Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 504 – 522
Received 25 April 2021; Accepted 08 May 2021.

Phenotypic and Molecular Detection of *Streptococcus Uberis* Isolated From Milk of Subclinical and Clinical Mastitis of Cow in Basrah City

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Abstract

Streptococcus uberis is found as commensals on the mucosa of the tonsils and intestinal tract of cows and responsible for approximately 20-30% of clinical mastitis cases in dairy cows .*S.uberis* is a gram positive coccus facultatively anaerobic and catalase negative and the key characteristics of *S.uberis* are the hydrolysis of esculine and production of acid on inuline ,*S.uberis* is responsible for infection in clinical and subclinical mastitis cows.Atotal of 100 milk samples was collected from clinical and subclinical mastitis cows from different dairy farm in Basrah province for detection of some phenotypic and genotypic characterization and detection of some virulence genes of *S.uberis* by PCR techniques and biofilm formation were assayed by quantitative microtiter plate. Antibiotic susceptibility performed using conventional assay method. Result revealed that ,The percentage of *S.uberis* isolated from the total examined milk sample was 46% in studding the phenotypic characteristic on sheep blood agar was found that alpha –hemolysis and negative camp factor like reaction and slime production as indictor for biofilm formation were detected in all the isolates .the study of virulence gene (sua 93.7%)(cfu 66.6%)(hasA 62.5%) in the present study usednine antimicrobial ,amoxillin(58.6%),ampicillin(100%),ciprofloxacin(69.5),ciftrixone(93.4),erythromycin(76%),trimethoprim(95%), gentamycin(100%),tetracycline(10.8),penicillin(45.6%).

Key words: *S.uberis;* mastitis; virulence factors; antimicrobial sensitivity; biofilm

Introduction

Bovine mastitis has been reported as the most important disease on dairy farms, because of the reduction of farm profitability, decreased milk production, discarded milk, treatment costs, and culling.[1]Essentially, there are two classes of mastitis: clinical and subclinical.Clinical mastitis is inflammation of the udder that can be observed by changes in the milk characteristics and in the normal constitution of the udder[2]Subclinical mastitis is inflammation of the udder that shows no external changes in the milk or on the udder[2].*S uberis* is a commensal bacteria present on the tonsil and intestinal mucosa of dairy cows in North America, Europe, and

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Australia. It is responsible for 20–30% of clinical mastitis cases in dairy cows.[3].

S. uberis is a Gram-positive coccus with a diameter of less than 2 m that grows by cell division in one plane, resulting in a linear array of nascent cells. Its nutritional requirements are complex and variable, indicating that it has evolved as a commensal or pathogen. It is facultatively anaerobic and catalase negative.[3].Colonies are convex, moist with dense centers. On sheep blood agar ,approximately 15% of isolates produce β-haemolysis. The hydrolysis of esculin is one of the most important biochemical diagnostic features of *S.uberis*.[4] and production of acid from inulin.*Streptococcus uberis* is the most common organism isolated from the mammary gland of non-lactating multiparous cows during the non-lactating period, and it is the cause of infection in clinical and subclinical mastitis cows..[5] It causes subclinical mastitis, which progresses to clinical mastitis, as well as acute and chronic mastitis in the infected mammary gland, which can last for more than one lactation.[6] During early lactation and at the end of lactaion, the occurrence of *S. uberis* mastitis is greatest.[7] Mastitis caused by *S. uberis* is also normal during the dry period before calving. *S. uberis* was found to be the cause of approximately 20% of mastitis cases at calving, and over 50% of quarters infected with *S. uberis* during the dry period developed clinical mastitis. Differences in the 16S and 23S rRNA genes differentiate *Streptococcus uberis* is distinct from the phenotypically similar but less common *Streptococcus* parauberis. As a consequence, it's well-equipped to deal with a variety of situations, including those encountered as a commensal. a pathogen of the mammary gland, a contaminant in the environment, or a pathogen of the bovine intestine[3]

This study is aimes to Isolation and identification of *Streptococcus uberis*, detection of some virulence genes from the isolated bacteria by PCR technique and characterization of biofilm formation .

Materials&Methods

Collection of milk samples

The current research contained a total of 100 milk samples, 50 of which were obtained from clinical mastitic cows and 50 from subclinical mastitic samples. Physical examination and a California mastitis test were used to obtain quarter milk samples from farms during a single visit during milking time (CMT).[8].

Bacteriological isolation:

Milk samples were streaked onchrom agarthen on 7 percent sheep blood agar, in a standard loop (Himedia, Mumbai, India). The plates were inoculated and incubated aerobically at 37°C. After 24-48 hours, the plates were tested for growth. The colony size, form, color, haemolytic

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characteristics, Grams staining, and catalase test, coagulase and oxidase were used to identify *Streptococci*, especially *S. uberis*.[9]

Phenotypic characterization of *S. uberis*:

1-Colony characteristic on Edward's media as selective medium for *S.uberis:*

Colonies that were primary identified as Streptococci were streaked on Edward's media plates as a selective medium, incubated at 37oC and examined after 24-48 h for growth and change in colour of the medium. The presence of growth, haemolysis and esculin hydrolysis (dark background) were indications of *S. uberis*. Then, colonies which grew on Edward's media were picked and streaked on macConkey agar, *S. uberis* was identified by the lack of growth on the agar. According to[9] the isolates were first described using standard biochemical tests.to . Since *S. uberis* is a fastidious bacterium, so it was sub-cultured on brain heart infusion agar for further PCR assays.

2-CAMP factor like reaction

Bacteria were screened for CAMP factor activity as previously described by[10]. Briefly, *S. uberis* strains were streaked perpendicular to a streak of β-haemolytic S. aureus on blood agar plates and after 6-20 h incubation at 37°C, they were observed for haemolysis.

Genotypic characterization of *S. uberis:*

1- DNA extraction from *Streptococcus* isolates:

The DNA was extracted and purified according to the instructions of the company (Geneaid, Lot No. FC26108-G/Korea). All *Streptococcus* speciesisolates had been grown in 5 ml of Brian heart infusion broth over night at 37 °C.

2- Detection of *HSP40* gene

Fourty six *S.uberis* isolated from animals were subjected for PCR assay to detect the presence of *Hsp40*, (Table 1) ,[11]

3- Detection of *SUA, CFU, HASA* virulence genes for *S. uberis*

All the isolates which were confirmed were subjected to PCR for the detection of genes that responsible for virulence *Sua, Cfu , HasA genes* in *S. uberis*, table (1),[12]

Primer name	Sequence 5-3 Reference	Target taxon/gene	T C	PRIMER SIZE

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Hsp40	AATTACGAGGTGCTGGACAA TTCTTGACCACTTGCCTCAG (chiang *et al*,2008)	*S.uberis*	62	119 bp
Sua	TCAACTTGACGAATCGCTTG TCAGCCATTGTTTCTGCTTG	Adhesion molecule	50	480pb
Cfu	TATCCCGATTTGCAGCCTAC CCTGGTCAACTTGTGCAACTG	CAMP factor	50	205bp
HasA	GAAAGGTCTGATGCTGATG TCATCCCCTATGCTTACAG	Hyaluronic acid capsule	44	319bp

Taple (1): Primer used in the study

Detection of slime production by Congo red agar method:

Cultivation of *S. uberis* isolates on Congo red agar (CRA) plates was used to assess slime production as a biofilm formation indicator. as described by[13]. The colony phenotypes of isolates were used to interpret them. Slime production was considered a positive indicator in black colonies with a dry consistency and rough surface and edges, whereas intermediate slime producers were black colonies with a smooth, round, and shiny surface and red colonies with a dry consistency and rough edges and surface. Colonies that are red and have a sleek, round look.

Quantification of biofilm formation on the microtiter plate

Biofilm formation was assayed phenotypically by the ability of cells to adhere to the wells of 96 –well microtiter plate as described by[14].Briefly ,A10ml of cell suspesion having 0.5OD600 was inoculated in 190 ml TSB medium in each well and 200ml was pourd into the wells .the negative control wells contained 200ml of TSB suplemented with 1% glucose.the tissue culture plates were incubated for 16 hours at 37C .after incubation plates are washed twice with phosphate buffer saline or sterile water and air dried .then added 200ml of crystal violet solution (0.2%)to all wells.after amint the excess crystal violet was removed and plates were washed twice and air –dried Finally, bound crystal violet was dissolved in 33% acetic acid.Biofilm,growth was monitored in terms of O.D(570nm) using,microplate reader .using ELISA reader .optical density cut off (ODc) was determined. It is defined as average ,OD of

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negative control +3*standard deviation(SD),of the negative control.Biofilm –production is considerd .

Non biofilm producer = (OD ≤ ODc),0

Weak biofilm producer = (ODc < OD ≤ 2* Odc),+

Moderate biofilm producer =(2*ODc < OD ≤ 4*Odc),++

Strong biofilm producer =(4* ODc < OD),+++

Antibiotic susceptibility testing of the isolated *S. uberis:*

Antimicrobial susceptibility of *S. uberis* strains to 14 antibiotics using Disk diffusion technique was performed according to the National Committee for Clinical LaboratoryStandards (NCCLS, 2008) on Mueller Hinton agar (Himedia, Mumbai, India) using commerciallyavailableantimicrobialtestdiscs.gentamycin,tetracyclin,pencilin,erythromycin,ceftrixone,amoxycillinResults were recorded by measuring the inhibition zonesand scored as sensitive, intermediate susceptibility and resistant according to the NCCLS recommendations.

Results

Bacteriological isolation:

A chromogenic medium for isolation and differentiation of *streptococcus* species as a specific bacteria that grow with different colony appearance. There are different variants of chromogenic agar which can be used for cultivation and sometimes for differentiation of bacteria. The results of bacterial isolation, show that *S. uberis* colonies appear as metallic blue fig (1). . After enrichment, the samples were streaked on sheep blood agar and incubated at 37 °C for 18 h. Subsequently, single colonies suspected to be *S. uberis* small and circular colonies with smooth edge , gives alpha –hemolysis fig (2). All sample were stained with Gram's staining and examined by alight microscope to notice Gram's reaction, shape and cell arrangements .The typical colonies that showed Gram-positive cocci occurring in long chain were taken as presumptive *streptococcus* fig (3)

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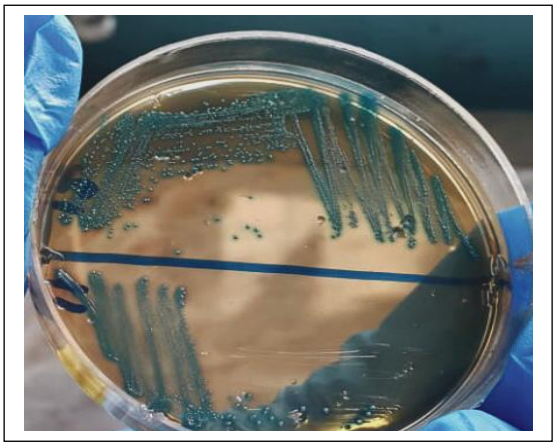
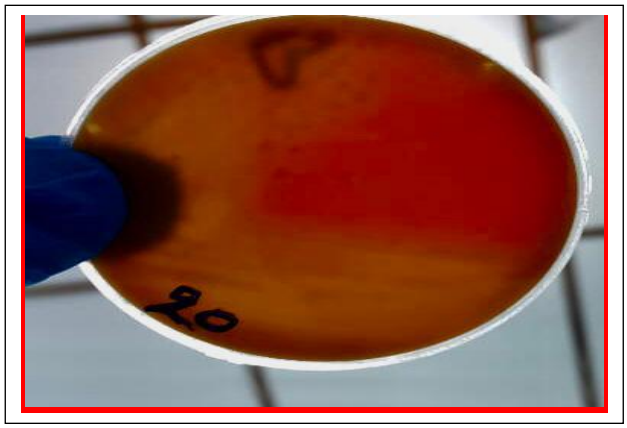
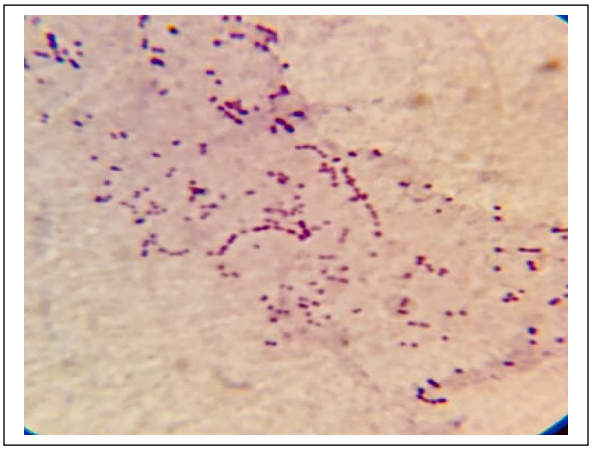


Fig (1) metallic blue colonies of *S. uberis* on chrom agar



Figure(2):*streptococcus uberis* on blood agar show alpha hemolysis



Figure(3) Gram staining of*streptococcus uberis*

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Biochemical test for the isolated bacteria .

Specific biochemical tests were used for detection of *streptococcui* these biochemical tests were catalase, oxidase ,coagulase all *streptococci* isolates showed negative catalase , coagulase and negative oxidase table(2)

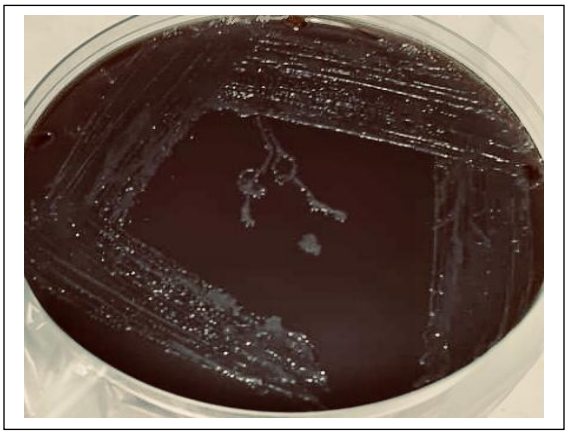
Table (2) biochemical test for the isolated bacteria

Bacteria	catalase	Coagulase	Oxidase
Streptococcus uberis	–	–	–

Phenotypic characterization of *S. uberis*:

1- Colony characteristic on Edward's media as selective medium for *S.uberis:*

The colonies that were identified on blood agar were streaked on Edward media plates and incubated at 37 ^{o}c and examined after 24-48 hours for growth and change in colour of the medium. The presences of growth, blue to metallic blue color and esculin hydrolysis (dark background) were one indication of *S. uberis* fig (4) .



Figure(4) *streptococcus uberis* on Edward media

2- CAMP factor like reaction

CAMP factor like reaction, *S. uberis* strains were streaked perpendicular to a streak of β-hemolytic *S. aureus* on blood agar plates . Enhanced hemolysis is indicated by an arrow head-shaped zone of beta-hemolysis at the junction of the two organisms , *S. uberis* show negative result , fig (5)

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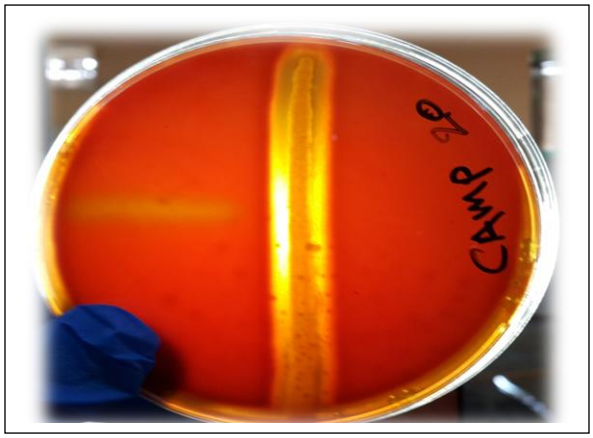

Fig (5) : Camp test in *Streptococcus uberis*(negative)

Numbers and percentage of *Streptococcus uberis*.

The results of bacterial identification depending on morphological ,microscopical and biochemical test ,show the percentage of ***Streptococcus uberis*** 56% from subclinical mastitis and 36% from clinical mastitis table (3) , fig (6)

Table (3):Numbers and percentage of *Streptococcus uberis* isolates from bovine mastitis

Sample source	NO.sample	NO.of *S . uberis*	%
Clinical mastitis	50	18	36
Subclinical mastitis	50	28	56
Total	100	46	92

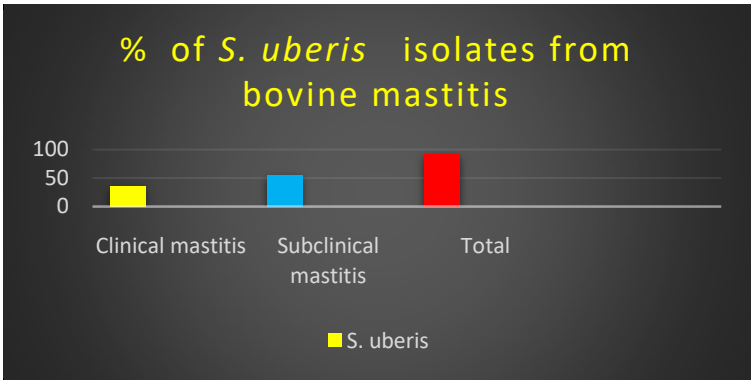

Fig (6) percentage (%) of *S. uberis* isolates from bovine mastitis

Genotypic characterization of *S. uberis:*

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1- DNA extraction from Streptococcus isolates:

A total of 46 *S. uberis* and 24 *S.agalagtia* isolates were identified by using conventional microbiological technique and biochemical tests , these isolates were subjected to DNA extraction fig (7) . The concentration of DNA was estimated by nanodrop

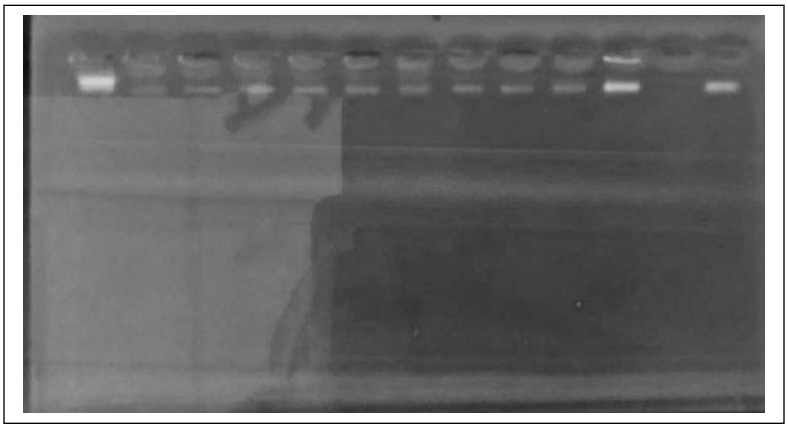

Fig (7) Total genomic DNA extracted from isolates using 2% agarose gel electrophoresis

2- Detection of *HSP40* gene

A total of 46 isolates were confirmed as *S. uberis*by detection of *HSP40* gene by PCR technique , the size of product was 119bp . The percentage of positive results for the genes was 100% , fig (8) .

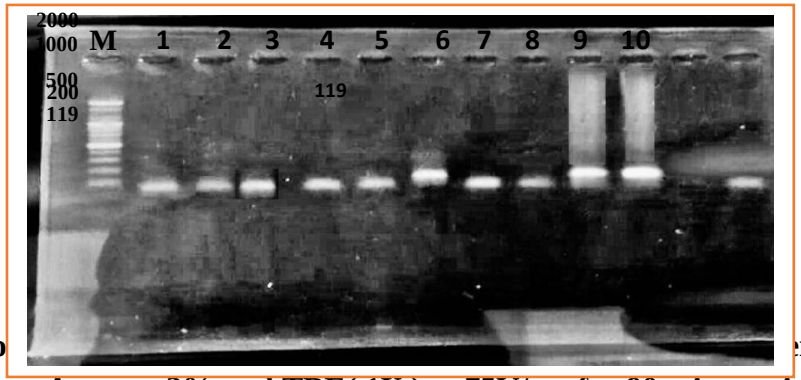

Fig (8) Gel electro ntional PCR (lan 1-12) .M. marker Agarose 2% and TBE(1X) at 75V/cm for 90 min . stained with safe stain, and on a UV transilluminator .

3- Detection of *SUA, CFU, HASA* virulence genes for *S. uberis*

All the isolates which were confirmed were subjected to PCR for the detection of genes that responsible for virulence *Sua, Cfu , HasA genes* in *S. uberis*The results revealed that *Sua* gene

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was found in 100% of *S.uberis* isolates ,displayed clear band of approximately 480bp . On the other hand the results of PCR amplification for *Cfu gene* and *HasA gene* were detected in 69.5 % and 65.2%,displayed clear band of approximately 205bp and 319bp respectively, fig (9,10,11) , table (4).

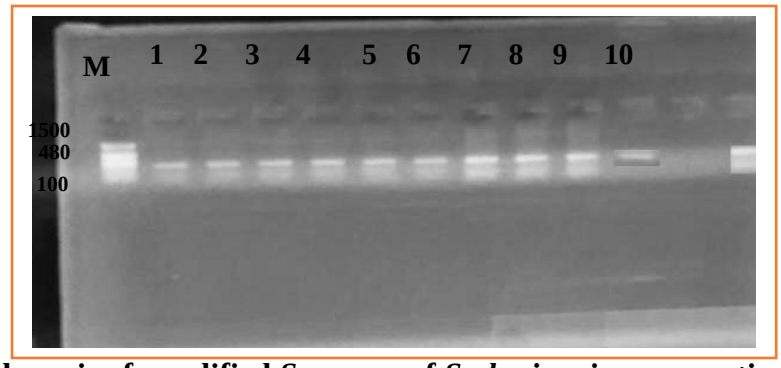

Fig (9) Gel electrophoresis of amplified *Sua* gene of *S.uberis* using conventional PCR (lan 1-12) .M. marker Agarose 2% and TBE(1X) at 75V/cm for 90 min . stained with safe stain, and on a UV transilluminator .

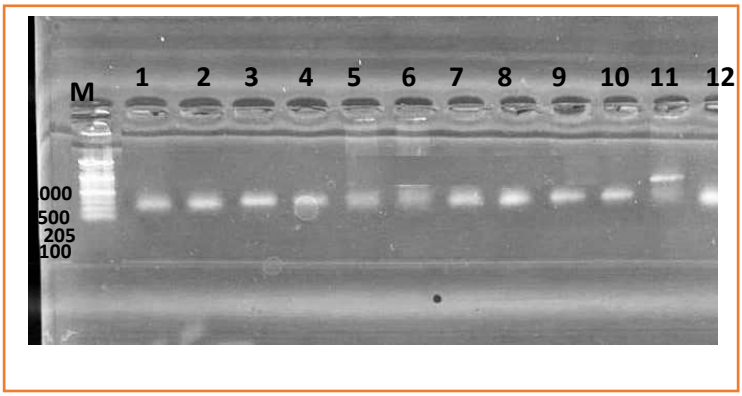

Fig (10) Gel electrophoresis of amplified *Cfu* gene of *S.uberis* using conventional PCR (lan 1-12) .M. marker Agarose 2% and TBE(1X) at 75V/cm for 90 min . stained with safe stain, and on a UV transilluminator .

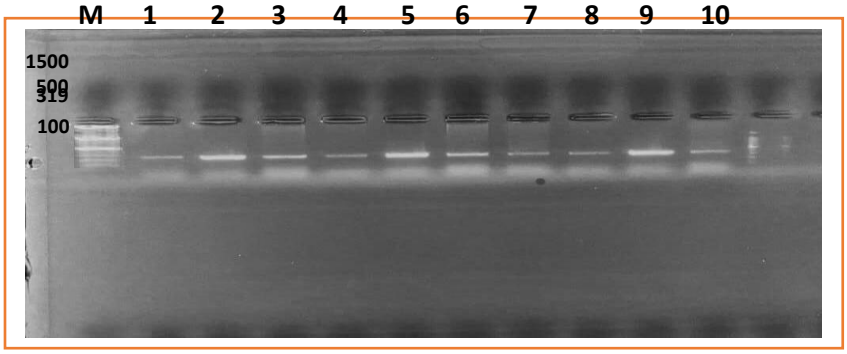

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Fig (11) Gel electrophoresis of amplified *HasA* gene of *S.uberis* using conventional PCR (lan 1-12) .M. marker Agarose 2% and TBE(1X) at 75V/cm for 90 min . stained with safe stain, and on a UV transilluminator .

Table (4): Number of samples and percentage of positive results for virulence genes detection in *streptococcus uberis* isolated from bovine mastitis.

The genes	No. of samples	No. of positive	%
Sua	46	45	97.8%
CFU	46	32	69.5%
HaSA	46	30	65.2%

Detection of slime production by Congo red agar method:

Slime production is an indicator for biofilm formation was evaluated by cultivation of *S. uberis* isolates on Congo red agar (CRA) plates . Isolates were interpreted according to their colony phenotypes. Black colonies with dry consistency and rough surface and edges were considered a positive indication of slime production, while both black colonies with smooth, round and shiny surface and red colonies with dry consistency and rough edges and surface were considered as intermediate slime producers fig (12) .

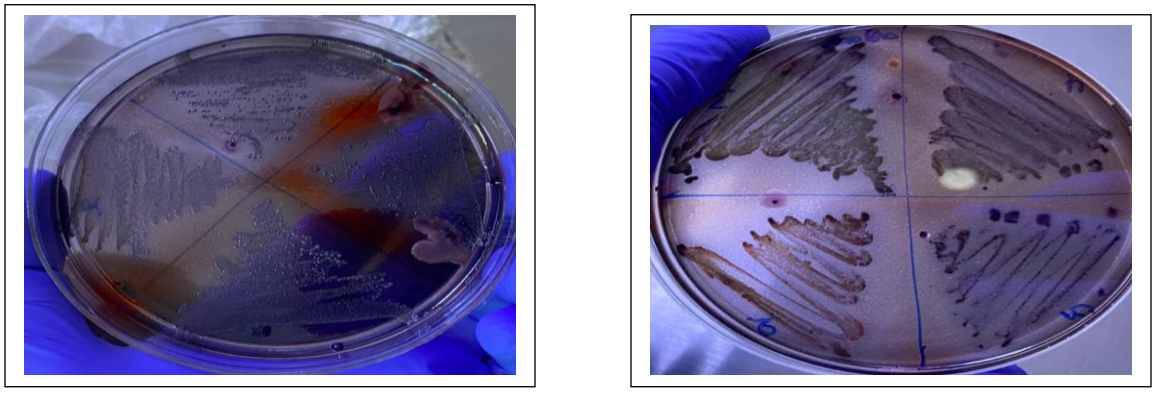

Fig (12) slime production by *S. uberis* on Congo red agar

Quantification of biofilm formation on the microtiter plate

The results of the microtiter plate assay of all *S. uberis* isolates, show different levels of biofilm production were detected as strong , moderate and weak ((42%, 35 %, and 28 %) respectively

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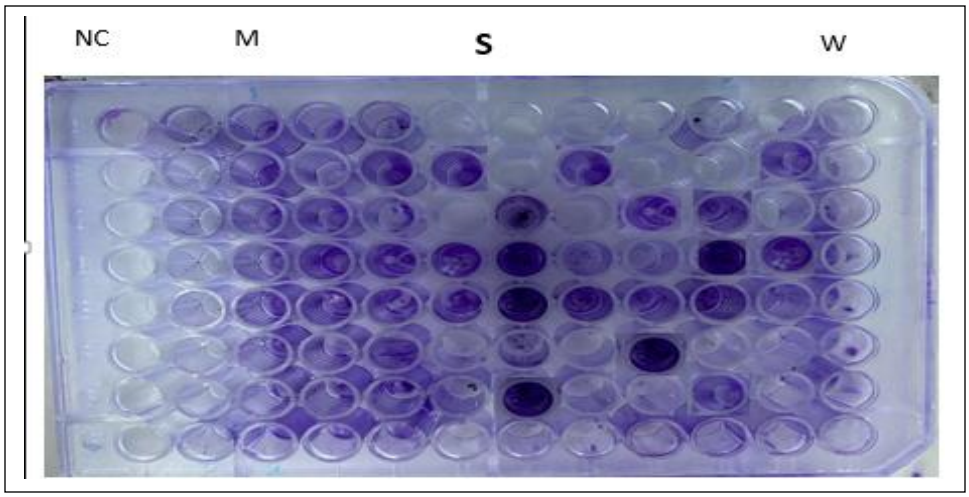
from Subclinical Mastitis , and (44% , 33% and 22%) from Clinical Mastitis figure (13) , table (5) .



NC = Negative control, S= strong, M= Moderate, W= weak

Fig(13) The biofilm formation by *S. uberis* on the microtiter plate.

Table (5): Results of microtiter plate assay of biofilm formation by *S. uberis* isolates.

Sample	**NO.of *S.uberis***	Biofilm production No./(%)		
		Total No.of biofilm producer		
		Weak producer	**Moderate producer**	**Strong producer**
Subclinical Mastitis	16	14(100%)		
		0	2(14.28%)	12(85.71)
Clinical Mastitis	6	6(100)		
		0	2(33.33%)	4(28.57%)

Non – biofilm produer (OD < ODc) , 0

Weak biofilm producer (ODc < OD < 4x ODc) , +

Moderate biofilm producer (2x ODc < OD < 4x ODc) , ++

Strong biofilm producer (4xODc < OD)+++

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Antibiotic susceptibility testing of the isolated *S. uberis:*

The results of antibiotic susceptibility testof 9 different antibiotics by disc diffusion methodagainst 24 *S. uberis*isolates Amoxillin ((sensetive 45.6%,tntermidiat 0%,resistent58.6%) , Ampicillin (senstive 4.3%,.intermediat 0% ,resistent 95.6%) ,Ciprofloxacin(sensitive 69.5%, intermediat 0%,resistent30.4%), Ciftrixone (senstive 93.4%,intermediat6.52%,resistent0%), Erythromycin (sensitive 21.7%%,intermediat 2.17%,resistent76%) , Trimethoprime (sensitive 4.3 %,intermediat 0%,resistent95.6%) ,Gentamycin (sensitive100%) ,Tetracycline (sensitive 10.8%,intermediat 0%,resistent 89.1%) , Pencillin (sensitive 4.3%,intermediat 0%,resistent 95.6%) fig (14 , 15) table (6) .

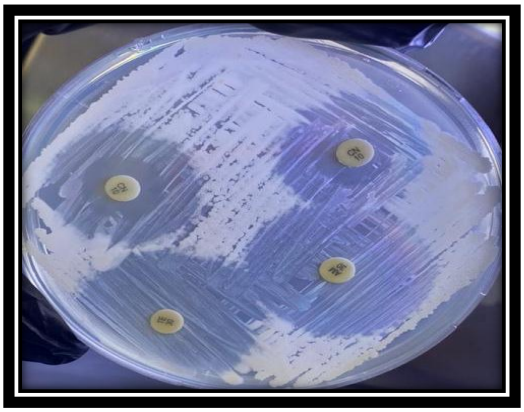

Fig (14) :Disc diffusion method with inhibition zones for some antibiotics against *S. uberis*

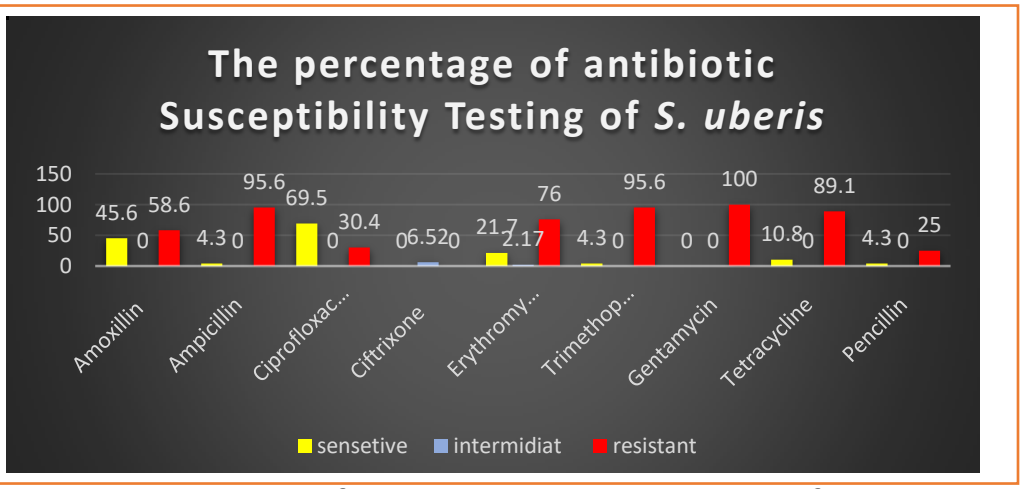

Fig (15) The percentage of antibiotic Susceptibility Testing of *S. uberis*

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Table (6) Antibiotic sensitivity test of 9 different antibiotics by disc diffusion test against 46 *S.uberis* isolates.

No	Antibiotic	Con.	No. of isolates		
			R	I	S
1	Amoxillin(Ax)	(30)	21	-	27
2	Ampicillin(AM)	(10)	-	-	46
3	Ciprofloxacin(cip)	(2)	14	-	32
4	Ciftriaxone(CRO)	(30)	-	3	43
5	Erythromycin (E)	(15)	10	1	35
6	Trimethoprime(TE)	(5)	2	-	44
7	Gentamicin (CN)	(10)	-	-	46
8	Tetracycline(TE)	(30)	41	-	5
9	Pencillin(P)	(10)	44	-	2

Con: Concentration; R: Resistant; I: Intermediate; S: Sensitive.

Discussion

Streptococcus uberis is a worldwide pathogen that causes intra-mammary infections in dairy cattle. *S. uberis* has been described as an opportunistic pathogen that utilizes nutritional flexibility to adapt to a range of ecological niches, including the mammary gland. It was suggested that cow-to-cow transmission of *S. uberis* potentially occurring in the majority of herds and may be the most important route of infection in many herds[15].

In this study, a total number of 100 different milk samples were collected from clinical, subclinical milk samples of different dairy cow farms aiming to isolate *S. uberis* that cause bovine mastitis to study its phenotypic and genotypic characteristics. Based on both phenotypic and genotypic identification, the number of Streptococcus spp. isolated from all tested milk samples was 46(92%).

In contrast to the total examined milk samples, the incidence of *S. uberis* was 46%. Nearly similar, [16] isolated *S. uberis* from normal, sub-acute and acute cow mastitic cases with a

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percentage of 18%. A higher incidence rate of Streptococcus spp. were isolated from mastitic cows (55 %) but a lower *S. uberis* was isolated with a percentage of 15.3% was detected by[17]. Also, a higher incidence of S. uberis as the predominant pathogen was recorded by[18] in cow's milk samples (46%). This variation in the results might be attributed to the difference in herd management between herds. Teat dipping before and after milking, washing milkers hands before and after milking, preparing a clean towel for each lactating cow, milking of infected cows, and finally, using dry cow therapy and treating clinical cases at an early stage can all help to reduce the occurrence [19].Antibacterial agents used to treat or prevent mastitis are usually effective against bovine mastitis organisms; however, chronic mastitis is difficult to treat. The ability of bacteria to form biofilms may be one explanation for their failure to react to treatment.[20]

Microorganisms are usually studied in broth cultures. The behavioral features of microorganisms have been discovered information to research focused on broth studies. Broth cultures, which allow organisms to move freely, have long been thought to reflect the dominant (physical and chemical) environment in which organisms live. Consequently, bacterial trends observed in broth cultures were viewed as characterizing an organism's

Conclusion:

growth and behavioral patterns (Monaghan, J. M., & Hutchison, M. L.*S. uberis* infections in the mammary gland cause significant economic losses in the dairy industry. Several studies have phenotypically and genetically characterized S.uberis strains in order to improve our understanding of virulence factors and biofilm formation in S. uberis strains. In the future, the results may be used to create more effective and acceptable treatment protocols.

(2012 alleviate the impact of mastitis caused by this environmental pathogen.

Acknowledgments

The author would like to thank the College of Veterinary Medicine, University of Basrah Iraq

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