Molecular Identification of Some Bacterial Species Causing Animal Mastitis in Basrah Province, Iraq

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ABSTRACT

Background: Mastitis, which refers to as infection of the mammary gland, is considered one of the important wide-ranging diseases that affect dairy animals that lead to decreasing of somatic cell contents (SCC) and milk amount. On the other hand, sub-clinical mastitis does not appear to produce any changes in the udder or milk. However, subclinical mastitis is a severe problem and acts as a pathogen pool that transmits udder infection across animals in the farm.

Objective: The aim of the current work was phenotypic and molecular identification of some bacterial species causing animal mastitis in Basrah province, Iraq.

Materials and methods: This cross sectional study was conducted in Basrah City from October 2018 to March 2019. A total of 50 milk samples were collected from cows with suspected clinical mastitis cases and transported immediately to laboratory unit and cultured on appropriate culture media like Blood agar, MacConkey agar (MA) and Mannitol salt agar (MSA) respectively. Thereafter, the pure isolate were identified by biochemical tests and molecular tests using 16S rRNA characteristics. **Results:** The most common causative agents were *Staphylococcus aureus* that isolated from cases and the other microorganisms were *Bacillus* spp., *Escherichia coli* and *Enterobacter*.

Conclusion: *S. aureus* and *E. coli* were the most predominant bacteria causing animal mastitis. The *16s rRNA* gene was a good tool for identification of bacteria causing mastitis for both Gram positive and Gram-negative bacteria where in concern with the morphological tests.

Keywords: Mastitis, E. coli, S. aureus, 16S rRNA, Basrah province, Iraq.

INTRODUCTION

Mastitis, disease considered as a severe infection in cattle mammary gland and represent one of the most commonly disease that affects dairy cattle [1]. The first type of the disease is called environmental mastitis, whereas contagious mastitis is spread by direct contact with other affected people or animals. *Escherichia coli*, *Klebsiella pneumonia*, *Enterobacter* spp., and *Staphylococcus* spp. are the most common environmental pathogens [2]. A long-term inflammatory condition with intermittent clinical flareups is chronic mastitis [3].

Although milk production decreases as the somatic cell count (SCC) rises, sub-clinical mastitis, disparate clinical mastitis, does not appear to produce any changes in the udder or milk. Clinical mastitis can be fatal in certain circumstances. Experts agree that subclinical mastitis is more expensive to the herd than clinical cases, despite the fact that it is impossible to quantify the financial damage it causes. Mastitis is most frequently caused by intra-mammary infections. Although several bacteria, including *S. aureus* and *Streptococcus agalactiae*, are typically categorized in the literature as the cause of contagious mastitis, it proposed that they can also be categorized as environmental pathogens [1].

The fact that these infections can spread via many channels, including bedding and infectious milk from affected cows, supports this viewpoint. The main source of ecological pathogens is feces. Furthermore, the teat end is constantly exposed to environmental pathogens between milking since the teat canal may remain open for one to two hours after milking. Most intra-mammary infections are developed during milking [4]. Clinical mastitis is illustrious by a fast onset, along with inflammation and udder edema. A quarter's milk is spoiled, has flakes or masses, and/or has a watery constancy. Cattle frequently develop a fever, a reduced appetite, and may appear physically sluggish. After a few hours, the somatic cell count (SCC) is larger than the typical counts, which are less than 200,000 cells /ml [3]. Subclinical mastitis is distinguished by the absence of outward symptoms in the udder or milk. Also, it causes a drop in milk production and rise in SCC. Shearer and others noted that subclinical Mastitis happens 15 to 40 times more frequently [5].

As a result, subclinical mastitis is further difficult to characterize, and the condition acts as a pathogen pool that transmits udder contamination across animals in the herd [6]. The aim of current work was morphological and molecular identification of some bacterial species causing animal mastitis in Basrah province, Iraq.

MATERIALS AND METHODS

Characterization of bacterial pathogen in concern of mastitis disease: About 1.5 ml of milk sample was placed in disinfected centrifuge tubes from each sample respectively, then centrifuged for five minutes in 100 rpm (round per minutes) at room temperature after discarding the supernatant, the pellet was inoculated in Mannitol salt agar (MSA), MacConkey agar and Nutrient (NA) agar, and then, incubated overnight at 37ºC. after that the colonies were sub cultured in nutrient agar plate media [7].

Identification of bacteria: Gram positive and Gram negative bacteria were distinguished using Gram stain technique which performed data for morphological information of bacterial isolates that examined under light microscope [8]. The catalase test was done according to **MacFaddin** [9], as the following: 2 to 3 colonies were

placed in a clean glass slide by using a sterilized wooden stick, after that a few drops of catalase reagent was added, then positive results showed formation of bubbles of oxygen that refers to a positive consequence. Oxidase test was achieved according to **Brown and Smith** [7], in which a sterile filter paper was saturated with oxidase reagent then fresh culture (18-24h) colonies were scrapped from plate culture and placed to the soaked paper with a disposable loop, the dark purple color indicates a positive reaction.

Molecular Study:

The DNA of suspected bacterial isolates was extracted according to procedure of Qiagen kit (USA) and the purity of genomic DNA was detected in 1% agarose gel electrophoresis then examined in UV light transilluminator. Agarose gel were prepared by dissolve 0.25 gram of agarose powder in 20 ml of Tris borate EDTA buffer (1-) after mixed slightly the suspension were heated until boiling and left to cooled until reached 50ºC after that ethidium bromide pigment were added and the mixture poured in appropriate casting plate with inserted comb to make the wells [10]. Then, the comb raised carefully and the gel were placed in electrophoresis tank which contain diluted TBE buffer after that 2 ml of bromothymol blue mixed with 4 ml of DNA that loaded in wells of agarose then supplied with electric current from power supply (120 mA 60 Volt) for 40 minutes [11].

The Oligonucleotide primer sequence of 16S rRNA gene was used [12]; [(F: 5′-AGA GTT TGA TCA TGG CTC AG-3′) and [R: 5′-GGT TAC CTT GTT ACG ACT T-3′)]. The DNA was amplified in 20μl PCR reaction mixture, and PCR products were electrophoresed in one percent agarose gel that stained by Ethidium Bromide with using 100 bp of DNA ladder at 70 Volt, for 40 minutes. The DNA bands of PCR products were visualized using transilluminator and photographed. PCR products of 1500 bp targeting 16S rDNA of isolate were selected, sent for sequencing, and subjected to alignment with known reference isolates in GenBank by BLAST at the National Centre for Biotechnology Information (NCBI).

Ethical approval:
The study was approved by the Ethics Board of University of Basrah.

Statistical Analysis
One-Way ANOVA in the GraphPad Prism (6.0.1) Software was used to detect significant differences between results of this study. P value ≤ 0.05 was considered significant.

RESULTS
Morphological and phenotypic tests of bacterial species that isolated from clinical Mastitis were illustrated in **Table 1** that showed 12 isolates were documented which correlated to 5 genera involved; *Staphylococcus aureus*, *Bacillus cereus*, *Enterobacter* spp. and *Escherichia coli*, with one insolate unidentified.

Table (1): Results of biochemical tests bacteria isolated from cattle mastitis.

Isolate No.	Grams stain test	Morphology	Catalase reaction	Oxidase reaction
1-	+	Coccus	+	-
2-	+	Coccus	-	-
3-	+	Coccus	+	+
4-	+	Rods	+	-
5-	-	Coccobacillus	+	+
6-	-	Coccobacillus	+	-
7-	-	Rods	+	-
8-	+	Coccus	+	-
9-	+	Coccus	-	-
10-	-	Rods	+	+
11-	-	Rods	+	-
12-	-	Coccobacillus	+	-

For all strains, the phenotypic traits of colonies developing on nutrient agar media were described. Gram-staining revealed that 50 percent of the isolates were Gram-positive and 50 percent were Gram-negative, these results were confirmed with 16S rRNA sequencing, the electrophoresis technique's results for genomic DNA extraction indicated clean and clear isolated DNA. Additionally, by using a set of universal primers, all isolates were examined for the amplification of 16S rRNA gene sequences, 27 F and 1492R generated a single amplification of about1500 bp for the whole isolates (**Figure 1**).

Based on partly sequenced of the 12 gained isolates, four genera were documented and characterized (**Table 2**). These composed *Staphylococcus aureus*, *Enterobacter*, *Bacillus cereus*, *E. coli and Staphylococcus* species, most dominant genera. Phenotypic characterization of about five isolates were confirmed by PCR and DNA sequencing using 16srRNA gene and the results of sequencing of 16srRNA analyzed using Blast program to identify the similarity with other sequence in the NCBI database (http://www.ncbi.nlm.nih).
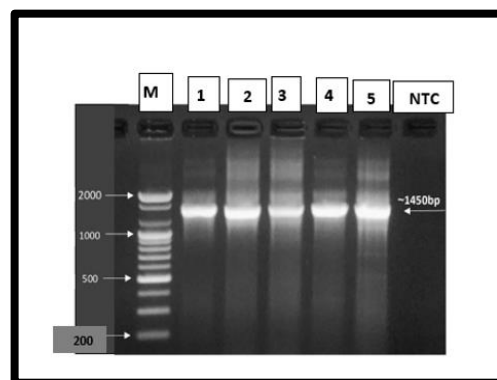

Figure 1: Results of electrophoresis revealed PCR Product Study of Bacterial isolates Lane M: Marker ladder (100 bp), lanes (1-5): 16S rRNA gene bacterial sp. of about 1450 bp. NTC: negative control.

Table (2): Molecular identification of bacterial isolates according to sequence results.

Sample code	Detection by PCR	Reference accession number	Maximum identity
1	*S. aureus*	MN889346.1	99.57%
2	Unidentified	-	-
3	*B. cereus*	KC441781.1	96.1%
4	*E. coli*	JQ3988451	99.64%
5	*Enterobacter*	JX102043.1	95.95%

DISCUSSION

Mastitis is considered one of the most common diseases that affect livestock, which causes huge economic losses in terms of mammalians. A wide variety of microorganism's cause Mastitis disease that result in a huge damage in dairy industry in otherwise caused decreased in milk production. Many microorganisms, especially bacteria, play a very big role in causing cases of mastitis that increasing costs of using antibiotics for treatment and culling of infected animals [13,14].

The present study recorded 12 isolates of pathogenic bacteria correlated to four species of bacteria which considered as the main causative agents of mastitis infection in cattle that consist of *Staphylococcus aureus*, *Escherichia coli* spp., *Bacillus cereus* and *Enterobacter* spp. Both *Staphylococcus aureus* spp. and *Escherichia coli* are dominants in this study, Samples of milk Mastitis were collected from infected cows that contain clinical symptoms as soon as possible with take in consideration all sterilization techniques .All isolates gained from this study showed catalase positive reactions but only three isolates revealed oxidase positive reaction that compactible with many research [15,16].

The results obtained from this study by using universal primer 16S rRNA showed that four bacterial species were dominated that compatible with many research [16,17].

These species were characterized by phenotypic and confirmed by molecular tests that quick, sensitive and accurate than routine diagnosis of bacterial identification [18]. Using sequencing alignment in this study was important to confirm similarity caused by structural, functional evolutionary links between references [19-21]. These bacterial species and other types were extensively isolated by several studies at similar area of Basrah [22-24]. Genetic identification and DNA sequencing were also used for similar studies for identifying bacterial species from different animal sources [15,25-27].

CONCLUSION

Staphylococcus aureus and *E. coli* were the most predominant bacteria causing animal mastitis. The *16S rRNA* gene was a good tool for identification of bacteria causing mastitis for both Gram positive and Gram-negative bacteria where in concern with the clinical cases.

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