

Group D was not found in any selected virulence genes.

Table 2
Comparative prevalence of five main virulence genes in phylo-groups A and B1.

Virulence gene	% Positive isolates across the various phylo-groups		Statistics
	Group A ($n = 25$)	Group B1 ($n = 41$)	
f17A	12.0	19.5	NS
irp2	24.0	9.7	$P < 0.05$
astA	8.0	12.2	NS
iucD	4.0	9.7	NS
colV	–	9.7	$P < 0.05$

NS: The difference is not significant.

4. Discussion

In the modern dairy industry, *E. coli* has frequently emerged in cases of bovine mastitis, and this bacterium was considered to be an environmental commensal [7]. However, mastitis caused by *E. coli* was generally a severe type [2,22]. Virulence factors and antimicrobial resistance likely played an important role in the development of *E. coli* infection and survival in these bovine hosts, and the distinctions between the pathogenicities observed among the various strains may have been due to the combination of virulence factors they produced and the individual cow's response [5,6,23]. Phylogenetic distribution of *E. coli* populations could be categorized into groups A, B1, B2 and D; phylogenetic groups B2 and D were mainly found in extra-intestinal infections and invasive strains, whereas groups A and B1 were mostly associated with commensal and diarrheagenic strains [7]. In our study, *E. coli* isolates associated with mastitis mainly belonged to groups B1 (58.6%) and A (35.7%), with only 4 (5.7%) isolates identified as group D and none as group B2. More recently, similar reports regarding phylogenetic groups have been published in other countries [4,16,33] and those findings support the role of environmental *E. coli* in such cases of mastitis.

Table 3
Antimicrobial resistance detected in mastitis *E. coli* isolates of phylo-groups A and B1.

Antibiotics	% Resistant isolates	
	Phylo-group A ($n = 25$)	Phylo-group B1 ($n = 41$)
Ampicillin	56.0	48.8
Cefalotin	44.0	39.0
Chloramphenicol	4.0	7.3
Ciprofloxacin	8.0	26.8
Gentamicin	24.0	39.0
Kanamycin	56.0	65.9
Nalidixic acid	28.0	51.2
Streptomycin	52.0	78.0
Sulfafurazole	16.0	24.4
Tetracycline	48.0	46.3

Table 4
Distribution of streptomycin resistance genes in *E. coli* isolates of phylo-groups A and B1.

Antimicrobial resistance genes	Phylo-group A ($n = 25$)			Phylo-group B1 ($n = 41$)		
	Resistant ($n = 13$)	Susceptible ($n = 12$)	Total	Resistant ($n = 32$)	Susceptible ($n = 9$)	Total
strA	3	2	5	10	1	11
strB	3	1	4	10	–	10
aadA	–	–	–	2	–	2
ant (3″)	1	1	2	3	1	4
aph(3″)	1	–	1	3	–	3
aph(6)-1d	–	–	–	1	–	1
aph(6)-1c	2	–	2	2	–	2
ant(6)	–	–	–	1	–	1

Our study showed that significant differences existed in the distribution of two virulence genes (*irp2* and *colV*) comparing groups A and B1. The same genes that were identified in the two groups included *f17A*, *irp2*, *astA* and *iucD*, with *irp2* being detected more often in phylo-group B1 compared to *colV* in group A. In group A, one isolate carried the *tsh* gene and another carried a *cnf2* gene, whereas only one isolate in group B1 was positive for *afa8E*. It is not unreasonable to hypothesize that the severity of mastitis mediated by *E. coli* depends on the combination of virulence factors they contain, but thus far, no specific known virulence factors that can be attributed to the clinical severity of *E. coli* mastitis have been confirmed, and no precise relationship(s) has/have been reported between current virulence factors and pathogenicity [27,36].

In establishing an infection, the first step required by the bacterium is binding to the host cell, followed by colonization of the surface of the target organ(s). The binding mechanism may be initiated by an adhesin such as F17 fimbriae (coded by the *f17A* gene cluster), which had the highest prevalence rate (15.7%). Nonetheless other genes of this type may play a role and the afimbrial adhesin AFA8 (coded by *afa8E*) was present in only one isolate (1.4%), whereas P fimbriae, S fimbriae, autoagglutinating adhesion and intimin (coded by *papC*, *sfaD*, *saa* and *eaeA*, respectively) were not identified in the present study. These results are in accordance with other published data from Iran [16], but are not in agreement with studies reported from southern Finland, where the prevalence ratex of the *f17A* and *papC* genes were reported as 4.2 and 16.7%, respectively [33]. Overall, this suggests that these known adhesins played only a limited role in the pathogenesis of *E. coli* associated with mastitis.

The *colV* gene, identified on ColV plasmids, may carry virulence genes associated with iron uptake and serum resistance, and these genes, including *iucD*, *irp2* and *iss* improved the virulence and pathogenicity of *E. coli* [32]. In this study, the *colV* gene appeared together with *iucD* in four isolates (5.7%) and all of the strains belonged to group B1. Similarly, *irp2* was detected in ten isolates (14.3%) in combination with other virulence genes, and *iss* was not found. However, 11 and 4% of *E. coli* from bovine mastitis in other countries contained the genes for aerobactin [21].

Enteroaggregative heat-stable toxin (coded by *astA*) was also found to be widely distributed among diarrheagenic and non-pathogenic *E. coli* strains originating from humans and animals. In this study, 8 isolates (11.4%) were positive for *astA*, which was present alone or in combination with *irp2* and *f17A*. In mastitis, the precise role of the temperature-sensitive hemagglutinin (coded by *tsh*) is uncertain; however, temperature-sensitive hemagglutinin has considerable homology with a subclass of the IgA protease family and was considered to be a mediator of adherence in the avian respiratory tract [12]. Similarly, *cnf2* was also detected in only one isolate and the role of this toxin in the pathogenesis of *E. coli* requires further study [1].

The results of antimicrobial susceptibility revealed that most of the *E. coli* isolates had high levels of resistance to the tested antibiotics. Most of the tested *E. coli* isolates were resistant to streptomycin, kanamycin and ampicillin. This could be explained by long-term use of these antibiotics in dairy herds [33]. Moreover, these data showed that isolates of phylo-group B1 (78.0%) were more resistant to streptomycin than those of group A (52.0%). This can be clearly seen in the prevalence of the resistance genes of streptomycin in the isolates of both groups, being higher in group B1 than in group A. Streptomycin resistance genes *strA* and *strB* were found together in many streptomycin-resistant isolates, in accordance with an earlier study [24], Other streptomycin resistance genes, including *aadA*, *ant* (3″), *aph*(3″), *aph*(6)-*1d*, *aph*(6)-*1c* and *ant*(6), were identified in some of these isolates. However, the fact that *strA*, *strB* and *ant* (3″) were present in streptomycin-susceptible isolates could be explained by the existence of defective genes or reduced expression of these markers [20].

In summary, *E. coli* isolates associated with bovine mastitis mainly belong to phylogenetic groups B1 and A. Moreover, isolates of phylo-group B1 contained more virulence factors and exhibited a higher level of antimicrobial resistance than isolates of group A. Therefore, the role of *E. coli* associated with phylo-group B1 in cases of bovine mastitis should be carefully evaluated.

Conflict of interest

The authors declare that they have no competing interest.

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