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Antimicrobial Resistance and Virulence Characterization of *E. Coli* Isolated from Subclinical Mastitic Sheep and Goats

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A B S T R A C T

Mastitis is one of the most serious healthy and economic problems in all dairy sheep and goat flocks. Examination of 200 milk samples collected from apparently healthy sheep and goats using California mastitis test (CMT) showed that 94 (47%) were CMT positive. Standard methods for isolation and identification of *E. coli* could identify 19 strains. Five different serogroups were successfully identified among 19 *E. coli* strains, the serogroups were O1 (21.1%), O26 (21.1%), O114 (15.8%), O148 (15.8%) and finally O125 (10.4%), in addition to 3 untypable strains. The antimicrobial susceptibility results for erythromycin and cefoxtin revealed all E. *coli* isolates were resistant to erythromycin (100%) followed by high frequencies of resistance observed to cefoxtin 18 isolates (94.7%). Phylogenetic grouping of *E. coli* by triplex PCR using two genes (*chu*A and *yja*A) and an anonymous DNA fragment *tsp*E4C2 indicated that 5/6 were B2 group and 1/6 was A group. Detection of virulence genes of 6 *E. coli*. isolates showed that 2/6 harbored *eae*A gene and none of 6 isolates harbored *CFA/I* gene. While, the *mph* A erythromycin resistance gene were present in 4/6.but 2/6 isolates harbored *bla* CTX cefoxitin resistance gene.

Keywords: *E. coli*, Mastitis, virulence, Antibiotic resistance, genes.

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1. INTRODUCTION:

Mastitis is an inflammation of the mammary gland. Unlike the gut, normally the mammary gland has no resident bacteria so the introduction of any foreign material or bacteria leads to a rapid and marked inflammation (Bramley, 1992). The diagnosis of subclinical mastitis is based on the somatic cells count (SCC), California Mastitis

Test (CMT) and the bacteriological examination Gebrewahid *et al*., (2012).

For identification of *E. coli*, the following approaches are mainly adopted; isolation of microorganism by traditional methods; including its isolation on MacConkey's agar plates, then streaking on eosin methylene blue and examination of typical colonies of *E. coli* by biochemical tests as sugar fermentation and IMVC Surendraraj *et al*., (2010). Different identification systems and serological identification of the isolates performed.

Phylogenetic grouping of *E. coli* was done by a rapid and simple technique based on triplex PCR. This method used a combination of two genes (*chuA* and *yjaA*) and an anonymous DNA fragment *TSPE4.C2* had shown that *Escherichia coli* was composed of four main phylogenetic groups (A, B1, B2, and D) and that virulent extra-intestinal strains mainly belonged to groups B2 and D. Clermont *et al*. (2000).

Intimin was a protein encoded by *eae* gene, attaching and effacing gene, Ghanbarpour and Oswald (2010). It was proven that the *eae*A gene in *E. coli* plays a definite role in induction of cattle mastitis Barbour *et al.,* (2015).

The colonization factor antigen (CFA) expressed by enterotoxigenic *E. coli* (ETEC) were identified as two fimbrial antigens, (CFA/I and CFA/II), which enable *E. coli* for attachment. (Evans *et al*.,1978)

Several studies showed that antibiotic resistant *E. coli* is increasing in our days Suojala *et al*. (2011). Therefore, identification of resistance genes of bacteria seems to be so essential in reduction of treatment cost.

The development of bacterial virulence and dissemination of antibacterial resistance had become a topic of concern due to its direct influence on public health, through elevating the morbidity, mortality, and treatment costs of infectious diseases. In view of these considerations, the present study was undertaken for isolation and identification of *E. coli* to record the prevalence of *E. coli* infection in subclinical mastitic sheep and goats and serotyping of the obtained isolates then performing the antibiogram susceptibility test on the isolates for erythromycin and cefoxtin, characterize *E.coli* isolates referring to their phylogenetic groups, presence of some virulence associated genes including*(eae*A, *CFA I*) and antibiotic resistance genes (*mph* A, *bla* CTX)among some isolates .

2. MATERIAL AND METHODS:

2.1. Collection of samples

Two hundred milk samples collected from135 apparently healthy animals (63 sheep, 72 goats) under a septic condition obtained from different localities in Sharkia and Dakahlia Governorates. The udder and teats were carefully cleaned then swabbed with 70% alcohol and discarded of the first 3 jets of milk, then approximately 5-10 ml of milk were draw from each half into sterile screw-capped bottles.

Samples were submitted to California mastitis test (CMT) to indicate the frequency of subclinical mastitis. By mixing two ml of Schalm reagent with an equal volume of milk. The reaction was measured visually for 10 seconds according to formation of precipitate or gel formation. Then sending the positive CMT ones to the bacteriology laboratory for *E. coli* isolation.

2.2 Isolation and identification of the causative agents

Milk samples were incubated aerobically at 37°C for 24hr then centrifuged at 3000 r.p.m.

for 20 minutes. The cream and supernatant fluid were discarded and a loopful from milk sediment was streaked onto MacConkey's agar plates and incubated for 24 hours at 37ºC. Suspected lactose fermented colonies were picked up and streaked on Eosin methylene blue (EMB) agar plates then incubated for another 24-48 hours at 37ºC. The suspected purified colonies were picked up for examination by IMVC and TSI biochemical tests.

2.3. Serological typing of E. coli

Nineteen isolates that were preliminary identified biochemically as *E. coli*, taken randomly, were subjected to serological identification (Edward and Ewing, 1972) using slide agglutination test.

2.4. Antimicrobial susceptibility testing

Antibiotic susceptibilities of *E. coli* isolates were determined by the standard disk diffusion method *(*Finegold and Martin, 1982*), E. coli* isolates were tested against antimicrobial agents: cefoxtin (FOX) and erythromycin (E) according to (CLSI, 2015).

DNA extraction:

Six *E. coli* isolates were inoculated into brain heart infusion broth and incubated for 24 hours for genotypic identification. DNA was extracted according to QIA amp DNA mini kit instructions.

Phylogenetic analysis:

E. coli phylogenetic grouping was accomplished by a rapid and simple method as previously described (Clermont *et al*., 2000).

Both *chu* A and *Tsp*E4.C2 negative and positive *E. coli* strains were grouped into group A and B2, respectively, and the *chu* A-negative and *Tsp*E4.C2-positive, and the *chu* A positive and *yja* A-negative *E. coli* strains were grouped into B1 and D, respectively. The used primers in this analysis are listed in Table 1.

Virulence Genotyping:

Polymerase chain reaction (PCR) was used to amplify genes of interest after DNA extraction. PCR amplification was performed with a PTC-100 programmable thermalcycler in a final volume of 25 µl consisting of 12.5 µl of Dream *Taq* TM Green Master Mix (2X) (Fermentas, USA), 1µl of each primer (Sigma, USA), 7 µl of template DNA and nuclease-free water up to 25 µl. Amplified PCR products was electrophoresed on 1.5% agrose gel in tris acetate EDTA and visualized by UV transilluminator. The virulence genes (*eae* A, *CFA/I*) and antibiotic resistance genes (*Mph* A, *bla* CTX) were detected by PCR. The primer sets used for this procedure are listed in Table (2).

3. RESULTS:

3.1. The prevalence of subclinical mastitis among examined sheep and goats:

The obtained results revealed that out of 91 examined sheep milk samples, 39 showed California mastitis test positive with a percentage of (42.8%) (on samples level) and 55 goat samples with a percentage of (50.4%) were CMT (+) out of 109 samples. From 200 samples 61 were bacteriological positive while 19 isolates (5 from sheep and 14 from goats) were identified as *E. coli* with percent 9.5 % from Sharkia and Dakahlia Governorates, as shown in Table (3).

3.2. Serological typing of E. coli isolates:

The results of serological identification of 19 *E. coli* Isolates revealed that the serogroups were O1 (21.1%), O26 (21.1%), O114(15.8%), O148 (15.8%) and finally O125 (10.4%), while 3 isolates were untypable. Table (4).

3.3. Antimicrobial susceptibility testing:

The results revealed that all isolates showed absolute resistance to Erythromycin (100%) followed by high frequencies of resistance observed to Cefoxtin (94.7%).

3.4. Phylogenetic grouping by PCR:

Six *E. coli* strains were assigned to two different phylogenetic groups. The majority of examined strains fell into Group B2 (5/6) while one strain was belonging to group A (Fig.1).

3.5. Virulence genotyping:

The six *E. coli* isolates were examined for the presence of 2 virulence associated genes and two antibiotic resistance genes. The virulence profiles showed that only two isolates harbored *eae*A gave an amplification product sizes of 248 bp., while, no isolates harbored *CFA/ I* as shown in (Fig.2). The Erythromycin resistance gene *mph* A gave a characteristic band at 403 bp. were present in four strains and also, the band at 593 bp. for *bla*CTX gene for Cefoxitin resistance were found in 2 strains (Fig.3). Distribution of virulence and antibiotic resistance genes in 6 *E. coli* isolates and the relationship between *E. coli* isolates phylogenetic group and their relationship with isolates serogroups represented in Table (5).

Table (1): PCR primers used for phylogenetic grouping of *E. coli* isolates:

Gene	Primer sequence (5'-3')	Amplified product	Reference
ChuA	GAC GAA CCA ACG GTC AGG AT TGC CGC CAG TAC CAA AGA CA	279 bp	
*Yja*A	TGA AGT GTC AGG AGA YGC TG ATG RAG AAT GCG TTC CTC AAC	211 bp	Jeong *et al*., 2012
*tsp*E4C2	GAG TAA TGT CGG GGC ATT CA CGC GYC AAC AAA GTA TTR CG	152 bp	

Table (2): PCR primers used for genotypic identification of *E. coli* isolates

Gene	Primer Sequence (5'-3')	Amplified product	Reference
eae A	ATG CTT AGT GCT GGT TTA GG GCC TTC ATC ATT TCG CTT TC	248 bp	Bisi-Johnson *et al*., 2011
CFA/I	GCTCTGACCACAATGTTGA TTACACCGGATGCAGAATA	364 bp	Ghosal et al., 2007
Mph A	GTGAGGAGGAGCTTCGCGAG TGCCGCAGGACTCGGAGGTC	403bp	Nguyen et al., 2009
*bla*CTX	ATGTGCAGYACCAGTAARGTKATGC TGGGTRAAR TAR GTS ACC AGA AYC AGC GG	593bp	Archambault *et al*., 2006

Table (3): The prevalence of CMT positive samples and the incidence of *E. coli* isolated from examined sheep and goats milk samples.

animals	No.of samples	(+ve) CMT Samples		Bacteriological positive samples		Identified *E. coli*	
		No.	%	No.	%	No.	%
sheep	91	39	42.8	23	25.3	5	5.5
Goats	109	55	50.4	38	34.9	14	12.8
Total	200	94	47	61	30.5	19	9.5

Table (4): The incidence and frequency distribution of *E. coli* serogroups

E. coli Serogroup	No. of isolates	Percent
O1	4	21.1%
O26	4	21.1%
O114	3	15.8%
O148	3	15.8%
O125	2	10.4%
Untypable	3	15.8%

Table (5): Relationship between *E. coli* isolates serogroup, phylogenetic group and gene prevalence.

Sample origin	Bacterial isolates	serogroup	Phylogenetic group	PCR result (virulence genes *eae*, *CFA/I*)	Resistance phenotype	PCR result (resistance genes for E&FOX)
sheep	*E. coli*	O1	B2	-	FOX, E	*mph*A, *bla* ctx
goat	*E. coli*	O26	A	*Eae*	FOX, E	*mph*A
sheep	*E. coli*	O26	B2	*Eae*	FOX, E	-
goat	*E. coli*	O114	B2	-	FOX, E	*mph*A, *bla* ctx

goat	*E. coli*	O125	B2	-	FOX, E	-
sheep	*E. coli*	O148	B2	-	FOX, E	*mph*A

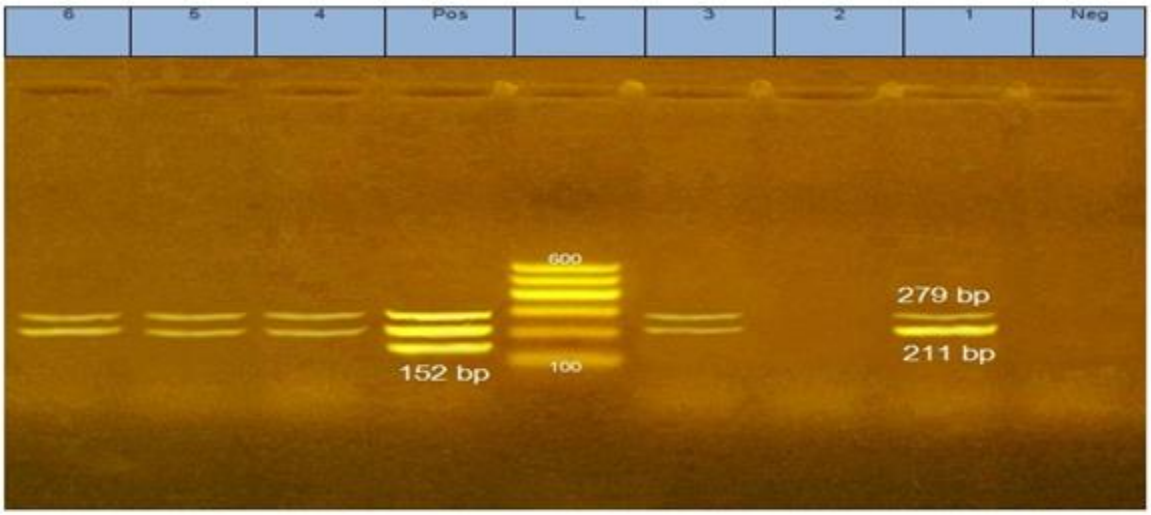

Fig. (1): Agarose gel electrophoresis of triplex PCR-for amplification products of. *chuA and yjaA* genes and an anonymous DNA fragment *TSPE4.C2*. of *E. coli* isolates. Lane L: 100 bp DNA Ladder (Pharmacia). Neg.: negative control. Pos.: positive control amplified at 152, 211 and 279 bp. for *TSPE4.C2, yiaA* and *chuA* genes respectively. Lane 1,3,4,5& 6: *E. coli chuA* and *yjaA* genes positive and negative for *TSPE4.C2*. Lane 2: *E. coli chuA, yjaA* and *TSPE4.C2 negative.*

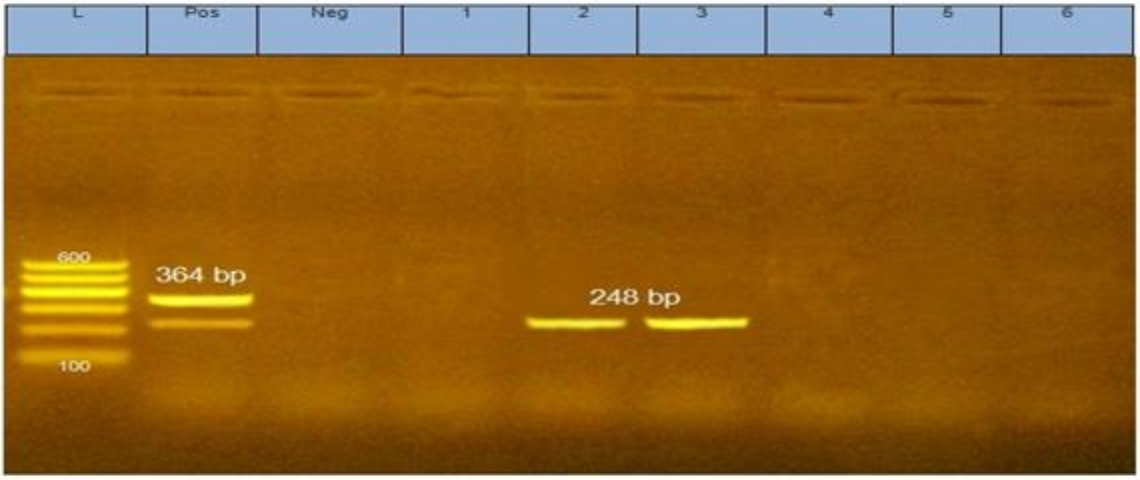

Fig. (2): Agarose gel electrophoresis of duplex PCR-for amplification products of. *eaeA* and *CFA/I* genes *E. coli* isolates. Lane L: 100 bp DNA Ladder (Pharmacia). Neg.: negative control. Pos.: positive control amplified at 248 and 364 bp. *eaeA and CFA/I* genes respectively. Lane 1,2,3,4,5& 6: *E. coli CFA/I* gene negative.Lane 2,3 :- *E.coli eaeA* gene positive .Lane 1,4,5,6 :- *E.coli eaeA* gene negative .

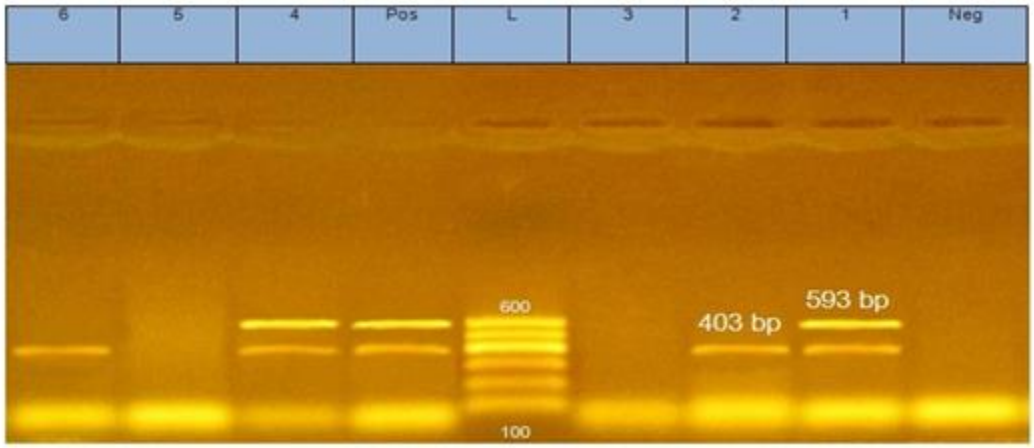

Fig. (3): Agarose gel electrophoresis of duplex PCR-for amplification products of *mph A* and *blaCTX* genes for *E. coli* isolates. Lane L: 100 bp DNA Ladder (Pharmacia). Neg.: negative control. Pos.: positive control amplified at 403 and 593 bp.*for mph A and blaCTX* genes respectively. Lane 1,2,4 & 6: *E.coli mph A* gene positive. Lane 3,5: *E. coli mph A* gene negative. Lane 1,4: *E. coli blaCTX gene* positive. Lane 2, 3, 5 & 6: *E. coli blaCTX* gene negative.

4. DISCUSSION

The subclinical mastitis cannot be detected by clinical methods so, the diagnosis of subclinical mastitis is based California Mastitis Test (CMT) and the bacteriological examination Gebrewahid *et al*. (2012). This test based on the reaction between DNA in the somatic cells and the CMT reagent. The high concentration of somatic cells leads to a higher CMT score. (Poutrel and Lerondelle 1983).

In the present investigation, examination of 63 sheep and 72 goats revealed that the prevalence of subclinical mastitis in sheep and goats were (42.8%, 50.4%) respectively according to "samples level" (Table 1). Nearly the same results were recorded by Windria *et al*. (2016) determined it 52.7 % among goat milk samples also, Hawari *et al.* (2014) recorded that 55.5% showed positive CMT from 220 milk sample in Awassi Sheep.

Other authors detected lower results than that in the present study as Moawad and Osman (2005) found it 31.63% in regard to examined glands of dairy ewes. Also, Schaeren and Maurer (2006) indicated it 40% of goat's halves and Rahman *et al*. (2016) 9.50% of its ewe's milk samples. These differences in incidence of mastitis may be attributed to different factors including climatic factors, sanitary condition in the farm and management factors Johsi and Gokahal (2006).

In the present investigation, out of 94 positive CMT examined milk samples representing different sheep and goat's flocks, results revealed that *E. coli* isolates were (5 and 14) with an overall prevalence rate (5.5% and 12.8%) from sheep and goats respectively. Nearly similar results Table (1). This result agree with Hussien (2003) isolated *E. coli* as a cause of colifrom mastitis in ewes from 8 milk samples (5.9%) in Egypt and Fotou *et al*.(2011) who reported the incidence of *E.coli* was 5% in subclinical mastitic sheep. And the nearly result reported by Moawad and Osman (2005) and de Garnica *et al*. (2013) that reported the incidence of

14.10 % and 17.4% in subclinical mastitic sheep respectively. But it disagrees with Acik *et al*. (2004) who reported the incidence 1.7 % in subclinical mastitic sheep.

As for goats, *E. coli* were (12.8%) in subclinical mastitic goats Table (1). These results agree with Bayoumi, Faten *et al.* (2005) and Najeeb *et al.* (2013) reported the incidence of 15 % and 10.96% respectively in subclinical mastitic goats. But it disagrees with El- Bassiony *et al*. (2008) that reported the incidence 5.13 % and Mugabe *et al*. (2017) who detected it with an incidence 36%.

The rate of prevalence of colifrom mastitis especially that caused by *E. coli* in the previous studies were differ from country to country according to the season, epidemiological determinants, management of animal flocks and other factors (White and Hinkley, 1999).

In the present study, O1 and O26 were the most predominant serogroup with a percentage 21.1% for each one, followed by O114 and O148 with percentage 15.8%; finally, O125 (10.4%). While 3 other strains were untypable. Many of *E. coli* isolates detected in this study belonged to classical Enteropathogenic *E. coli* (E.P.E.C.) serogroupes as O114 and O26 (36.8%) that were previously detected by Lira *et al*. (2004); Mosherf (2004) and Sabry *et al*. (2006) among serotyped *E. coli* strains from cases of mastitis.

O26 was one of the most important serogroup of *E. coli* isolated from cases of mastitis that previously reported by several investigations Murphy *et al.*, (2007), Osman, Kamelia *et al.*, (2012) and Lamey, Amira *et al*., (2013)

Virulence factors of the bacterial strain can give it a chance for colonization, multiplication and survival in udder in the face of host defense mechanism Kaipainen *et al*. (2002)

Phylogenetic assay and virulence genes detection by PCR had classified (5/6) *E. coli* strains into B2 group which *eae* gene was positive in one of that strains and (1/6) of the examined strains belong to A group which harbor *eae* gene. But, none of examined isolates harbor *CFA/I* gene that mediate bacterial adhesion to the intestinal cells and bind to the intestinal cell lining which was characterized the Enterotoxigenic *Escherichia coli* (ETEC). While, Sjöling *et al.* (2007) can determined *CFA/I* by PCR in one strain and also can detected other colonization factors (CS2, CS3, CS6b, CS7, CS12 CS14, CS17and CS19b) in Egyption *E. coli* strains. In this study, O26 was the only stains that had the virulence *eae*, attaching and effacing, gene. This finding ensured with Murphy *et al.,* (2007) that found *eae* gene in eleven *E. coli* O26 isolates (2 from caprine milk and 9 from bovine Milk) from totally seventeen O26 starins.

E. coli O26 isolates which harbor the *eae* gene may be considered as enteropathogenic *E. coli* (EPEC) which implicated in developing countries as the cause of infantile diarrhoea (Nataro and Kaper, 1998).

Presence of some pathogenic strain in group A which are nonpathogenic group of *E. coli* suggests that this strain could have a commensal origin but may became pathogenic through a horizontal acquisition of virulence –related genes (Ewers *et al*., 2007).

High level of Erythromycin resistance was also reported in many previous studies as Isnel and Kirkan, (2012) 65% from goats, Bourabah *et al*., (2014) 66.66% in Tiaret province and El Ayis and Fadlalla, (2017) 80% from ewes, as well as a very high level of resistance to Cefoxtin was

detected by another investigator Barbour *et al*., (2015) who found different percentage of resistance B-lactam antibiotics Cephalothin, Cefotaxime and Cefoxitin with 100%, 97% and 21% respectively.

*Mph*A is a phosphotransferase that phosphorylates and so inactivates erythromycin Noguchi *et al.,* (2000). This gene was indicated in the present study (4/6) and this finding agree with Nakamura *et al.,* (2000) who can discovered *mph* A in 3/6 erythromycin resistant strains in Japan. While, present of *bla*CTX gene in (2/6) represented the Cefoxitin resistance by using PCR. Resistance to this type of extended-spectrum b-lactam antibiotics was occurred also due to extended-spectrum b-lactamases (ESBLs) such as *bla*SHV, *bla*CTX-M and *bla*TEM. (Paterson & Bonomo, 2005).

5. Conclusion

In conclusion, Data from this study revealed a high prevalence of B2 pathogenic phylogroup *E. coli* strains. Present of virulence and antibiotic resistance genes, such as *eae* A, *Mph*A and *bla*CTX genesshows that the increase and spread of antibiotics resistance is alarming to limit the random use of antibiotics in the veterinary field.

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