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ORIGINAL ARTICLE

Antimicrobial and Virulence Characteristics of *Escherichia coli* Isolated from Mastitis and Endometritis Cases in Sheep and Goats

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Abstract

This study was focused on the prevalence of *E. coli* in mastitis and endometritis of sheep and goats in Beni-Suef government with a total number of 238 samples as follow; 122 of native breed lactating ewes (n=90) and does (n=32) from private farms suffered from mastitis and a total of 116 of native breed of ewes (n=83) and she-goats (n=33) from endometritis cases in which137 isolates of *Escherichia coli* (*E. coli*) categorized as 68 isolates were isolated from mastitis and 69 isolates from endometritis with a prevalence rate of 55.7 % and 59.5 %, respectively. Antibiogram for *E. coli* isolates recovered from ovine and caprine mastitic cases showed resistance to amoxicillin-clavulanic acid (67.65%), cephalexin (64.71 %), amoxicillin (58.82%), cefotaxime (57.42 %), ceftriaxone (54.42 %) and gentamicin (44.11%). On the contrary, the recovered *E. coli* isolates from endometritis of ewes and does showed resistance to amoxicillin-clavulanic acid (69.57%), cefotaxime (68.11 %), amoxicillin (66.66 %), cephalexin (65.22%), ceftriaxone (62.32 %), colistin (46.38%) and gentamicin (44.93%). Sixty *E. coli* isolates (27 isolates from ovine mastitis milk samples and 33 isolates from endometritis samples were inoculated onto Yeast extract-casamino acids Congo red agar (YESCA CRA) revealed that 39 of *E. coli* isolates showed positive biofilm formation. Multidrug resistance was detected in 70 *E. coli* isolates out of 137 isolates (51.09%) (31 *E. coli* isolates from mastitis milk samples and 39 isolates from endometritis samples). The prevalence of virulence and resistance genes of *E. coli* as *fim*H, *iut*A, *qnr*A and *bla*$_{TEM}$ was 100%, 25%, 50% and 100%, respectively. This work was designed for detection of virulence and antibiotic resistant *E. coli* isolates from mastitis and endometritis cases in sheep and goats.

Keywords

E. coli, Endometritis, Mastitis, Sheep and Goats, Virulence

1. Introduction

Mastitis is the inflammation of one or both halves of mammary glands which may cause partial or full damage to udder, loss of body weight; reduce the growth rate of their offspring and deaths **(Haenlein, 2004).** Clinical and subclinical mastitis (CM and SCM) cause severe inflammatory signs due to traumatic, pathological, and bacteriological changes in mammary glands lead to permanent blockage of milk ducts **(Monsang et al., 2014).**

Endometritis is an inflammation of uterine tissue. Uterine infections in female domestic ruminants are often caused by bacterial pathogens, especially *E. coli*, which has frequently been isolated in ewes **(Manes et al., 2010; Martins et al., 2009)** and goats **(Ababneh and Degefa, 2006).** Endometritis causes infertility and reduces productivity with clinical signs which may include vaginal discharge **(El-Arabi et al., 2013).** *E. coli* was the common bacteria causing endometritis in ewes in the tropical zone of Nigeria with a prevalence rate of 32% and the bacterial population in the vagina (64%) was significantly higher than that in the uterus (34%) **(Mshelia et al., 2014).** The prevalence rate of *E. coli* isolates recovered from ovine endometritis samples was 57.2% **(Martins et al., 2009).** Multi drug resistant bacteria have become a great problem that affect the health of animal and human **(WHO, 2000).**

Pathogenicity of bacteria can be detected by biofilm formation that becomes a main constituent of bacterial virulence **(Otto, 2006).** Important factors as presence of resistance genes and production of high amount of extra polymeric substance (EPS) that produced during the process of biofilm help in bacterial protection **(El-Feky et al., 2009).** Curli fimbriae, type I pili, and flagella are surface organelles and extracellular molecules that donate to biofilm formation of *E. coli* **(Vidal et al., 1998)**. Some molecules as slime exopolysaccharides help in formation of three dimensional structures and depth of biofilm formation, also flagella help in cell attachment during *in-vitro* biofilm formation in bacteriological media **(Danese et al., 2000)**. *E. coli* has some pathogenic constituents as epithelial cell adhesion, flagellar motility and toxin production that lead to great inflammatory response in mammary gland and uterus **(Moori Bakhtiari et al., 2018)**. Biofilm formation in some microorganisms helps in antibiotic resistance; increasing antimicrobial resistance up to 1000 folds, and high antibiotic concentration needed to inactivate microorganisms with biofilm formation **(Thien-Fah and George, 2001).** The prevalence level of *fim*H was 100% in mastitic cases and was associated with adhering, invading and surviving within the epithelial cells of the mammary gland resulting in persistent intra mammary infections **(Fernandes et al., 2011)**.

2. Materials and Methods

2.1. Sampling and Samples Processing

Under aseptic conditions, a total of 122 mastitis milk samples from 122 sheep and goats that mainly suffered from subclinical mastitis that detected by California mastitis test and 116 uterine samples from 116 sheep and goats including uterine discharges and vaginal swabs from does and ewes suffering from endometritis were collected from Beni-Suef Governorate, Egypt, during the period from July to October, 2021. Collected samples were immediately transferred to the laboratory of Bacteriology, Mycology and Immunology of Faculty of Veterinary Medicine, Beni-Suef University in an ice box for bacteriological examination.

2.2. Morphotypic Characterization of *E. coli*

Milk samples were centrifuged at 3,000 rpm for 20 minutes. The cream and supernatant fluid were discarded. Loopfuls from the uterine samples and sediment of milk samples were inoculated into Tryptone soya broth (TSB**) (**Oxoid) and incubated at 37°C for 16-18 hrs. A loopful from each TSB culture was streaked on MacConkey agar medium (Oxoid) and incubated at 37°C for 24 hrs. After that, pink colonies were selected for morphological and biochemical identification by using oxidase, indole production, methyle red, vogues Proskauer , citrate utilization tests and urease tests. Also *E. coli* were streaked on Eosin methylene blue (EMB) agar medium and triple sugar iron agar (TSI) **(Quinn et al., 2011)**.

2.3. Antimicrobial Susceptibility Testing of *E. coli*

The disc diffusion method was applied according to the clinical and laboratory standards institute **(CLSI, 2021).** The antimicrobial discs used in treatment of ovine and caprine mastitis and endometritis under field conditions as amoxicillin-clavulanic acid (30µg), amoxicillin (30µg), cephalexin (30µg), cefotaxime (30µg), ciprofloxacin (5µg), ofloxacin (5µg), doxycycline (30µg), tetracycline (30µg), gentamicin (10µg), amikacin (10µg), apramicin (15µg), sulfamethoxazole-trimethoprim (25µg),co-trimethoprim (25µg), fosfomycin (200µg), chloramphenicol (30µg), and colistin (10µg) were used in which diameter of the inhibition zones around antimicrobial discs were measured, then *E. coli* were classified into sensitive (S), intermediate (I) and resistant (R) according to **(CLSI, 2021).**

2.4. Biofilm Formation of *E. coli* Isolates on YESCA CRA Medium

Congo red (CR) assay for *E. coli* was used, as described by **(Zhou et al., 2013)**, through pre-enrichment of the isolates by cultivation on Luria–Bertani agar medium and incubation at 37°C for 24 hr. then inoculation on YESCA CR agar plates at 26°C for 48 hr. for good induction of Curli formation that detected by color of the colony as follow; dark red color indicates positive result and white color as a negative result according to **Hammar and Normark (1996)**.

2.5. Polymerase Chain Reaction (PCR) for Detection of Virulence and Resistance Genes of *E. coli*

Four *E. coli* isolates (two isolates from mastitis milk samples and two isolates from endometritis samples) were chosen for detection of genotypic characters by using (PCR) for detection of some virulence and resistance genes as *fim*H, *iut*A, *bla*$_{TEM}$ and *qnr*A using their specific forward and reverse primers as shown in **Table (1).**

Table (1). Oligonucleotide primers used for detection of virulence and resistance genes.

Target genes	Annealing temperature	Product	Primer sequence (5′-3′)	References
*fim*H	50°C	508-bp	TGCAGAACGGATAAGCCGTGG GCAGTCACCTGCCCTCCGGTA	Ghanbarpour and Salehi (2010)
*iut*A	63°C	300-bp	GGCTGGACATGGGAACTGG CGTCGGGAACGGGTAGAATCG	Yaguchi et al., (2007)
*qnr*A	55°C	516-bp	ATTTCTCACGCCAGGATTTG GATCGGCAAAGGTTAGGTCA	Robicsek et al., (2006)
bla$_{TEM}$	54°C	516-bp	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	Colom et al., (2003)

3. Results

3.1. Prevalence of *E. coli* in Sheep and Goats Mastitis Milk Samples

The prevalence of *E. coli* in the collected samples was 55.7% (68 isolates) in sheep and goats mastitis milk samples and 59.5 % (69 isolates) in endometritis samples.

3.2. Antimicrobial Susceptibility Testing of *E. coli*

Antimicrobial susceptibility testing of *E. coli* isolates revealed that 72.99%, 72.26%, 70.8%, 70.07%, 68.61%, 64.23% , 63.5%, 62.04% and 61.31 % of them were sensitive to co-trimethoprim, ofloxacin, doxycycline, ciprofloxacin, fosfomycin, sulfamethoxazole-trimethoprim, chloramphenicol, amikacin and tetracycline , respectively. While 69.34, 66.43, 64.96, 63.45 and 62.77 % were resistant to amoxicillin-clavulanic acid, cephalexin, amoxicillin, ceftriaxone and cefotaxime, respectively as showed in **Table (2).**

3.3. Detection of Multidrug Resistance *E. coli* Isolates

Furthermore, 54.26% (70 out of 129) of *E. coli* isolates showed multi-drug resistance with a prevalence rate of 54.26%.

3.4. Biofilm Formation of *E. coli*

Biofilm formation of *E. coli* isolates recovered from mastitis milk samples showed that 66.67% of *E. coli* isolates showed positive results and 33.33% as negative results as in the **Table (4).** Whereas, for endometritis samples; 63.64% and 36.36% of *E. coli* isolates revealed positive and negative results, respectively as showed in the **Table (5).**

Table (2). *In-vitro* Antimicrobial susceptibility testing of the recovered *E. coli* isolates.

Class		Type	S		I		R	
			No	%	No	%	No	%
β –lactams	β–lactamase stable	Amoxicillin - Clavulanic acid	26	18.96	16	11.7	95	69.34
		Amoxicillin	29	21.17	19	13.9	89	64.96
	Cephalosporins	Cephalexin	36	26.27	10	7.3	91	66.43
		Cefotaxime	40	29.19	11	8.03	86	62.77
		Ceftriaxone	32	23.35	18	13.2	87	63.45
Fluoroquinolones		Ciprofloxacin	96	70.07	16	11.7	25	18.23
		Ofloxacin	99	72.26	15	10.9	23	16.79
Tetracyclines		Doxycycline	97	70.8	11	8.03	29	21.17
		Tetracycline	84	61.31	16	11.7	37	27.01
Aminoglycosides		Gentamicin	82	59.85	15	10.9	40	29.25
		Amikacin	85	62.04	17	12.4	35	25.56
		Apramicin	68	49.64	14	10.2	55	40.16
Potentiated Sulfonamide		Sulfamethoxazole-trimethoprim	88	64.23	19	13.9	30	21.87
		Co-Trimethoprim	100	72.99	13	9.5	24	17.51
Fosfomycins		Fosfomycin	94	68.61	17	12.4	26	18.99
Phenicols		Chloramphenicol	87	63.5	18	13.1	32	23.4
Lipopeptides		Colistin	73	53.28	19	13.9	45	32.84

R: Resistant isolates, I: Intermediate sensitive isolates, S: Sensitive isolates, No: Number of *E. coli* isolates, %: Percentage were calculated in relation to the total number of the tested *E. coli* isolates (n=137).

Table (3). Multidrug resistant *E. coli* isolates recovered from mastitis and endometritis samples.

MDR isolates	No.	%	Milk and uterine samples (129 isolates)			
			Sheep		Goat	
			No.	%	No.	%
E. coli	70	54.26	46	35.66	24	18.60

Table (4). Biofilm formation of *E. coli* isolates recovered from sheep and goats mastitis milk samples.

	Source of isolates	No. of tested isolates	Biofilm formation							
			Positive						Negative	
			Strong		Intermediate		Total positive			
E. coli			No.	%	No.	%	No.	%	No.	%
	Sheep	18	11	61.11	2	11.11	13	72.22	5	27.77
	Goats	9	3	33.33	2	22.22	5	55.56	4	44.44
	Total	27	14	51.85	4	14.81	18	66.67	9	33.33

Table (5). Biofilm formation of *E. coli* isolates recovered from sheep and goats endometritis samples.

	Source of isolates	No. of tested isolates	Biofilm formation							
			Positive						Negative	
			Strong		Intermediate		Total positive			
E. coli			No.	%	No.	%	No.	%	No.	%
	Sheep	21	11	52.38	3	14.28	14	66.67	7	33.33
	Goats	12	5	41.67	2	16.67	7	58.33	5	41.67
	Total	33	16	48.48	5	15.15	21	63.64	12	36.36

%: was calculated according to the corresponding number (No.) of tested isolates

3.5. Relation between Antimicrobial Sensitivity Test and Biofilm Formation

Twenty- nine *E. coli* isolates (17 isolates from mastitis milk samples and 12 isolates from endometritis samples) out of 39 positive biofilm isolates with rate of 74.36% were considered MDR in which some *E. coli* isolates showed antibiotic resistance and biofilm formation that increase the *E. coli* virulence **(Table, 6).**

3.6. Prevalence of Resistance and Virulence Genes in Selected *E. coli* Isolates by PCR

Four *E. coli* isolates (two isolates from mastitis milk samples and two isolates from endometritis samples) were tested by using (PCR) for detection of presence of some virulence and resistance genes as *fim*H, *iut*A, *bla*TEM and *qnr*A with prevalence rates of 100%, 25%, 100% and 50%, respectively **(Figs. 1-4).** Four *E. coli* isolates showed multi drug resistance were selected and tested for some resistance genes as *bla*TEM, *and qnr*A. The results showed in the **Table (7).** Obtained results showed prevalence rates of *fim*H and *iut*A were 100% and 25%, respectively as showed in the **Table (7).**

Table (6). Relation between antimicrobial sensitivity test and biofilm formation of *E. coli* isolates.

E. coli	Total No. of positive biofilm isolates	No. of positive biofilm and MDR isolates	%	Milk samples (17 isolates) and Uterine samples (12 isolates)			
				Sheep		Goat	
				No.	%	No.	%
	39	29	74.36%	18	46.15	11	28.21

Table (7). Prevalence of different genes of *E. coli* isolates from ovine and caprine mastitis and endometritis samples.

	Target genes	Endometritis				Mastitis			
		Sheep		Goat		Sheep		Goat	
		Positive		Positive		Positive		Positive	
		No.	%	No.	%	No.	%	No.	%
E. coli (*n=4*)	*qnr*A	1	25	1	25	-	-	-	-
	*bla*TEM	1	25	1	25	1	25	1	25
	*fim*H	1	25	1	25	1	25	1	25
	*iut*A	1	25	-	-	-	-	-	-

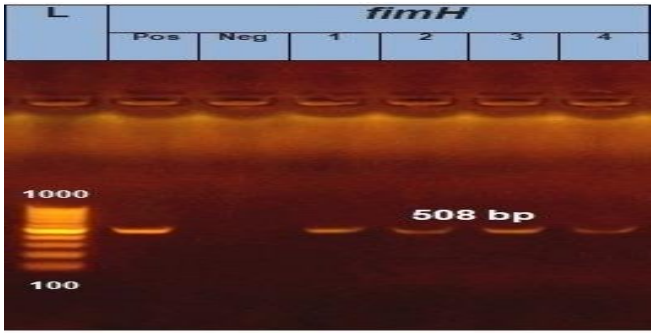

Fig. (1). PCR results of *fim*H at amplicon of 508 bp. Neg: Control negative, L: ladder, Pos: Control positive (*E. coli* isolate). Lane (1-4): positive *E. coli* isolates for *fim*H.

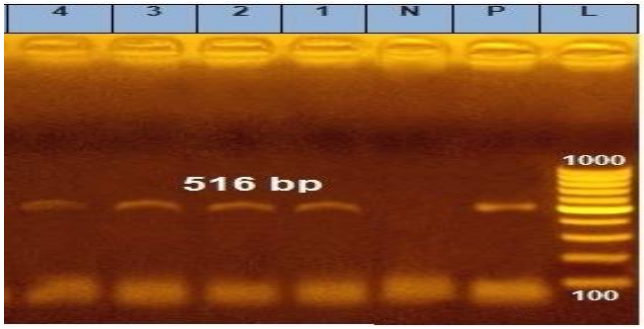

Fig. (2). PCR results of *iut*A at amplicon of 300, respectively. Neg: Control negative, L: Molecular size DNA ladder, Pos: Control positive. Lanes (1) for *iut*A: positive *E. coli* isolates for *iut*A.

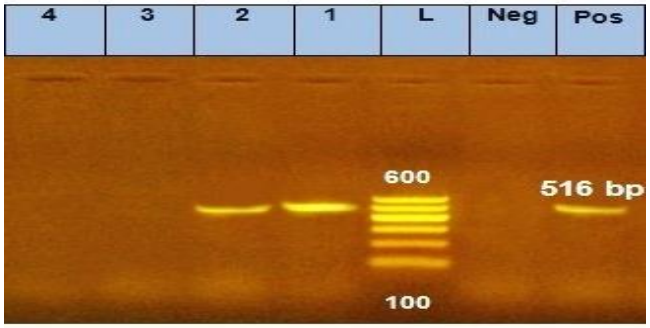

Fig. (3). PCR results of *bla*TEM at amplicon of 516 bp. Neg: Control negative, L: Molecular size DNA ladder, Pos: Control positive. Lanes (1-4): positive *E. coli* isolates for *bla*TEM.

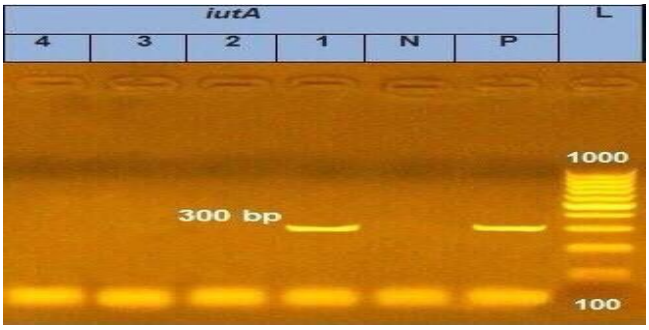

Fig. (4). PCR results of *qnr*A at amplicon of 516 bp. Neg: Control negative, L: Molecular size DNA ladder, Pos: Control positive. Lanes (1-2): positive *E. coli* isolates for *qnr*A.

Fig. (5). Positive biofilm formation (dark red color) of *E. coli* on YESCA/CR agar plates at 26°C for 48 hrs.

4. Discussion

Mastitis may be either clinically (symptomatic) or sub clinically (asymptomatic), but the last one being more prevalent in goats **(Mishra et al., 2018).** Mastitis; mainly subclinical mastitis (SCM) is the most prevalent disease between dairy animals that reducing the development of animal production sector worldwide **(Haggag et al., 2019)** in which the prevalence of *E. coli* as a bacterial pathogen causing mastitis of sheep and goats was 55.7%. **Moshref, (2004)** recovered *E. coli* from ovine mastitis milk samples with a prevalence rate of 41.02%**. Mahlangu et al., (2018**) isolated *E. coli* in a high proportion of 64.5% from mastitis milk of goat in thika-East sub country of Kenya**. Abdallah et al., (2018)** reported that *E. coli* were the most predominant bacterial isolates from ewes' and does' milk samples with a rate of 45.5%. *E. coli* isolates recovered from clinical mastitis milk samples from dairy herds with a prevalence rate of 52% **(Longo et al., 2001).**

Concerning endometritis; the prevalence of *E. coli* was 59.5% and this agreed with a study conducted in Red Sokoto and West African Dwarf Does in Makurdi of Nigeria in which the prevalence of *E. coli* was 79% that was more common than other bacterial isolates **(Mshelia et al., 2014).** The causing endometritis in ewes with a prevalence rate of *E. coli* 37.83% **(Manes et al., 2010).** *E. coli* was the common bacteria causing endometritis in ewes and does with a prevalence rate of 44.4% and 15.4% respectively **(Safana et al., 2019).**

The antibiogram of *E. coli* isolates recovered from sheep and goats mastitis and endometritis samples had shown in the **Table (2).** In our study, *E. coli* isolates recovered from ovine and caprine mastitis milk samples showed higher resistance against amoxicillin-clavulanic acid (67.65%) and cefotaxime (57.35%) and these results were agreed with those reported by **İşnel and Kırkan (2012),** who isolated *E. coli* isolates from goats with sub clinical mastitis that showed resistance against ampicillin (100%), penicillin (100%), amoxicillin-clavulanic acid (90%) and cefotaxime (67.3%). Also, *E. coli* isolates recovered from mastitis milk samples showed high resistance to amoxicillin with a percentage of (76%) **(Decimo et al., 2016). Kundukad et al., (2017)** stated that administration of penicillins intra mammary enhances the biofilm formation due to the negative relationship between pH and biofilm formation, so, some chemical agents as sodium bicarbonates can be used that increase pH resulting in reduction of infection and biofilm formation. *E. coli* isolates from uterine samples were sensitive to ciprofloxacin at a rate of 63.45% and these results coincided approximately with findings of **Martins et al., (2009)** who reported that vaginal isolates of *E. coli* were highly susceptible to ciprofloxacin (100%)**.** Moreover, the recovered *E. coli* isolates from endometritis samples of goats in southern Nigeria were sensitive to ofloxacin with a rate of 95.3% **(Goncuoglu et al., 2010).** Whereas, these results were disagreed with those reported by **Mshelia et al., (2014)** who recovered *E. coli* isolates from endometritis in ewes that showed resistance against ofloxacin and ciprofloxacin at a rate of 64.1% and 56.4%, respectively**.** Multidrug resistance (MDR) was assessed by phenotypic resistance to three or more different antimicrobial classes as defined by **Magiorakos et al. (2012).** Based on the finding of this study, *E. coli* isolates were classified as MDR *E. coli* (54.26%). The excessive use of antimicrobial compounds in veterinary medicine or treatment of animals with antibiotics without application of sensitivity test milk samples may develop antimicrobial resistance (AMR) in different bacterial pathogens **(Abed et al., 2021).**

Biofilm formation of *E. coli* isolates had shown in the **Tables (4, 5).** Biofilm helps *E. coli* to resist adverse factors and is the main cause for failure of repeated antibiotic treatment also these bacterial isolates have the ability for forming biofilms and become less susceptible to antibiotics that used in dairy farms as β-lactamase **(Nasr et al., 2012).** *E. coli* has the ability to produce a viscous extracellular polysaccharide layer (slime) as a virulence factor that contributes to adhesion of bacteria to tissue of mammary gland and protection against phagocytosis and opsoniozation**,** so it is a main cause of failure of antibiotic treatment and chronicity of mastitis as mentioned by **Dubravka et al., (2010). Reichhardt et al., (2015)** explained biofilm formation by binding of CR dye to curled whole cells, without inhibition of the growth, also *E. coli* isolates exhibit extracellular adhesive amyloid fibers called Curli that enables the adhesion of bacteria and stimulates biofilm formation. *E. coli* isolates had shown resistance to different classes of antibiotics due to presence of resistance genes as *bla*$_{\text{TEM}}$ and *qnr*A with a prevalence rate of 100% and 50% as showed in the **Table (7).** The most prevalent resistance genes in *E. coli* isolates recovered from caprine mastitis as follow; *bla*$_{\text{TEM}}$ (94%), *qnr*A (52%) **(Guerra et al., 2006).** In the current study, four *E. coli* isolates (two isolates from mastitis milk samples and two others from endometritis samples)

were selected for detection of virulence associated genes by PCR as showed in the **Table (7)** which detected that virulence associated *fim*H gene was detected in all tested *E. coli* isolates that were positive for biofilm formation. *Fim*H is a type 1pilus helps in adhesion and invasion of epithelial cells of the uterus and mammary gland **(Bien et al., 2012).** Also, *fim*H gene is associated with *E. coli* adhesion, invasion and survival in the epithelial cells of the mammary glands. Pilli have conjugative function that allows cell to cell adhesion and induction of colonic acid by natural incompatibility group F (incF) plasmid allows cell surface adhesion, so promoting biofilm formation **(May et al., 2010).** In the current study, *iut*A *was* detected in 25% of the tested isolates of endometritis of sheep only. These results were coincided with those reported by **Safana et al., (2019)** who recorded *iut*A gene was a virulence factor of *E. coli* isolates with a prevalence rate of 38% of the uterine *E. coli* isolates and 20.3%. **(Ratledge and Dover, 2000);** Also, all tested *E. coli* isolates harbored *fim*H virulence genes (100%). These results were similar to those reported by **Preethirani et al., (2015);** while, it was 92% **(Mahlangu et al., 2018),** 89% **(Mshelia et al., 2014; Mishra et al., 2018)** of uterine cases

5. Conclusion

Mastitis and endometritis in sheep and goats constitute an enormous animal health problem, in which *E. coli* is the main etiological agent. There is a strong association between the phenotypes and genotypes of AMR in *E. coli* isolates that can produce biofilm, and it is a fundamental problem for dairy farms where it affects the udder health and uterine tissue. The prevalence of virulence genes and high levels MDR reinforce the huge significant role of *E. coli* infections.

6. Authors Contributions

All authors contributed equally to study design methodology, interpretation of results and preparing of the manuscript.

7. Conflict of Interest

The authors declare no conflict of interest.

8. References

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