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Escherichia Coli associated with Environmental Mastitis in Sheep and Goats Farms: Probable Sources, antibiogram profile and Molecular characterization

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A B S T R A C T

The current study was conducted to determine the role of E. coli in causing subclinical mastitis in sheep and to determine the role of bedding and water as sources of E. coli associated with environmental mastitis in sheep and goats. In addition, determination of the antibiotic sensitivity of some E. coli isolates to select the suitable antibiotics for treatment of mastitis and finally, application of PCR for detection of some virulence genes. Escherichia coli were isolated from milk, bedding and water of sheep farms at the rate of 8.67, 16 and 10%, respectively while it were isolated from milk, bedding and water of goats farms at the rate of 15.33, 24 and 10%, respectively. Moreover, Twenty E. coli isolates were serologically identified revealing the presence of serotypes; O114:H21, O111:H4 and O124. Results of antibiogram test performed for E. coli O114:H21 clarified that it was highly sensitive for Ciprofloxacin (CIP 5 µg), Enrofloxacin (5 µg) and Oxytetracycline (30 µg). E. coli isolates were tested by multiplex PCR for detection of stx1 (555 bp) and eaeA (425 bp) genes for characterization of Enteropathogenic E. coli successfully. Finally, it was concluded that, E. coli could be isolated from the examined samples of milk, bedding and water of different farms under investigation. Also, it was noticed that the isolation rates of bacteria was higher in goats farms compared to sheep farms which may be due to the defects in the hygienic conditions inside these farms.

Keywords: Escherichia Coli, Environmental Mastitis, Sheep and Goats.

1. Introduction

Small ruminant production plays an important role as an income generating activity, particularly for the smallholders, whilst being a source of animal protein to support the national program (Windria et al., 2016).Mastitis is an important disease of sheep and goats because it decreases the amount and quality of the milk produced by a dairy animal and reduces weight gain in lambs and meat kids. Also, it can affect animal wellbeing. Furthermore, mammary infections are the primary causes of 'Milk-drop syndrome in ewes' (>85% of all causes), the syndrome has been defined as a pathological entity at flock level, characterized by reduced milk yield of lactating ewes, with no clinical signs specific to a disease (Giadinis et al., 2012). Mastitis is a term describes the inflammation of mammary glands. It is usually observed shortly after lambing until the post-weaning period and leads to some physical changes in the affected udder including; swelling, warmth and sometimes painful sensation when touch. In severe cases, blood supply to the udder is affected and a blue discoloration may result, hence it is named "blue bag" (Khan and Khan, 2006).Mastitis can either be clinical or subclinical. Clots or serum in the milk are signs of clinical mastitis. In addition the udder may become swollen, hot and/or tender to the touch (Minguijón et al., 2015). Subclinical mastitis is only detectable using a test such as the California Mastitis Test (CMT) or counting inflammatory cells in the milk or culturing milk in the laboratory (Gelasakis et al., 2015).

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In lactating dairy goats, the inflammation of the mammary gland is one of the most common infectious diseases. It is responsible for important economic losses and it can reduce yield and quality of the milk (Zaninelli et al., 2015), therefore, mastitis is one of the important pathologies in goats with serious financial consequences (Bourabah et al., 2013).

Premature culling of ewes is an inevitable result of mastitis due to loss of udder function or reduced milk yield or quality, the same reasons may result also in decreasing the performance of lambs and kids. Moreover, mastitis is a direct cause of death of severely affected females and result in high costs for diagnoses and treatment (Giadinis et al., 2012).

Environmental mastitis refers to the intra-mammary infections caused by organisms that survive in the female's surroundings such as soil, manure, bedding, water, or on body sites of the female other than the mammary gland. This type of infection is usually opportunistic; in another word it occurs when circumstances enhance environmental contamination and change the mammary gland's susceptibility to infection.

Mastitis occurred more frequently in farms managed under an intensive or semi-intensive system. Management system includes various aspects of flock husbandry and organization (e.g. housing facilities, nutrition, milking routine, animal breed); many of these have been described to affect development of mastitis in ewes. Also, "management system" encompasses many factors, which can contribute to subclinical mastitis or its control (Vasileiou et al., 2019).

There are several bacteria which are known to cause mastitis in sheep and goats including Streptococcus sp., Staphylococcus sp., Pasteurella sp., and coliforms, such as E. coli. The exact type of bacteria that is causing the mastitis can only be determined by laboratory analysis (Tolone et al., 2016). Genera classified as coliform are E. coli, Klebsiella and Enterobacter. Other Gram-negative bacteria frequently isolated from intramammary infections include species of Serratia, Pseudomonas, and Proteus (Koneman et al., 1983). The point sources of coliform bacteria that cause infections include bedding materials, soil, manure and other organic matter in the environment of cows (Hogan and Smith, 2003).

Good udder and teat conformation can help to reduce the risk of mastitis. One way to help prevent mastitis is to keep milking and living areas clean. Also, post dipping teats after milking can greatly reduce the risk of mastitis in milking does and ewes.

Treatment of mastitis is generally done with the use of either injectable or intramammary antibiotics. There are no antibiotics that are labeled for use in sheep or goats for the treatment of mastitis. Therefore all treatment of mastitis for sheep and goats is considered extra-label and must be done on the advice and under the supervision of a veterinarian.

So the aim of the current paper was determination of the frequency of isolation of E. coli causing environmental mastitis in sheep and goats beside determination of the role of soil and water in transmission of these bacteria.

2. Materials and Methods

2.1. Collection of samples:

2.1.1. Milk samples:

A total of 455 milk samples collected from sheep farms were screened by California mastitis test (CMT) to obtain 150 positive milk samples. Also, 485 milk samples collected from goats' farms were screened by CMT to obtain 150 positive milk samples to be examined bacteriologically for isolation of E. coli. Udders were cleaned before sample collection then

each teat end was scrubbed with a pledged of cotton moistened with betadine. A separate pledged of cotton was used for each teat. The first few streams of milk were discarded. After testing, positive CMT samples were placed in an ice box and transported directly to the laboratory. In a sterile test tube, 20 ml of each milk sample were centrifuged at 10.000 rpm for one minute then sediments were obtained and 5 ml of nutrient broth were added. Finally, they were incubated at 37°C for 24 hours for microbiological examination (Schalm et al., 1971).

2.1.2. Bedding samples:

A total of 100 bedding samples were collected from different sites of animal's yards (50 samples from sheep yards and 50 samples from goats' yards) particularly from wetted areas with high moisture and organic matter load. Samples were taken at depth of 5 cm from soil surface in sterile glass bottle fitted with sterile glass stopper according to Clegg et al., (1983). Samples were transferred to the laboratory where each sample was subjected to thorough mixing, and then one gram was weighed and triturated well in a sterile mortar with 99 ml of sterile buffered peptone water (BPW) then aseptically strained through sterile gauze. The obtained filtrate was incubated at 37°C for 24 hours for microbiological examination.

2.1.3. Water samples:

A total of 100 water samples were collected from different sites of animal's yards (50 samples per each species) where 10 representative samples were taken from water in front of the animals per visit. Samples were collected by using sterile plastic syringes (20 ml capacity) (Moubarak, 1989). Each sample was labeled and identified to its source, site and type of watering system.

2.2. California mastitis test (CMT) (Quinn et al., 2004):

A small amount of milk from each quarter is squired into shallow cups in the CMT paddle, an equal amount of 3% CMT reagent was added to each cup and mixed well. A gentle circular motion was applied to a mixture in a horizontal plane for 15 seconds. Finally, the reactions were graded as negative, trace, 1+, 2+, and 3+.

CMT score	Visible reaction	Interpretation
0	Milk fluid and normal	Negative
±	Slight precipitation	Trace
1+	Precipitation but no gel formation	Weak positive
2+	Gel formation	Distinct positive
3+	Increased viscosity and gel cohesive with the surface	Strong positive

2.3. Isolation and identification of E. coli:

The collected samples and swabs were incubated in nutrient broth at 37°C for 18-24 hours. A loopful from each incubated sample was streaked directly onto MacConkey Agar and Eosin methylene blue Agar (EMB). The inoculated plates were incubated at 37°C for 24-48 hours under aerobic conditions (Cruickshank et al., 1975). Suspected colonies that grow on incubated plates were picked up and subculture onto nutrient agar slants and incubated at 37°C for 24 hours then stored in refrigerator for more identification. Isolates were subjected to biochemical identification according to Quinn et al., (2011). The isolates were serologically identified according to Kok et al. (1996) by using rapid diagnostic E. coli antisera sets (DENKA SEIKEN Co., Japan).

2.4. In vitro sensitivity of E. coli isolates to certain antimicrobials:

Nine commonly used antibiotics for control of mastitis were used in the present study to determine the antibiogram pattern of the obtained isolates of bacteria including; Ciprofloxacin (CIP) (5 µg), Flumequine (30 µg) (UB), Enrofloxacin (5 µg) (ENR), Doxycycline (30 µg) (DO), Sulphamethoxazole-Trimethoprim (23.7 + 1.25 µg) (SXT), Oxytetracycline (30 µg) (OT), Amoxicillin (10 µg) (AML) and Erythromycin (15 µg) (E). The antimicrobial susceptibility test was performed for E.coli serotypes O114:H21 and E.coli serotypes O124 isolates. Standard agar disk diffusion method was employed according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS), (2012) using commercial antibiotic disks (Oxoid).

2.5. Multiplex PCR for characterization of virulence factors of E. coli:

2.5.1. Oligonucleotide primers sequence required for PCR:

Virulence factors of E. coli, including, stx1 and eaeA. Amplification was performed with consensus primers for ampC, which recognize any plasmid-borne cephamycinase genes derived from the chromosomal ampC gene of Citrobacter freundii (Winokur et al., 2001). Sequence of primers was listed in the following table;

Virulence factor	Primer sequence (5'-3')	Size of product (bp)	Reference
stx1	TTCGCTCTGCAATAGGTA	555	Franck et al., (1998)
	TTCCCCAGTTCAATGTAAGAT		
eaeA	ATATCCGTTTTAATGGCTATCT	425	
	AATCTTCTGCGTACTGTGTTCA		

3.2.4.1. Extraction of DNA from bacteria isolates (Sambrook et al., 1989): It was performed by using the boiled cell method for extraction of DNA from bacterial isolates. Bacterial isolates were cultured in nutrient broth at 37oC for 12 hours and then 1 ml of distilled water was added. Bacterial suspension was centrifuged at 10000 xg for 5 minutes then bacterial pellet was resuspended in distilled water by using vortex and boiled in water bath for 15 minutes to ensure lysis of cells and complete denaturation of DNA. Finally, centrifugation at 10000 xg for 5 minutes then the supernatant was collected as DNA template.

2.5.2. Preparation of PCR reaction:

Oligonucleotide primers set were dissolved in nuclease free water to obtain 20 pmol / µL concentration (Lee et al., 2008). The melting temperature™ of the primers was given by the manufacturer. The reaction was included in a total volume of 25 µl in 0.5 µl Eppendorf tube according to iNtRON´s Maxime PCR PreMix Kit as follow;

PCR reaction mixture	Volume/reaction
2x PCR Master mix Soln.	12.5 µl
Template DNA	5 µl
Forward primer (20 pmol)	1.25 µl
Reverse primer (20 pmol)	1.25 µl
PCR grade water	5 µl
Total	25 µl

2.5.3. PCR cycling program:

The tubes were placed into the thermal cycler that already programmed. The PCR cycle conditions were optimized for each primer set. Briefly, the PCR cycling conditions consisted of initial denaturation at 94° C for 5 min, followed by 30 cycles each of denaturation at 94° C for 30 s, annealing at the temperature optimal for each primer set for 45 s, and extension at 72° C for 45 s. At the end of cycling, the tubes were stored at - 20 °C until needed for electrophoresis.

2.5.4. Detection of PCR products (Sambrook et al., 1989):

Once the PCR was completed, the prepared agarose was melted, left to cool till 55˚C and poured into the assembled horizontal gel tray after proper installation of the desired comb. Accurately, 10µl of each PCR products and negative control were loaded on the gel wells. One well of the gel was loaded with 10µl of DNA ladder. The run for electrophoresis was done at 100 volt for 1 hour in TBE buffer. The gel was stained with 0.5µg/ml ethidium bromide for 30-45 minutes at room temperature. Finally, Specific amplicons were observed under ultraviolet trans illumination compared with the DNA ladder and photographed.

4. Discussion

Mastitis in small ruminants is predominantly subclinical so bacterial culturing provides a standard tool in the diagnosis of mastitis (Contreras et al. 2007) and for economic and practical reasons; only one sample of milk was taken to diagnose mastitis (Olechnowicz and Jaskowski 2014).

Gram-negative bacteria that commonly cause mastitis are classified as environmental pathogens. The point sources of coliform bacteria that cause infections include bedding materials, soil, manure and other organic matter in the environment. Rates of coliform mastitis increase during climatic periods that maximize populations in the environment. The portal of entry into the mammary gland for Gram-negative bacteria is the teat canal. E. coli is the predominant coliform species causing intramammary infections.

Data presented in Table (1) illustrated the isolation of E. coli causing mastitis from the examined samples of sheep and goats farms. E. coli were

isolated from milk, bedding and water of sheep farms at the rate of 8.67, 16 and 10%, respectively while it were isolated from milk, bedding and water of goats farms at the rate of 15.33, 24 and 10%, respectively.
The recorded results were in harmony with those of Mork et al. (2007) who could isolate E. coli at a percentage of 7.3% and Gebrewahid et al. (2012) who found that the frequency of E. coli was 17.0%. Also, they were in accordance with the results of Abdallah et al., (2018) who isolated E. coli from SCM ewes' milk samples with a percentage of 44.4%, and isolated E. coli from SCM does' milk samples with a percentage of 26.8% and Majeed, (2020) who could isolate E. coli with a percentage of 37.2% from milk of SCM sheep.
Isolation of E. coli from milk, water and soil confirmed the role of environment in causation of SCM in sheep and goats. Virdis et al., (2010) decided that poor management and bad hygiene are known to be extrinsic factors to the entry of infectious agents
Serological identification of E.coli isolated from sheep and goats farms was presented in Table (2). Twenty E. coli isolates obtained from the examined samples of sheep and goats farms were serologically identified revealing the presence of serotype O114:H21 that was identified from milk, bedding and water samples of sheep farms and milk and water samples of goats farms, serotype O111:H4 that was identified from milk and bedding samples of sheep farms and milk and water samples of goats farms, serotype O124 that was identified from milk and water samples of sheep farms and milk, bedding and water samples of goats farms, beside two isolates that could not be identified.
Development of natural bacterial resistance to different types of antibiotics could lead to arising of multidrug-resistant bacteria that causing financial and economic implication, due to non-effective treatment and spread drug-resistant bacteria from animals to animals. Although the new generation of advanced antibiotics was produced by pharmacological cooperation, still drug-resistant bacteria have been increasing.
Conventional treatment of mastitis using antibiotics is costly and has led to the emergence of antimicrobial resistance (AMR) against most of the commonly used antibacterial agents (Jingar et al., 2017). Not only AMR is regarded as a serious threat to global public health and food security, but it also increases animal suffering and production losses (Alekish et al., 2013).
Antimicrobial sensitivity patterns are an important component of the decision making process in determining appropriate antimicrobial therapy against bacterial infection of the mammary gland.
Results of antibiogram test performed for E. coli O114:H21 obtained from sheep and goats farms were illustrated in Table (3). They clarified that E. coli O114:H21 was highly sensitive for Ciprofloxacin (CIP 5 µg), Enrofloxacin (5 µg) and Oxytetracycline (30 µg) while it was quite sensitive for Flumequine (30 µg), Doxycycline (30 µg), Sulphamethoxazole-Trimethoprim (25 µg), Amoxicillin (10 µg) and Erythromycin (15 µg). On the other hand, E. coli O124 was found to be highly sensitive for Doxycycline (30 µg) and Erythromycin (15 µg) while it was quite sensitive for Enrofloxacin (5 µg), Amoxicillin (10 µg) and Oxytetracycline (30 µg) and it was moderately sensitive to Flumequine (30 µg), Sulphamethoxazole-Trimethoprim (25 µg) and Ciprofloxacin (CIP 5 µg).
The antibacterial sensitivity test results in this study indicated the widespread resistance among E. coli isolates against most commonly used antibacterial agents. All isolated strains of E. coli were found resistant to several different antibacterial agents. Recent studies involving dairy cows and sheep in Jordan indicated an alarmingly increasing incidence of AMR (Zuhair, 2017). Multidrug resistance patterns of E. coli similar to the one reported here have been reported previously in many parts of the world (Jahan et al., 2015).
The recorded results were nearly similar to that recorded by Mahmoud et al., (2015) who found that the most effective antimicrobials affecting E.coli isolated from mastitis were lincospectine (56.6%), danofloxacin (56.6%), Florphenocol (43.3%) ceftifure (40%) and enrofloxacin (40%). While, the least effective antibiotics were oxytetracycline, ampicillin as the E.coli was completely resistant to them.
Differentiation of pathogenic strains from normal flora strains depends on the identification of virulence characteristics. Virulence factors associated with strains of E. coli include adhesions, toxins, cell wall, capsule production, and serum resistance (Gyles, 1993).
In the present study virulence factors were discussed because it thought to play an important role in pathogenicity of E. coli. Isolates of E. coli were tested by multiplex PCR for detection of stx1 (555 bp) and eaeA (425 bp) genes for characterization of pathogenic E. coli. The results photographed in Photo (1) showed that PCR products of isolates include positive control, resulted in Lanes 1, 2 and 3 E. coli positive strains for eaeA gene, Lanes 4, 5 and 6 showed E. coli positive strain for stx1gene. This finding agreed with the results of Kaipainen et al. (2002); Zaki et al. (2004); Fernandes et al. (2011) and Lamey et al. (2013).

5. Conclusion:
From the results presented in this study it could be concluded that, environmental bacteria causing mastitis including; E. coli in sheep and goats could be isolated from the examined samples of milk, bedding and water of different farms under investigation. Also, it was noticed that the isolation rates of bacteria was higher in goats farms compared to sheep farms which may be due to the defects in the hygienic conditions inside these farms.
In addition, variations observed in the activity of the antibiotics tested against bacterial isolates confirmed the importance of antibiotic susceptibility tests, performed together with the identification of the bacterial agents for the selection of antibiotics of low probability of resistance to the suspected pathogenic organisms that cause mastitis. Moreover, PCR is a useful diagnostic tool for detection of virulence genes because it sensitive and specific so this technique should be adopted as a complementary tool to conventional tests.

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Table (1): Rate of isolation of E. coli from sheep and goats farms

Escherichia coli	**Milk**			**Bedding**			**Water**			**Total**		
	No.	**+ve**	**%**	**No.**	**+ve**	**%**	**No.**	**+ve**	**%**	**No.**	**+ve**	**%**
Sheep	150	13	8.67	50	8	16.0	50	5	10.0	250	26	10.4
Goats	150	23	15.33	50	12	24.0	50	5	10.0	250	40	16.0

Table (2): Serological identification of E.coli isolated from sheep and goats farms

Source of isolates / Serotypes	**Sheep (10 isolates)**			**Goats (10 isolates)**		
	Milk (n=5)	**Bedding (n=3)**	**Water (n=2)**	**Milk (n=5)**	**Bedding (n=3)**	**Water (n=2)**
O_{114}:H_{21}	4	1	1	1	0	1
O_{111}:H_4	1	1	0	1	0	1
O_{124}	1	0	1	1	2	1
Non serotyped	0	0	0	0	1	1

Table (3): Results of antibiogram test performed for E. coli O114 and E. coli O124 serotypes obtained from sheep and goats farms

***E.coli* serotypes / Antimicrobial discs**	**O_{114}**		**O_{124}**	
	Inhibition Zone	**Sensitivity**	**Inhibition Zone**	**Sensitivity**
Ciprofloxacin (CIP 5 µg)	12 mm	++++	9 mm	++
Flumequine (30 µg)	11 mm	+++	9 mm	++
Enrofloxacin (5 µg)	13 mm	++++	11 mm	+++
Doxycycline (30 µg)	8 mm	+++	13 mm	++++
Sulphamethoxazole -Trimethoprim (25 µg)	11 mm	+++	7 mm	++
Oxytetracycline (30 µg)	12 mm	++++	10 mm	+++
Amoxicillin (10 µg)	10 mm	+++	10 mm	+++
Erythromycin (15 µg)	11 mm	+++	14 mm	++++

- = Resistance + = weakly sensitive ++ = moderately sensitive
+++ = quite sensitive ++++ = highly sensitive.

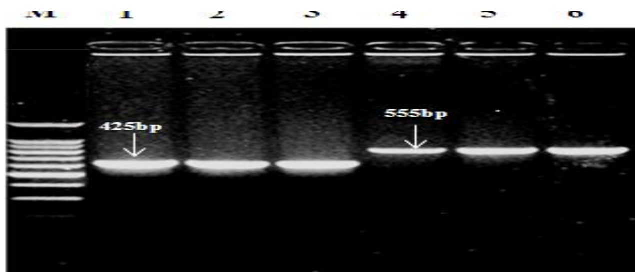

Photo (1): Sampler of Agarose gel electrophoresis of multiplex PCR of stx1 (555bp), and eaeA (425bp) genes for characterization of pathogenic E. coli. Lane M: 100 bp ladder as molecular size DNA marker,
Lanes 1-3: Positive strains for eaeA gene,
Lanes 4, 5 and 6: Positive strains for stx1 gene.