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ERIC-PCR for Genotyping of *Staphylococcus aureus* Isolated from Clinical and Subclinical Mastitis Cow's Milk

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ABSTRACT

The present study was designed to genotype *Staphylococcus aureus* isolated from mastitis cows. One hundred cow's milk samples were gathered (50 clinical and 50 subclinical mastitis milk samples) from various dairy farms in various regions of Egypt's El-Gharbia governorate. *S. aureus* was isolated and identified from the samples using bacteriological and molecular examination. Genotyping was subsequently carried out using the Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR). According to our findings, the prevalence of *S. aureus* was 30% and 12% from clinical and subclinical mastitis milk samples respectively. Discriminatory index of ERIC-PCR was 0.977. The dendrogram analysis of the isolates showed one cluster, two sub clusters, and four separate isolates. The same cluster contained some strains obtained from various regions, while some strains collected from the same region and also the same farm were located in different clusters. This suggested the possibility of infection transmission between these regions, requiring increased control measures, management practices and decreasing the transmission of the diseased animals between these regions to prevent the transmission of different genotypes between them and increase the cure rate of the disease. In conclusion, ERIC-PCR was a reliable genotyping approach for *S. aureus* isolates with high genetic diversity and a high discriminating index. ERIC-PCR is a useful molecular epidemiology approach for *S. aureus* isolates as there was a genetic similarity between some strains gathered from several regions at the El-Gharbia governorate in Egypt.

1. INTRODUCTION

Milk is a significant part of the global human diet, but it also provides a favorable environment for the growth of numerous germs, particularly pathogenic harmful bacteria that cause mastitis. Mastitis is inflammation of the mammary gland causing both local and systemic symptoms (Grima *et al*., 2021).

Mastitis is an important disease which causes high economic costs in the dairy business since it results in milk waste, high treatment costs and culling of cows. It has a significant impact on public health due to the direct transfer of food-borne pathogens and/or the consumption of the generated toxins and enterotoxins (Guimarães *et al*., 2013 and Dalanezi *et al*., 2020).

There are two types of mastitis: clinical and subclinical. Clinical mastitis manifests as visibly abnormal milk, udder enlargement and inflammation, or discomfort, while subclinical mastitis shows no overt symptoms (Campos *et al*., 2022). Yet, the amount of milk produced falls and the somatic cell count rises in subclinical mastitis.

One of the most significant causing pathogens of bovine mastitis is *S. aureus* (Kaczorek-Łukowska *et al*., 2022). It capable of expressing a broad range of pathogenic components that are employed to attach, colonize, invade, and infect the host. *S. aureus* has the ability to induce subclinical infections, which raise the somatic cell count (SCC), as well as clinical mastitis (Monistero *et al*., 2018). Mammary epithelial cells, endothelial cells, and fibroblasts are invaded by *S. aureus*, which escapes from the phagosome into the cytoplasm of mammary epithelial cells, where it is encased in membrane-bound vacuoles causing cell necrosis (Dego *et al*., 2002).

Polymerase Chain Reaction (PCR) is used to exponentially amplify a certain preselected DNA fragment utilized for bacterial species identification (Al-Obaidi *et al*., 2018).

The PCR approach is more accurate, more sensitive as it can distinguish between closely related species, less expensive and faster than the classic cultured method as it can identify bacterial infections in hours as compared to days (Abd El-Tawab *et al*., 2016).

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Genotyping techniques utilizing DNA found in the genomes are essential epidemiological tools for differentiating bacterial isolates from the same species, which in turn provides significant data for tracing infection sources and managing diseases. Groundbreaking efforts have been made to discriminate between different types and subtypes of bacteria at the molecular level. Notably, genotyping techniques have led to the development of creative tools for better surveillance and epidemic investigation. Thus, early detection and genotyping of microorganisms are essential tools to control the therapeutic treatment and the rapid expansion of clinically significant bacteria (Al-Obaidi *et al*., 2018).

There are numerous molecular biology methods currently in use. These methods included Ribotyping, Amplified Ribosome DNA Restriction Analysis (ARDRA), Amplified Fragment Length Polymorphism (AFLP), different PCR methods, microarrays, Pulsed Field Gel Electrophoresis (PFGE), and Random Amplified Polymorphism Deoxyribonucleic acid (RAPD). The approach used in any study is in line with the study's economic facilities, although some methods are discriminating and sharp with great accuracy, they are excessively pricey. Among the aforementioned techniques, ERIC PCR has an acceptable result, plus it is quick, simple to use, and inexpensive (Ranjbar *et al.,* 2017). Bacterial species have varied ERIC patterns and numbers, which make them useful as genetic markers for bacterial genetic diversity (Barus *et al*., 2013).

The *S. aureus* strains were described, and successfully categorized using ERIC-PCR to create efficient defense mechanisms against mastitis (Arslan and Mutlu, 2016). Distinctive banding patterns acquired through ERIC-PCR demonstrated impressively the genetic diversity of *S. aureus*, isolated from various milk samples with high discriminative power (Akindolirea *et al*., 2018).

The current study aimed to determine the genotyping of *S. aureus* strains causing mastitis in cows using ERIC-PCR fingerprinting.

2. MATERIAL AND METHODS

Benha University Institutional Animal Care and Use Committee (BU-IACUC) had been approved this study, giving it Ethical approval number of "BUFVTM 02-06-23". Farm owners gave informed verbal or written consent to participate in this study.

2.1. Sampling

One hundred cow's Milk samples were collected from 50 cows with clinical mastitis and 50 cows with subclinical mastitis, which were positive for California Mastitis Test (CMT)) according to the method described by Quinn *et al*. (1999). The samples were aseptically collected from several dairy farms in various locations throughout the Egyptian governorate of El-Gharbia in summer and winter seasons. Each milk sample (15-20 ml) was placed in a sterile, clean, screw-capped and labeled bottle and preserved in a sterile, chilled container then quickly transferred to the laboratory of the Animal Health Research Institute (Tanta branch, Egypt) for the bacteriological examination.

2.2. Bacteriological examination:

Isolation of Staphylococci according to Quinn et al., (2002)

Milk samples were added to nutrient broth (Oxoid, CM0001B) and incubated aerobically for 18–24 hours at 37°C. A loopful of the cultured broth was streaked across the surface of nutrient agar (Oxoid, CM0003) and selected agar medium for *S. aureus*; mannitol salt agar (Oxoid, CM0085) and Baired parker agar for(Oxoid, CM0275) to isolate *S. aureus*. The inoculation plates underwent an aerobic incubation period of 18–48 hours at 37°C.

Detection of hemolysis (Bailey and Scott, 1978): A loopful of the cultured broth was streaked across the surface of sheep blood agar plates and incubated at 37ºC for 24 h for detection of hemolysis.

Identification of Staphylococci isolates according to Quinn *et al.* (2002)

The suspected colonies were inspected by microscopic examination of Gram's-stained films and by detection of bacterial motility.

Biochemical Identification of S. aureus:

Coagulase production test (APHA, 2001), and detection of other biochemical reactions (Quinn *et al*., 2002) using Catalase test, Indole test, Methyl Red test, Voges Proskaur test, Citrate utilization test, sugar fermentation test, Urease test and Oxidase test.

2.3. Molecular identification of the isolates

The DNA of *S. aureus* isolates was extracted according to the QIAamp DNA Mini Kit (Catalogue No.51304) manufacturer's instructions. The 23S rRNA gene was amplified in order to confirm the biochemically identified *S. aureus* isolates (Bhati *et al*., 2016). The primer was created by the German company Metabion and the sequence is illustrated in Table (1).

Table (1): Oligonucleotide primers sequences for *S. aureus* isolates.

Target	Primer sequence	Amplified segment (bp))	Reference
S. aureus 23Sr RNA	F-5'-ACGGAGTTACAAAGGACGAC-3' R-5'-AGCTCAGCCTTAACGAGTAC-3'	1250	Bhati *et al*., 2016
ERIC	ERIC1: 5'-ATG TAA GCT CCT GGG GAT TCA C-3' ERIC2: 5'-AAG TAA GTG ACT GGG GTG AGC G-3'	Variable	Versalovic *et al*., 1991

Genotyping of isolated S. aureus using ERIC-PCR

ERIC-PCR was used to assess genotypes and the genetic connection between the isolates. Representative 10 isolates of *S. aureus*, from the different localities of samples collection, were subjected to ERIC-PCR fingerprinting using a single amplification profile, and primers from Metabion, Germany (Table 1), (Versalovic *et al*., 1991).

Data from fingerprinting was converted into a binary code based on the presence or absence of each band. The unweighted pair group method with arithmetic average (UPGMA) and Ward's hierarchical cluster methodology were used to create the dendrogram. The dendrogram was created and the cluster analysis was carried out using Statistical Package for the Social Sciences (SPSS) version 22 (IBM Corp. 2013, Armonk, NY). Simpson's index of diversity (D), which measures the average probability that a typing method will give a different type to two unrelated strains randomly picked from a population, was used to measures the discriminatory power of ERIC-PCR (Hunter, 1990). Good differentiation is indicated by a D value greater than 0.9.

3. RESULTS

Prevalence of *S. aureus* isolated from milk samples:

The bacteriological examination of milk samples revealed that the number of *S. aureus* isolates was 15 out of 50 clinical mastitis milk samples (30%) and 6 isolates out of 50 subclinical mastitis milk samples (12%) as in table (2).

Table (2) Isolation rate of *S. aureus* strains isolated from cow's milk.

Type of mastitis samples	Number of samples	*S. aureus* isolates	
		No	%
Clinical mastitis	50	15	30%
Subclinical mastitis	50	6	12%
Total	100	21	21%

S. aureus showed round, convex and golden yellow colonies on nutrient agar, yellow colonies surrounded by a halo zone on mannitol salt agar, black and shiny colonies with narrow white margins surrounded by a clear zone on Baired parker agar. The isolates were positive for complete blood hemolysis on blood agar. On microscopic examination of Gram's-stained films, *S. aureus* showed gram positive cocci arranged in grape -like clusters. It was non-motile.

Biochemical reactions of *S. aureus*: Coagulase +ve, Catalase +ve, Indole -ve, Methyl Red test +ve, Voges Proskaur +ve, Citrate +ve, sugar fermentation test +ve, Urease +ve and Oxidase -ve.

Molecular identification of the isolates:

S. aureus isolates (21) were confirmed by cPCR using a specific primer for 23S rRNA gene that amplified at 1250 bp (figure, 1).

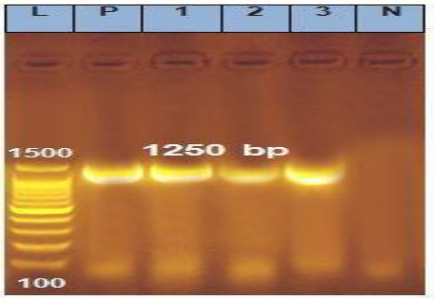

Fig.1: Agarose gel electrophoresis pattern of PCR for detection of *S. aureus* 23S rRNA gene at 1250 bp. L: Ladder from 100 bp to 1500 bp P: Positive control: *S. aureus* ATCC 8096 N: Negative control Enterococcus faecalis (ATCC 29212), Lanes 1, 2, 3: Number of *S. aureus* isolates (positive for23S rRNA gene at 1250 bp)

ERIC-PCR genotyping:

The isolates' ERIC-PCR fingerprinting patterns were examined. The position and number of the amplified segment were used to distinguish between different ERIC-PCR profiles.

The banding patterns could be visually compared to identify several DNA segments with sizes between 148 and 1095 bp (figure2). ERIC-PCR primer for the isolates produced 9 profiles (referred to as E1 (3-4) to E9). The discriminatory power of the ERIC-PCR was 0.977. The dendrogram analysis of the ten examined isolates showed one cluster (2 sub clusters) and four separate isolates (figure 3). Some strains, which were gathered from different regions, were present in the same cluster and were genetically related to each other. For example, strains number 3&4 were collected from different regions and were located in the same profile in the same cluster (cluster I). Also strains 2-6-9, in cluster I, were collected from different regions. Some strains which were gathered from the same region, even from the same farm, differed genetically and were present in different clusters as for example, strains number 1 and 7 were gathered from the same region but strain1were located in cluster I and strain 7 was located as a separate isolate not in the same cluster. On the other side, some strains, collected from the same region as strains number 1, 4, were present in the same cluster (cluster I) and some strains, collected from different regions as strains number 5,8,10, were present as separate isolates.

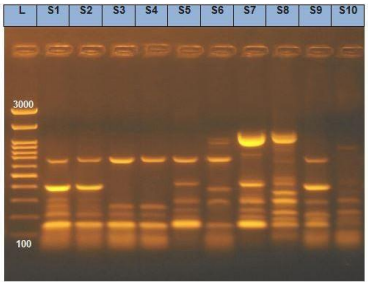

Fig. 2. ERIC-PCR fingerprinting *of S. aureus* isolates in a 1 % agarose gel. Lane L, 100-bp ladder (Range: 100-3000 bp) and lanes S1-S10 *S. aureus* isolates the size of the bands ranged between 148 and 1095 bp.

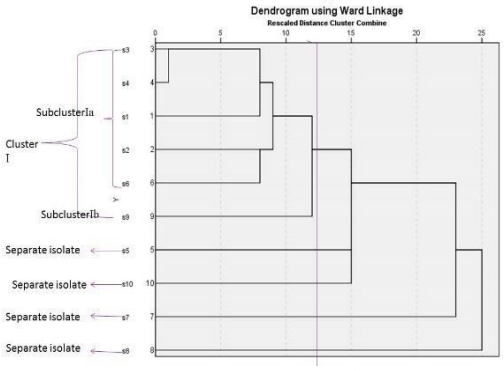

Fig. 3. Dendrogram showing the relatedness of *S. aureus* samples isolated from different dairy farms at different centers of Gharbia governorate (Isolates 1-4-7 - el santa center, 3- tanta center, 2- basuon center, 6- elmahalla center, 9- kotoor center, 5-zefta center, 10- kafer elzayat center, 8- samanood center), as determined by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) fingerprinting using the SPSS computer software program. Strains number 1,2,3,4,6 present in cluster I and strains 5,7,8,9,10 present as separate isolates.

4. DISCUSSION

For the dairy sector, production of high-quality products from healthy cows with healthy udder is one of the primary challenges (Lücken *et al.*, 2021). Mastitis is the most prevalent and costly production condition in dairy herds (Dalanezi *et al*., 2020).

From an economic and animal welfare perspective, mastitis should be reduced and to do this, it is necessary to better understand the causes so that preventive measures may be implemented (Lücken *et al.*, 2021).

Pathogens causing mastitis can be either contagious (spreading during the milking process) or environmental microbes. *S. aureus* is categorized as a contagious pathogen. Poor farm cleanliness contributes to its spread among the herd (Kaczorek-Łukowska *et al*., 2022). It is responsible for both clinical and subclinical mastitis in cows causing high economic losses for the livestock industry (Brahma *et al*., 2022).

Mastitis-affected dairy herds can introduce *S. aureus* into the milk supply, causing human food poisoning (Xavier *et al*., 2017).

In the present research, fifteen *S. aureus* isolates were detected from 50 clinical mastitis milk samples (30%), (Table, 2) and this to a certain extent is near to the results obtained by Hussein *et al.* (2018) who recovered *S. aureus* in clinical mastitis by 29.41 % at Damietta governorate, Egypt.

Lower prevalence of *S. aureus* in clinical mastitis in cows was detected in previous studies by Grima *et al.* (2021) who detected *S. aureus* by 21.05% from a total of 57 clinically mastitic cows in Sebeta town dairy farms in Ethiopia. Moreover, Dalanezi *et al.* (2020) in Brazil isolated *S. aureus* by 4% from cows with clinical mastitis.

On the other hand, a higher isolation rate of *S. aureus* in clinical mastitis in cows was detected in previous studies by Tassew *et al*. (2016) who detected *S. aureus* by73.3% in clinical mastitis in cows in and around Kombolcha, Ethiopia, and Abd El-Tawab *et al.* (2016) isolated *S. aureus* by 48.8% in clinical mastitic cows at El-Kaliobia governorate in Egypt

In subclinical mastitis milk samples (n=50), the isolated *S. aureus* were 6 isolates by 12%, (Table 2), which agreed with Saeed *et al.* (2022) who isolated *S. aureus* by 12% in subclinical mastitis in Pahang in Malaysia, and Sayed *et al.* (2014) isolated *S.aureus* by14.8% in subclinical mastitis in Egypt.

A higher prevalence of *S. aureus* in cows with subclinical mastitis was detected by Tassew *et al*. (2016) who recovered *S. aureus* by 42% in and around Kombolcha, Ethiopia. Moreover, 43.9% reported by Abd El-Tawab *et al.* (2016) at Al-Qaliobia governorate in Egypt. Also, Dos Reis *et al.* (2011) isolated *S. aureus* by 30.8% in the states of São Paulo and Minas Gerais, Brazil.

A lower isolation rate of *S. aureus* isolates in subclinical mastitis in cows was detected by Zaatout *et al.* (2019) who recovered nine isolates of *S. aureus* from 167 milk samples of lactating cows with subclinical mastitis in Algeria by 5.3%.

The results showed that the incidence of *S. aureus* in clinical mastitis was higher than in subclinical mastitis and this in agreement with previously reported results by Tassew *et al*. (2016)

The high incidence of *S. aureus* in mastitis is due to its contagiousness and ability to live in the udder causing chronic and subclinical infections. It is also due to the absence of stringent farm biosecurity as, cleaning the floor properly, washing the bodies of the animals, and failure in following the correct protocol for milking cows (Grima *et al.*, 2021). On the other side, the prevalence of *S. aureus* in milk decreased and its transmission to humans ceased by improving the sanitary conditions of the milking area and/or utensils. In addition the prevalence of *S. aureus* differs according to the geographic regions (Daka *et al.*, 2012).

Due to the limitations of cultural and biochemical methods for isolating bacteria, another method (PCR) based on the amplification of DNA coding for 16S rRNA and 23S rRNA, has been effectively employed for the identification of bovine mastitis pathogens (Riffon *et al*., 2001). This technique identifies bacterial pathogens in hours as opposed to the days that the traditional cultured method requires. Plus it can differentiate between closely related organisms as it is precise, more sensitive, and affordable (Abd El-Tawab *et al.*, 2016). In this research, cPCR is used to confirm the isolates using a specific *S. aureus* primer for 23S rRNA gene. The tested *S. aureus* isolates gave amplified products at 1250 bp (Figure, 1).

The process of typing is intended to support numerous public health, microbiological, and epidemiological investigations (Ramadan, 2022). Typing is used to describe the species and characteristics of organisms, with the discrimination at the strain level being made both phenotypically and genetically. Traditional typing methods include serotyping, biotyping, and phage typing have been used for a long time. However, molecular level typing is very essential nowadays because of its specificity, which is frequently used to support the associated phenotypic traits. For instance, one species may have numerous subtypes or subpopulations and one of them may be more pathogenic than the others (Al-Obaidi *et al*., 2018). Genetic approaches can be used to get information on the relatedness of strains, possible sources of infection, and routes of transmission (McMillan *et al*., 2016).

Compared to phenotypic approaches, genotypic methods have higher reproducibility, more reliability, and more discriminatory power. Selecting the best approach usually depends on a variety of conditions and elements, including the cost, scope, and size of the study (Ramadan, 2022). ERIC-PCR is a straightforward PCR-based DNA fingerprinting technique that has been widely employed and demonstrated to be quick, simple to use, and cost- effective for gathering data on the genetic similarity of bacterial strains, belonging to a bacterial species, in order to trace the source and path of microbial contamination (Ranjbar *et al.*, 2017 and Alsultan *et al.*, 2022).

The epidemiology of bovine mastitis has been studied at the level of subspecies using a variety of DNA typing techniques, including genome sequencing, library typing, and comparative typing based on common electrophoretic fingerprint bands (Xavier *et al*., 2017). Among the fingerprint-based typing methods, ERIC-PCR has been used for typing and differentiating the *S. aureus* strains isolated from cows with mastitis, facilitating successful description and classification of the *S. aureus* strains. Also it might be used to create a database by creating genotypes, identify the cause of the epidemic, create efficient mastitis protection techniques and conduct studies on the creation of vaccines by creating genotypes (Arslan and Mutlu, 2016). Vaccination programs against bacterial infections protect dairy farm animals by avoiding or lowering bacterial illnesses and can contribute to the safety of the dairy sector and animal vaccines are a valuable defense against a variety of bacterial infections that can impair the quality of milk produced by cows (Sedky *et al*. 2020). ERIC-PCR is used to create bacterial genotypes which help in the creation of effective bacterial vaccines.

For the aforementioned reasons, we used ERIC-PCR in the current investigation to determine the genotypes of *S. aureus* strains causing mastitis in cows.

Our results of ERIC-PCR revealed banding patterns ranging in size between 148 and 1095 bp (Figure 2). This range was included within the range obtained by Akindolirea *et al*. (2018) who used ERIC-PCR for *S. aureus* isolated from different milk samples and gave band sizes ranging from 250 to 2000 bp.

To facilitate any epidemic study, a suitable typing technique must have the discriminatory power to distinguish all unrelated isolates epidemiologically (Al-Obaidi *et al*., 2018).

In our study, ERIC-PCR results for the isolates produced 9 profiles which indicated high genetic diversity and heterogeneity of the strains with high discriminatory power (0.977). This was in line with the findings of Akindolirea *et al*. (2018), who discovered a high genotypic diversity of *S. aureus* isolated from various milk samples by employing ERIC-PCR and provided highly reproducible results with high a discriminative power to differentiate genetic dissimilar *S. aureus* isolated from the same sample. On the same aspect, Ye *et al*. (2012) classified 35 *S. aureus* isolates into 28 ERIC types with discrimination indexes (D) of 0.984. They concluded that *S. aureus*' genetic diversity and its sources in the food chain could be determined using the ERIC-PCR approach. Also, Arslan and Mutlu (2016) genotyped *S. aureus* strains isolated from bovine mastitis with high discrimination indexes (*D*) of 0.96.

In this study, the presence of some *S. aureus* strains which were obtained from the same region in multiple clusters, and some strains which were gathered from various regions in one cluster (Figure, 3) indicated the potential spread of infection between the regions under study and inadequate control measures on the farms that are being investigated. Similarly, Montso *et al*. (2019) reported high level of genetic relatedness between *K. pneumoniae* isolated from different collection sites. That suggested cross contamination, and this highlights the need for the region's farming management standards to be raised. Zhang *et al*. (2018) found that some *K. pneumonia* isolates from the same city were divided into different clusters of the ERIC type indicating the genetic heterogeneity of these isolates.

According to Guimarães *et al*. (2011), the animals that came from different places may have contributed to the dissemination of a wide range of bacterial genotypes. Sellyei *et al*. (2017) reported that the clusters could be explained by the movement of breeding animals.

5. CONCLUSIONS

In conclusion, the ERIC-PCR was found to be a powerful genotyping technique for *S. aureus* isolates in cows with mastitis, with significant genetic diversity and high discriminatory power. There was a genetic similarity between some strains gathered from various regions in the El-Gharbia governorate, Egypt, which demonstrated the spread of infections between the regions that were under research. So, to increase the mastitis cure rate, we advised restricting the movement of animals between these regions and increasing the control and management practices to prevent the spread of diverse bacterial genotypes.

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