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Molecular characterization of *Escherichia coli* isolated from mastitis in dairy cattle.

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ABSTRACT

Escherichia spp. is reported as the major important environmental agent causing mastitis in lactating cattle herds because of its wide distribution and rapid dissemination all over the herd animals, accompanied by high economic losses. This study was conducted for the detection and determination of the resistance genes in *Escherichia coli* (*E. coli*) strains isolated from the milk of cattle suffering mastitis. The incidence rate of *E. coli* infection (4.6%) was represented by eight isolates/175 milk samples. *E. coli* isolated strains were identified by applying the traditional methods of isolation and identification, following confirmation by PCR, that reported the amplification of 16S rRNA alkaline phosphatase (*phoA*) gene at 720 bp. On the identified isolates, the antibiotic sensitivity test was carried out against six antibiotics that belong to 6 antibiotic groups: ciprofloxacin (quinolones), erythromycin (macrolides), streptomycin (aminoglycosides), norfloxacin (fluoroquinolones), clindamycin (lincosamides) and sulphamethoxazole + trimethoprim (sulphonamides). The results showed that the isolates had complete resistance (100%) to erythromycin (macrolides), streptomycin (aminoglycosides), clindamycin (lincosamides) and sulphamethoxazole + trimethoprim (sulphonamides). PCR detected the presence of 4 resistance genes, sulphonamide resistance (*sul1*), macrolide 2′-phosphotransferase (*mphA*), aminoglycoside nucleotidyltransferase (*aadA1*), and Erythromycin Esterase (*ereA*), in all tested isolates, while trimethoprim resistant dihydrofolate reductase (*dfrA*) resistance gene was detected in 5 isolates (62.5%). This study concluded that *E. coli* isolated from the milk of cattle suffering from mastitis showed multidrug resistance activity and carried several resistance genes, which play an essential role in the resistance activity effect toward the antibiotic drugs of choice for the treatment of diseased animals.

1. INTRODUCTION

Escherichia coli is the most common major pathogen that causes environmental mastitis, affecting the high-producing cow during the first third of lactation season. Differentiation and classification of pathogenic strains from commensal microorganisms depend upon the detection of the virulence factors. This microbe is recorded as the common facultative anaerobic, gram-negative rods, motile by peritrichous flagella and opportunistic microorganisms. This agent is almost the common flora in the environment and intestines of humans and animals (Mariat et al., 2009; Desmarchelier and Fegan, 2016).

The pathogenic strains are responsible for the majority of disease conditions for different wildlife, farm animals, and human beings (zoonotic disease) (Debroy et al., 2018; Ribeiro et al., 2019). Specific virulence factors of *E. coli* were determined as attachment, hemolytic activity, and secretion of enterotoxins (extra-intestinal factors). *E. coli* affects the udder tissue, inducing a high percentage of environmental cattle mastitis, especially in lactating cows during the first lactation season (Burvenich et al., 2003).

However, in the case of environmental cattle mastitis, caused by opportunistic *E. coli*, it multiplies in the udder tissue and is secreted without needing attachment phenomena. So, the pathogenesis of *E. coli* mastitis is different than other infectious conditions such as colibacillosis in neonatal calves (Gyles et al., 1993; Burvenich et al., 2003).

The essential factors affecting the rate and severity of cattle *E. coli* mastitis are the health condition of cattle, environmental hygienic measures, management of cattle, and bacterial factors (Burvenich et al., 2003; Lehtolainen, 2004).

The establishment of the antibiotic sensitivity test for isolated strains is important for the limitation of antibiotic drugs that are used in the treatment of infected cases (Ismail and Abutarbush, 2020). PCR technique was objected to for differentiation of the encoded genes on the DNA as a result of the degree of variability being very high, depending on the fingerprint (Lipman et al., 1995 and Lam et al., 1996).

This research aimed to study the molecular characterization of *E. coli* microorganisms for the detection of resistance genes in *E. coli* isolated from the milk of cattle suffering mastitis.

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2. MATERIAL AND METHODS

Ethical Approval

The study was done according to an approved protocol by the Ethical Committee, Faculty of Veterinary Medicine, Benha University (Approval number BUFVTM 34-09-23).

2.1. Sampling:

Milk samples (n=175) were collected from Friesen-Holstein cattle from different regions of Cairo Governorate through the period between January 2023 and March 2023. The cattle were diagnosed with clinical mastitis; about 100 ml of milk was taken from each animal under an aseptic condition. All samples were put in ice bags and sent to the lab as soon as possible.

2.2. The laboratory examination of milk samples:

From clinical cases, samples were streaked on blood agar (Oxoid CM0271) plates and incubated aerobically at 37°C for 18-24 hr. Then, the colonies were re-cultivated on MacConkey agar (Oxoid CMO0115) plates and Eosin Methylene Blue (EMB) agar (HIMEDIA M317-500G) plates and incubated at 37 °C for 24 hr. The inoculated plates were examined for colonial growth and morphological properties in combination with biochemical identification, according to Quinn et al., (2002). The isolated *E. coli* strains were identified with gram stain (HiMedia Laboratories Pvt. Ltd. India), catalase, coagulase, and oxidase tests. The obtained suspected isolates of *E. coli* were re-cultivated on nutrient agar slants that incubated at 37 °C for 24 hr. The slants were stored in the refrigerator for further examination (Boerlin et al., 2003).

2.3. Antibiogram sensitivity test:

This test was achieved for *E. coli* isolated strains, according to the Standard agar disk diffusion technique, under the recommendations and guides by the National Committee for Clinical Laboratory Standards (NCCLSI, 2012 and 2021).
The antimicrobial sensitivity for this work, by the use of six antimicrobial groups: quinolones (ciprofloxacin five µg), macrolides (erythromycin 15µg), aminoglycosides (streptomycin ten µg), fluoroquinolones (norfloxacin ten µg), lincosamides (clindamycin two µg) and sulphonamides (trimethoprim+sulphamethoxazol 25µg). The results were interpreted following EUCAST (2019).

2.4. Molecular identification:

The isolated strains were subjected to the extraction of DNA using a Qiagen Mini Kit (Hombrechtikon, Switzerland). For molecular identification, the Primers set encoded for the 16S rRNA gene (*phoA*) of *E. coli* (Hu et al. 2011) was used (Table 1). The PCR reaction carried out using a total volume of 25µl contained 3µl of DNA template, 5pmol of each primer, and 5µl of 1X PCR master mix (Cat. No. 51304 Jena bioscience, GmbH, Germany). In a thermal cycler (Perkin-Elmer, Waltham, USA), the PCR mixtures were then subjected to the following thermal cycles: 50 °C for two min., (1 cycle), 95°C for 5min., (1 cycle), 40 cycles of 95°C for 45 s, 50°C for one min., and 72 °C for 1 min, and 72°C for seven min. (1 cycle). Then, the *E. coli* isolates were subjected to PCR for detection of resistance genes: sulphonamide resistance (*sul1*), macrolide 2′-phosphotransferase (*mphA*), aminoglycoside nucleotidyltransferase (*aadA1*), and Erythromycin Esterase (*ereA*) (Table 2).

Table 1 The Primers sequences, target genes, amplicon sizes and thermal cycle.

Target agent	Target gene	Primers sequences	Amplified fragment (bp)	Primer Denaturation	Amplification (35 cycles) Sec. den.	Ann.	Ext.	Final extension	Reference
E. coli	*phoA*	*F* CGATTCTGGAAATGGCAAAAG	720	94˚C	94˚C	55˚C	72˚C	72 ˚C	**Hu et al.,2011**
		R CGTGATCAGCGGTGACTATGAC		5 min	30 sec.	40 sec.	45 sec.	10 min.	

Table 2 The Oligonucleotide primers sequences Source (Metabion Germany).

Primers	Sequences	Amplified product	References
*Sul*1	F CGGCGTGGGCTACCTGAACG	433 bp	Ibekwe *et al*.,2011
	RGCCGATCGCGTGAAGTTCCG		
*aad*A1	F TATCAGAGGTAGTTGGCGTCAT	484 bp	Randall *et al.,* 2004
	R GTTCCATAGCGTTAAGGTTTCATT		
*mph*A	FGTGAGGAGGAGCTTCGCGAG	403 bp	Nguyen *et al*., 2009
	RTGCCGCAGGACTCGGAGGTC		
*ere*A	FGCCGGTGCTCATGAACTTGAG	420 bp	Nguyen *et al*., 2009
	RCGACTCTATTCGATCAGAGGC		
*dfr*A	F TGGTAGCTATATCGAAGAATGGAGT	425 bp	Grape *et al*., 2007
	R TATGTTAGAGGCGAAGTCTTGGGTA		

3. RESULTS

The result of *E. coli* isolation was eight isolates out of 175 milk samples (4.6%). The isolates appeared as gram-negative rods under the microscope. Biochemically, they were coagulase-positive, catalase-negative and oxidase-negative. PCR showed amplification of the 16S rRNA gene of *E. coli* at the (720) bp (Figure 1). Antimicrobial sensitivity test showed that the isolates were highly sensitive to ciprofloxacin and norfloxacin (100 %), while they showed complete resistance (100%) to erythromycin, streptomycin, clindamycin, and trimethoprim + sulphamethoxazole (Table 3). Also, PCR detected the presence of 4 resistance genes, *sul1, mphA, aadA1,* and *ereA,* in all tested isolates, while *dfrA* was detected in 5 isolates only (62.5%) (Table 4 and Figure 1-7).

Table 3 Antibiogram activity of different antimicrobial agent on E. coli.

antimicrobial agent Class	Name	Disk conc.	sensitive No.	%	Intermediate No.	%	Resistance No.	%	A.A
Quinolones	(Ciprofloxacin) CIP	5 µg	8	100%	0	0	0	0	S
Macrolides	(Erythromycin) E	15 µg	0	0	0	0	8	100%	R
Aminoglycosides	Streptomycin S	10 µg	0	0	0	0	8	100%	R
Fluoroquinolones	(Norfloxacin) NOR	10 µg	8	100%	0	0	0	0	S
Lincosamides	(Clindamycin)CLN	2 µg	0	0	0	0	8	100%	R
Sulphonamides	(Trimethoprim Sulphamethoxazol) SXT	25 µg	0	0	0	0	8	100%	R

Table 4 Virulence genes screening results of *E. coli* strains isolated from milk source.

Sample ID	*sul*1	*aad*A1	*mph*A	*ere*A	*dfr*A
14	+	+	+	+	+
18	+	+	+	+	+
49	+	+	+	+	-
71	+	+	+	+	-
81	+	+	+	+	+
82	+	+	+	+	+
84	+	+	+	+	-
88	+	+	+	+	+
Total NO.	8	8	8	8	5
%	100	100	100	100	62.5

Sulphamethoxazole (*Sul*1) positive (100%), streptomycin (*aad*A1) positive (100%), erythromycin (*mph*A) positive (100%), clindamycin (*ere*A) positive (100%) and trimethoprim (*dfr*A) positive (62.5%).

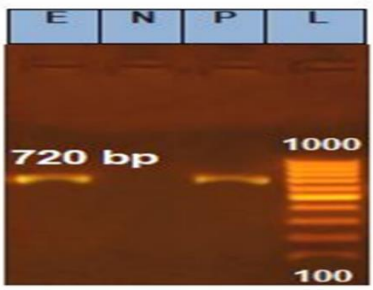

Figure 1 Agarose gel electrophoresis of phoA gene (720 bp) *E. coli* L: 100 bp ladder, P: Control positive phoA (720, bp) (N: Control negative, E: Positive *E. coli* strain isolated from mastitis cattle.

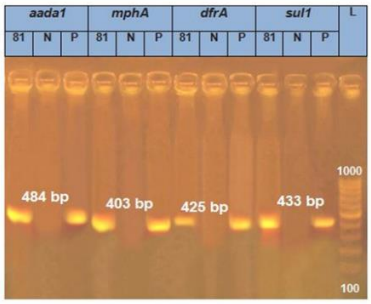

Figure 2 Agarose gel electrophoresis of aadA1, mphA, dfrA and sul1 resistance genes (484,403,425 and 433 bp, respectively) of E. coli, L: 100 bp ladder, P+: Control positive (aadA1, mphA, dfrA and sul1), N: Control negative, 81: Positive *E. coli* strains isolated from mastitis cattle, for aadA1, mphA, dfrA and sul1 resistance genes.

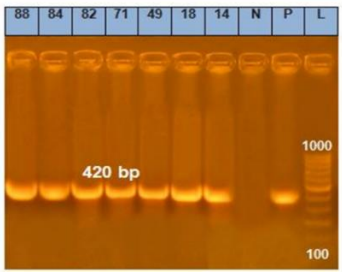

Figure 3 Agarose gel electrophoresis of ereA resistance gene (420bp) of E. coli, L: 100 bp ladder, P: Control positive ereA (420) bp, N: Control negative, 14, 18, 49, 71, 82, 84, and 88: Positive *E. coli* strain isolated from mastitis cattle, for ereA resistance gene.

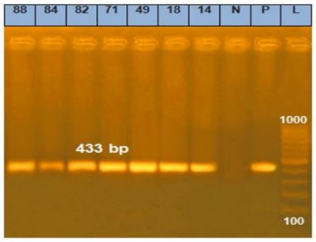

Figure 4 Agarose gel electrophoresis of sul1 resistance gene (433bp) of E. coli, L: 100 bp ladder, P: Control positive sul1 (433) bp, N: Control negative: 14, 18, 49, 71, 82, 84, and 88: Positive *E. coli* strains isolated from mastitis cattle, for sul1 resistance gene.

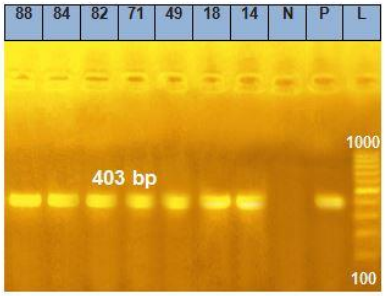

Figure 5 Agarose gel electrophoresis of mphA resistance gene (403bp) of E. coli, L: 100 bp ladder, P: Control positive mphA (403) bp, N: Control negative: 14, 18, 49, 71, 82, 84, and 88: Positive *E. coli* strains isolated from mastitis cattle, for mphA resistance gene.

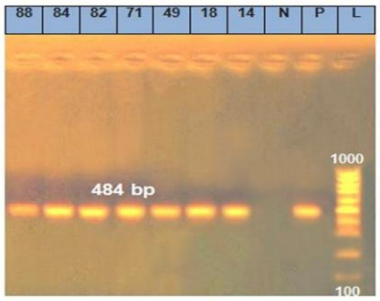

Figure 6 Agarose gel electrophoresis of aadA1 resistance gene (484bp) of E. coli, L: 100 bp ladder, P: Control positive aadA1 (484) bp, N: Control negative: 14, 18, 49, 71, 82, 84, and 88: Positive *E. coli* strains isolated from mastitis cattle, for aadA1 resistance gene.

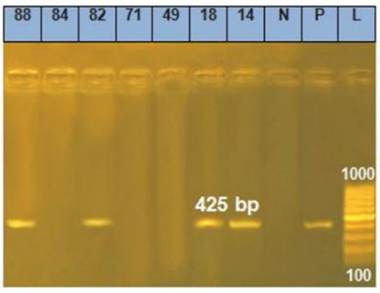

Figure 7 Agarose gel electrophoresis of dfrA resistance gene (425bp) of E. coli, L: 100 bp ladder, P: Control positive dfrA (425) bp, N: Control negative: 14, 18, 49, 71, 82, 84, and 88: Positive *E. coli* strains isolated from mastitis cattle, for dfrA resistance gene.

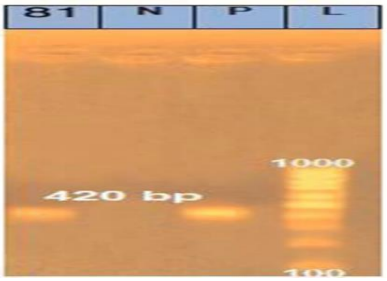

Figure 8 Agarose gel electrophoresis of ereA resistance gene (420bp) of E. coli, L: 100 bp ladder, P: Control positive, N: Control negative, 81: Positive *E. coli* strain isolated from mastitis cattle, for ereA resistance gene ز

4. DISCUSSION

The isolated *E. coli* strains are related to the environmental pathogens causing mastitis, and it is the most common member of coliform spp. The main sources of coliform microbes that induce mastitis are water suppliers, bedding of yards, bedding (sewages), and environmental residues. Incidence rates of coliform mastitis were increased during the climatic periods in which microbes of maximum populations in the environment. The usual entrance route of infected environmental bacteria into the udder tissue is through the teat orifice (Elie et al., 2015). The resulted data tabulated, including total rates of *E. coli* isolation, is (4.6%),

represented by eight isolates from the total 175 cattle milk samples. Eight *E. coli* was isolated from 8 /175 milk samples (4.6%). This isolation rate agreed with that of Mork et al. (2007) (7.3%), while disagreed with Gebrewahid et al. (2012), Abdallah et al. (2018), and Ombarak (2019), who recorded higher recovery rates; 17.0%, 44.4%, and 9.3%, respectively. The notification of clinical cases of mastitis caused by *E. coli* infection was lesser than the multidrug resistance bacteria.

The use of any antibiotic drugs for treatment, with a low concentration, short course time, and improper interval times, leads to a bad accuracy result of treatment. Consequently, high economic losses (Alekish et al., 2013). Therefore, the dissemination and distribution of infected microbes with the transmission of resistance genes to another microbe results in multidrug-resistant strains (Jingar et al., 2017). Here, the sensitivity of the isolated *E. coli* was determined against six antibiotic agents that belong to six antibiotic groups: ciprofloxacin (quinolones), erythromycin (macrolides), streptomycin (aminoglycosides), norfloxacin (fluoroquinolones), clindamycin (lincosamides) and sulphamethoxazole + trimethoprim (sulphonamides). The results showed that the isolates were completely sensitive to the drugs of two groups (quinolones and fluoroquinolones). Thus, the susceptibility was (100 %), while they showed 100% resistance to erythromycin (macrolides), streptomycin (aminoglycosides), clindamycin (lincosamides), and sulphamethoxazole + trimethoprim (sulphonamides). In this objective, the reported results of the antibiogram sensitivity test showed that the isolated strains were highly sensitive to ciprofloxacin and norfloxacin. In the case of the sulphamethoxazole-trimethoprim and erythromycin were recorded of high resistance action in (100 %). That is more varied than the results stated by Awad and Awad (2021), who found moderate resistance of *E. coli* against sulphamethoxazole + trimethoprim and erythromycin. In the case of ciprofloxacin, reporting a high sensitivity and quietly sensitive to norofloxacin. On the other hand, streptomycin and clindamycin showed a complete resistance pattern (100%) in this research. While Kindu et al. (2019) tabulated that streptomycin (50%) showed resistance action. Also, Briscoe et al. (2005) and Bharathi et al. (2008) reported that sulfamethoxazole-trimethoprim (60%), clindamycin (80%), and erythromycin (60%). (Memon et al., 2013) and (Bedada and Hiko, 2011) detected the susceptibility of *E. coli* to streptomycin, norfloxacin, and ciprofloxacin was 100%, but the isolates were completely resistant to erythromycin. Depending on the definition of multidrug-resistant (MDR), it has been known as acquired resistance to at least one agent in three or more antimicrobial categories (Groups). Thus, the *E. coli* isolates of the current study expressed MDR activity (Jamali et al., 2018). PCR was applied for the determination of resistant genes (*sul1*), (*mph*), (*aadA1*), and (*ereA*). The specific bands of gene amplification for each resistant once were illuminated at the bp 433, 403, 484, and 420 bp, respectively, in an incidence rate of (100%). While *dfrA*, at 425 bp, was detected in 5 isolates only with a percent of (62.5%). In this trial, the *aadA1* gene is the only resistant gen that is demonstrated as guide in studying the molecular characterization of resistance activity of *E. coli* isolated strains from cattle suffering mastitis (Ameen et al., 2019). The most important cause of dissemination for the MDR strains in dairy farms is udder inflammation (mastitis). The limitation of drug efficacy is a result of the high frequency of antibiotic-resistant pathogenic agents, which leads to the suppression of the sensitivity of the used antibiotic drugs (Jones-Dias et al., 2013). Finally, the isolated *E. coli* from cattle with mastitis represented a highly dangerous state, resulting in the spreading of a powerful and harmful infectious microbe.

5. CONCLUSIONS

The current study achieved the illustration of different resistant genes encoded in the DNA of isolated *E. coli* that control the resistance activity effect toward the antibiotic drugs choice in the treatment of diseased animals and recording that the isolation of MDR *E. coli* from milk samples obtained from cattle with clinical singes of mastitis in different country and localities. Therefore, preventive measures for treatment and control must be recommended to avoid contamination with pathogenic strains and discarding the infected milk. On the other hand, pay attention to bacteria that cause the disease, workers or breeders and cattle to take health measures, including sterilizing and disinfecting the environment. Consequently, it may cause major health concerns and problems for both humans and animals.

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