Journal Homepage: -www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI:10.21474/IJAR01/7910
DOI URL: http://dx.doi.org/10.21474/IJAR01/7910

RESEARCH ARTICLE

STAPHYLOCOCCUS AUREUS ANDESCHERICHIA COLICAUSING SUBCLINICAL MASTITIS IN SHEEP AND GOATS.

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Manuscript Info

Manuscript History
Received: 12 August 2018
Final Accepted: 14 September 2018
Published: October 2018

Key words:
Mastitis,*S.aureus*,*E.coli*,prevalence, PCR, Antibiotic resistance

Abstract

Mastitis is one of the most serious economic problems in all dairy sheep and goats flocks. Examination of 110 milk samples collected from apparently healthy sheep and goats using the California mastitis test (CMT) showed that 49 (44.5%) were CMT positive. Standard methods for isolation and identification of *S.aureus*and *E. coli* could identify 3 and 9 strains respectively. Five different serogroups weresuccessfully identified among 9 *E. coli* strains, the serogroups were O26 (33.3%), O1 (22.2%), O114 (22.2%), O148 (11.1%) and finally O125 (11.1%). The antimicrobial susceptibility results for erythromycin and Cefoxitin revealed that all *E.coli* isolates were resistant to erythromycin (100%) followed by high frequencies of resistance observed to Cefoxitin 8 isolates (88.9%). All *S.aureus* isolates wereCefoxitinresistance while no resistance pattern was detected to erythromycin. The 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 the B2 group and 1/6 was A group. The genotyping identification using *nuc*can identify 2 strains only of *S.aureus* isolates considered it of low identification power than use the traditional biochemical tests and Vitek 2 System identification in vitro.

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 diagnosisof 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.

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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) .

The specific target in the identification of *S. aureus* by PCR is using the*nuc*gene, that encodes thermonuclease (Maes*et al*., 2002; Louie *et al.,* 2002).

The present study was undertaken for isolation and identification of *S. aureus*and *E. coli*to record the prevalence of theirinfection in subclinical mastitic sheep and goats. The serotyping of *E.coli*isolates was occurred and characterized referring to their phylogenetic groups by PCR. Using of *nuc* gene for genotypic identification of *S.aureus* isolates.Then performing the antibiogram susceptibility test on the isolates against cefoxitin and erythromycin.

Material And Methods:-

Collection of samples

One hundred and ten milk samples collected from apparently healthy sheep and goats under aseptic condition. 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 drawn from each half into sterile screw-capped bottles. Samples were submitted to the Californiamastitis test (CMT) to indicate the frequency of subclinical mastitis.By mixing two ml of a Schalm reagent with an equal volume of milk. The reaction was measured visually during 10 seconds according to the formation of a precipitate or gel formation. Then sending the positive CMT ones to the bacteriology laboratory for*S.aureus*and *E. coli* isolation.

Isolation and identification of the causative agents

Milk samples were incubated aerobicallyat 37°Cfor 24hr thencentrifuged at 3000 r.p.m. for 20 minutes.The cream and supernatant fluid were discarded and a loopful from milk sediment was streaked onto blood and MacConkey's agar plates and incubated for 24 hours at 37ºC. Suspected lactose fermented *E.coli* colonies were picked up and streaked on Eosin methylene blue (EMB) agar plates while the grower colonies on blood agar were streaked on mannitol salt agar specific for *S.aureus* then incubated for another 24-48 hours at 37ºC. The suspected purified *S.aureus* colonies were picked up for examination by biochemical testsaccording to (Quinn *et al*., 1994) especially using Coagulase test (slide and tube methods) then confirmed by the Vitek 2 System. The purified *E.coli*colonies were for examined by biochemical testsaccording to(Quin*et al*., 2002).

Serological typing of E.coli

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

Antimicrobial susceptibility testing

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

DNA extraction:

Six *E.coli* and 3 *S.aureus*isolates were inoculated into brain heart infusion broth and incubated for 24 hours for genotypic identification. DNA was extracted according to QIAamp 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*Aand *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.**

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	**Jeong*et al*., 2012**
***Yja*A**	TGA AGT GTC AGG AGA YGC TG ATG RAG AAT GCG TTC CTC AAC	211 bp	
***tsp*E4C2**	GAG TAA TGT CGG GGC ATT CA CGC GYC AAC AAA GTA TTR CG	152 bp	

Genotyping identification of *S.aureus* strains:
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 were electrophoresed on 1.5% agarosegel in tris acetate EDTA and visualized by UV transilluminator.The gene (*nuc*) was detected by PCR. Theprimer sets used for this procedure are listed in **Table(2).**

Table 2:-Primer Used For Pcr Amplification And Amplified Product.

Gene	Sequence (5`→3`)		Amplified product (bp)	Reference
Nuc	F	ATATGTATGGCAATCGTTTCAAT	395 bp	**Gao *et al*., 2011**
	R	GTAAATGCACTTGCTTCAGGAC		

Results:-

The prevalence of subclinical mastitis and bacteriological identification of S.aureus and E. coli:
The obtained results revealed that out of 110 examined milk samples, 49 showed California mastitis test (CMT) positive with a percentage of (44.5%) (on samples level). CMT positive samples were examined for bacteriological identification of *S.aureus* and *E. coli* as shown in **Table (3)**. 3 *S.aureus* and 9 *E. coli* isolates were detected with a percentage of 6.1% and 18.4% respectively.

Serological typing of E. coli isolates:
The results of serological identification of 9 *E. coli* Isolates revealed that the serogroups were O26 (33.3%), O1 (22.2%), O114 (22.2%), O148 (11.1%) and finally O125 (11.1%). **Table (4).**

Antimicrobial susceptibility testing:
The results revealedthat all *E.coli* isolates showed absolute resistance to Erythromycin (100%) followed by high frequencies of resistance observed to Cefoxitin (88.9%). All *S.aureus* isolates wereCefoxitin resistance. Cefoxitin is tested as a surrogate for oxacillin; which is reported susceptible or resistant based on the cefoxitin result to detect methicillin resistance *S.aureus* (MRSA). While no resistance pattern was detected to erythromycin.

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 belonged to group A **(Fig.1)**.
Genotypic identification Staphylococcus aureus by PCR:
Only two strains were detected by using *nuc*gene as showed in **(Fig. 2)**.

Results:-

The subclinical mastitis cannot be detected by clinical methods, so the diagnosis of subclinical mastitis is based on 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, the prevalence of subclinical mastitis in sheep and goats were 43.8% and 45.2% respectively according to "samples level" **(Table 3).** Similar results were recorded byMoawad and Osman (2005) who found it as 31.63% in regards to examined glands of dairy ewes. Also,Schaeren and Maurer (2006) recorded it

as 40% of goats halves. Windria*et al*. (2016) determined it as52.7 % among goat milk samples. Similar results also were reported by Hawari*et al.* (2014) as 55.5% in milk samples from Awassi Sheep. Different resultswere recorded by other authors as Rahman*et al*. (2016) as 9.50% and Mugabe *et al.* (2017) as 13.5% in goats.These differences in the incidence of mastitis may be attributed to some factors including climatic, a sanitary and managemental factors (Johsi and Gokahal2006).

In the present investigation, out of 49 positive CMT examined milk samples from sheep and goats flocks,*E.coli* isolates were 4 and 5 with an overall prevalence rate as 19% and 17.9% from sheep and goats respectively. Similar resultswerereported by Moawad and Osman (2005) and de Garnica*et al*. (2013) who recorded the incidence as 14.10 % and 17.4% in subclinical mastitic sheep, but disagrees with Acik*et al*. (2004) who reported the incidence of 1.7 % in subclinical mastitic sheep,Hussien(2003) as 5.9% in Egypt andFotou*et al*.(2011**)** who reported the incidence as5%.

Regarding E coli, the current results agreed with Bayoumi, Faten*et al.* (2005) and Najeeb*et al.* (2013) who reported the incidence of 15 % and 10.96% in subclinical mastitic goats. But it disagreeed with El- Bassiony*et al*. (2008) who reported the incidence of 5.13 % and Mugabe *et al*. (2017) who detected E. coli with an incidence of 36%.The rate of prevalence of coliformmastitis especially that caused by *E. coli* in the previous studies were differed 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, O26, O1, and O114 were the most predominant serogroups with a percentage of 33.3%, 22.2% and 22.2% respectively, followed by O125 and O148 with percentage 11.1% for each. Many of *E. coli* isolates detected in this study belonged to classical Enteropathogenic.*E.coli* (E.P.E.C.) serogroups as O114 and O26 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. The most important serogroups of *E.coli*(026) was previously reported by several investigations as Murphy *et al.*, (2007), Osman, Kameli*et al*.,(2012) and Lamey, Amira*et al*.,(2013).

Phylogenetic assay and virulence genes detection by PCR had classified 5/6*E.coli* strains into the B2 group while 1/6 of the examined strains belong to A group. Presence of some pathogenic strains in group A which is a non-pathogenic group of *E.coli* suggests that this strain could have a commensal origin but may become pathogenic through a horizontal acquisition of virulence-relatedgenes (Ewers *et al*., 2007).

The low incidence of isolates from subclinical mastitic sheep and goats in this study by Gram-positive cocci *S. aureus*as 9.5% and 3.5% respectivelywas agreed with Hussein, (2003); Arsenault *et al.*,(2008) and Aydin*et al.*, (2009). Thislow incidence may be due to the presence of CoNS can tend to occupy attachment sites in the teat duct of small ruminant required by coagulase-positive staphylococci Markey *et al.,* (2013).

In this work, results showed that the genotyping identification using *nuc*can identify 2 strains of *S.aureus* which may generate false-negative results. While by the traditional bacteriological identification and using of the Vitek 2 System can be detected 3 strains of *S.aureus*. This difference may be due to the variations in the DNA sequence of the *nuc* genethat leads to misidentification of the *nuc* gene of *S.aureus* isolate by PCR(Hoegh*et al*., 2014).

High level of Erythromycin resistance in *E.coli* isolates was also reported in many previous studies.Isnel and Kirkan (2012) reported it as 65% from goats, Bourabah*et al*.,(2014) as 66.66%in Tiaret province and El Ayis and Fadlalla, (2017) as 80% from ewes. A very high level of resistance to Cefoxitin was detected by another investigator Barbour*et al*., (2015) who found different percentages of resistance B-lactam antibiotics Cephalothin , Cefotaxime and Cefoxitin as 100%, 97% and 21% respectively.In the present work, antibiotic susceptibility test was performed on *S.aureus* isolates using disc diffusion method revealed that all *S.aureus* isolates were MRSA (100%) confirmed the use of cefoxitin for rapid detection of MRSAwhich agreed with Mohammed (2011).

Conclusion:-

In conclusion, Data from this study revealed a high prevalence of B2 pathogenic phylogroup*E.coli* strains in the milk samples. Using of traditional identification of *S.aureus* in vitro including the tube and slide coagulase tests and Vitek 2 System were more accurate than using of *nuc* gene for the identification.From the clinical view, the presence of highly antibiotic patterns in isolates showed that the increase and spread of antibiotics resistance are alarming to limit the random use of antibiotics in the veterinary field.

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		Identified *E.coli*		Identified *S.aureus*	
		No.	%	No.	%	No.	%
Sheep	48	21	43.8	4	19	2	9.5
Goats	62	28	45.2	5	17.9	1	3.5
Total	110	49	44.5	9	18.4	3	6.1

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

***E. coli* Serogroup**	**No. of isolates**	**Percent**
O1	2	22.2%
O26	3	33.3%
O114	2	22.2%
O148	1	11.1%
O125	1	11.1%

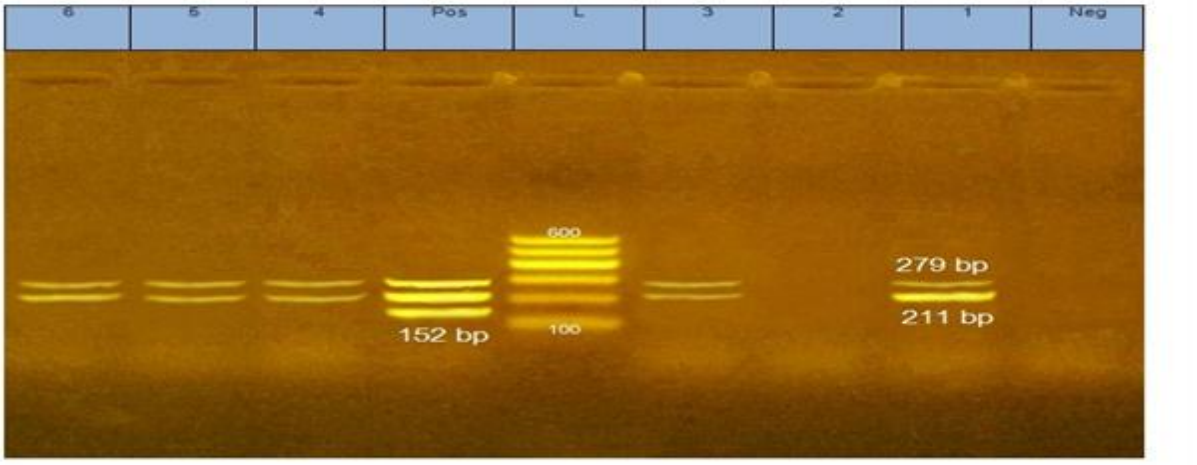

Fig. 1:-Agarose gel electrophoresis of triplex PCR-for amplification products of. *chuA* , *yjaA*genes and an anonymous DNA fragment *TSPE4.C2*. of*E.coli* isolates.

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.colichuA*and*yjaA*genespositive and negative for *TSPE4.C2*.
Lane 2 :*E.colichuA* , *yjaA*and *TSPE4.C2 negative* .

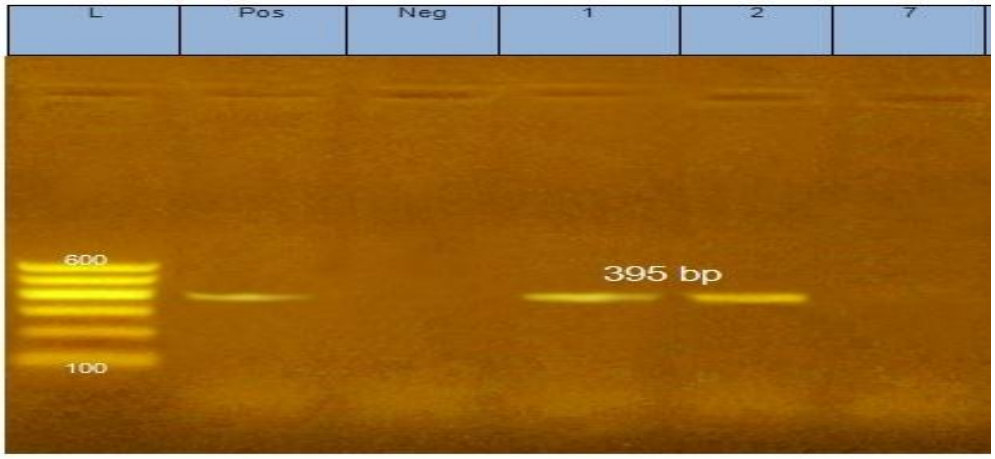

Fig. 2:-A: Agarose gel electrophoresis of PCR-for amplification products of *nuc*gene from *S. aureus*.**Lane L:**100 bp NA Ladder (Pharmacia). **Pos.:** positive control for *nuc* amplified at 395bp. **Neg.:** negative control.
Lane 1:S.*aureusnuc*. gene positive.
Lane 2: S.*aureusnuc*. gene positive, while
Lane 7:S.*aureusnuc*. gene negative.

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