Al-Qadisiyah Journal of Veterinary Medicine Sciences
(P-ISSN 1818-5746/ E-ISSN 2313-4429)
www.qu.edu.iq/journalvm

Phylogenetic analysis and molecular identification of Staphylococcus species isolated from cheese

Qasim Zamel Bneed, Orooba Meteab Faja
Department of Public Health, College of Veterinary, University of Al-Qadisiyah, Al-Diwaniyah City, Iraq
Received: January 10, 2024; Accepted: March 28, 2024; Published: May 7, 2024

Abstract

The purpose of the study was to identify and characterize the microbes found in the cheese samples that were collected from various Al-Diwaniyah City markets using molecular methods. In Al-Diwaniyah City, 75 cheese samples were collected from three different sources. The samples were cultured on the culture media. Detection of the isolated bacteria were done by using the staining, the culture, and the biochemical examination such as catalase tests, as well as PCR. 75 cheese samples tested positive for *S. aureus* 37(48%), *S. epidermidis* 20 (26.6%) and *S. intermedius* 18(24%). A 16S rRNA gene was used to amplify twenty isolates. All isolates were identified by PCR by using 16S rRNA gene and DNA sequencing. All of the examined isolates showed similarity of (93-99) % to those in the Gen Bank of NCBI.

Keywords: cheese, Staphylococcus Spp., gene 16s rRNA, Phylogenetic analysis.

Introduction

Production of the cheese was common use during the last three decade due to the cheeses consist of high level of protein and calcium, bioactive substances and the probiotic bacteria (1, 2). At the moment, only few scientific articles have been published in the world on the microbiological quality of soft cheese that made from pasteurized or raw cheese of goat and cow (3, 4, 5,6). *S. aureus* is third pathogens in the world for bacterial causes of foodborne illness (7). Consumption of heat-stable enterotoxins of *S. aureus* contaminated foods is a cause of food poisoning, abdominal cramps, nausea, diarrhea, endocarditis, vomiting, toxic shock, pneumonia, and skin infections (8).Milk and milk products are even being researched for possible health benefits. Evidence indicates its beneficial benefits on immunity, moderate hypertension, tumor prevention (8), suspending system routine precautions, and increasing dieter satiety, among other things. It is, however, a perfect medium for the pathogen's growth (10). Foodborne infections are a public health problem in poor countries. There are over two hundred fifty kinds of foodborne diseases. A common bacterium causes the bulk of these foodborne infections (11)

Materials and Methods

the study's toolkits included

Table 1 lists the commercial kits employed in the molecular investigation.

Type of Kits	Company/country
DNA extraction kit	AddBio /Korea
PCR master mix kit	AddBio /Korea

Primers

According to Table (2) the precise primer that targets the 16S rRNA gene was drawn from (12).

Table (2) Study-related primers

Primer name	Primer sequence	Size of bp	Target gene	Reference
F	5-AGA GTT TGA TCM TGG CTG AG-3	1500bp	16S rRNA	Sambrook and Russel, 2006)
R	5-TAC GG TAC CTT GTT ACG ACT-3			

Sample collection

The markets of Al-local Diwanyia provided a total of 75 different samples of cheese for testing. A thorough study was carried out following the collection of the samples, which took place between (the ninth - the eleventh) month of 2022. In order to conduct bacteriological analysis, the gathered cheese samples were brought to Microbiology Department, public health Laboratory, Veterinary College.

Bacterial isolation and identification

The cheese samples were diluted ten times in 0.1 percent peptone water before even being inoculated onto NA, BA, MA and BHI A, agar using the pour plate method and incubated at 37 C^0 overnight. The cells were then streaked over EMB and MC agar. These isolates were saved in case the bacteria needed to be recognized. The strains were detected by gram stain, colony shape, biochemical examination (Sugar fermentation, MR-VP assays, and indole), and coagulase and catalase tests. The isolates were amplified based on 16S rRNA gene.

Molecular identification

Al-Qadisiyah Journal of Veterinary Medicine Sciences
(P-ISSN 1818-5746/ E-ISSN 2313-4429)
www.qu.edu.iq/journalvm

1- Extraction of the gDNA

Following the instructions of gDNA Purification Kit, Promega, genomic DNA was extracted from bacterial culture as follows:

1 ml of culture on BHI for 2 minutes at 13000 rpm is used for pellet cells. Supernatant was then thrown away. 100 l from DNA rehydration and 100 l from Lysozyme added to the pellet and vortex for gram positive bacteria. 30 minutes of 37°C water bath incubation. Centrifuge the samples for 2 minutes at a speed of 13000 rpm after incubation. The extra was subsequently thrown away. 600 l of the Nuclei Lysis Solution was added, and the mixture was gently pipetted. All mixtures underwent a 5-minute incubation at 80 °C followed by a cooling to room temperature. 3 l of RNase is needed for RNA lysis. After mixing, it was incubated for 15 minutes at 37°C.

Cell lysate was mixed with 200 l of protein precipitation for protein precipitation. Then thoroughly combined by the vortex. followed by -30 °C deep freeze incubation. centrifuged for 5 minutes at 13,000 rpm after that. Transfer of diluted DNA to another tube have 600 l of isopropanol at room temperature. Gently combine, centrifuge as in "Pellet Cells" above, and then decant the supernatant. 600 l of 70% ethanol at ambient temperature, then, the mixture was centrifuged for 120 seconds at (13) thousand rpm. Drying of the pellet by air after ethanol aspiration. Rehydration of the DNA pellet was place in 100 l of Rehydration Solution at 65 °C for 1 hour.

DNA Amplification:

The electrophoresis examination of DNA recovered from each of the tested isolates confirmed the amplification findings. According to this study, the DNA strands were produced as a consequence of certain primers successfully attaching to recovered DNA from isolates. These successful connections were seen as distinct bands under UV light using ethidium bromide for DNA staining. The DNA mass was also determined by electrophoresis using the DNA marker 2000 bp DNA Ladder.

3-PCR Conditions:

PCR conditions were done by PCR device as table (3).

Table 3 PCR Thermo-cycler Conditions

Steps	°C	Min.: sec.	Cycle
Initial Denaturation	95°C	05:00	1
The denaturation Stage	95°C	00:30	30
The annealing stage	Varied	Varied	
The extension stage	72°C	01:00	
The final stage (extension)	72°C	07:00	
Hold	10°C	10:00	1

4- Gel electrophoresis

Gel electrophoresis test was carried out by combining 10 L of the amplicon with 2 L of gel loading dye and electrophoresing the mixture in pre-stained 1.0% (w/v) agarose (Sigma Aldrich, USA) for an hour at 80–90 volts in 1x Tris–Acetate–Ethylenediaminetetraacetic acid. There were both positive and negative controls. As a molecular size indicator, the 100 bp ladder used. Using the GeneSys G: BOX EF2 gel documentation system (Syngene, USA), the gels were seen under UV light.

Results and discussion

Ethical approval

The present study was conducted according to the standards for animal care and use and was approved by the Ethical Committee at University of Al-Qadisiyah.

Isolation of bacteria

A total of 75 bacterium strains were recovered from cheese. All isolates were cultivated in aerobic conditions at 37°C. Various forms of bacterial colonies developed on the surface of the agars after each isolate was incubated on different agars for 24–48 hours at 37°C. The 75 isolates have Gram-stain and biochemical qualities, as well as phenotypic characteristics such as spherical shape with irregular clusters (grapes bunch) for evaluate the bacterium isolation, the isolates were collected and bacteriologically tested. Isolated of Staph Spp. From Local cheese various colony morphologies were grow many medium. A total of (36) isolates were effectively acquired from each isolating plate. Staphylococcus aureus identified from three sources of cheese samples distributed as 10(50%), 3 (15%) and 7 (35%), from AL-Diwaniyah city center, AL-Daghara and AL-Sannia samples respectively. Additionally, Staph. epidermidius at incidence of 4(20%), 3 (15%) and 2(10%) were obtained from AL-Diwaniyah city center, AL-

Al-Qadisiyah Journal of Veterinary Medicine Sciences
(P-ISSN 1818-5746/ E-ISSN 2313-4429)
www.qu.edu.iq/journalvm

Daghara and AL-Sannia samples respectively, Staph. Intermedius at incidence of 3(15%), 1(5%) and 3(15%) for local cheese showed in Table (4).

Table 4. Number of isolated of *Staph. Spp*. From Local cheese

NO. of samples	*Staph.* Spp.	Location	No. (%)	Calculated X2	Calculated P
20	*Staph. aureus*	Diwaniyah city center	10(50)	5.55	0.062
		AL-Daghara	3(15)		
		AL-Sannia	7(35)		
9	*Staph. epidermidis*	AL-Diwaniyah city center	4 (44.44)	1	0.607
		AL-Daghara	3(33.33)		
		AL-Sannia	2(22.22)		
7	*staph. intermedius*	AL-Diwaniyah city center	3(42.85)	1.71	0.424
		AL-Daghara	1(14.28)		
		AL-Sannia	3(42.85)		

The present study detected the occurrence of *Staph. aureus* in cheese samples. This comes agreement with the literature that found that occurrence rate of *Staph. aureus* identified in samples of milk was found to be 24.8% (31 out of 125), which aligns with the incidence of *Staph. aureus* observed in cow samples of milk in Turkey (28%), as well as milk samples collected from instances of acute mastitis in Jordan. The incidence of mastitis in milk samples from bovine sources varied across different countries. Specifically, the occurrence was found to be lower in the United States (46.6%), Indonesia (57%), and Bangladesh (72.7%). However, it was higher compared to samples taken from cows in Jordan (13.7%), Ethiopia (12.5%), and South Africa (13,14). The observed variations in study outcomes may be ascribed to disparities in geographical location, characteristics of the investigation population numbers, farm hygiene and management protocols, milking techniques, and tools utilized (13,14,15,16,17).

Genotyping of most common isolates

Eight Staphylococcus spp. isolated (Qoqc07, Qoqc65, Qoqc29, Qoqc39, Qoqc03, Qoqc48, Qoqc13 and Qoqc55) that previously diagnosed were selected for further identification confirm using 16S rRNA gene Sequencing method which include amplification of the referred gene with the conventional PCR technique. To identify the selected isolates, PCR products obtained from the total genomic DNA were used to amplify the 16S rRNA gene. The PCR products were ap-proximately 1500 kb in size corresponding to these group of bacteria Figure (1).

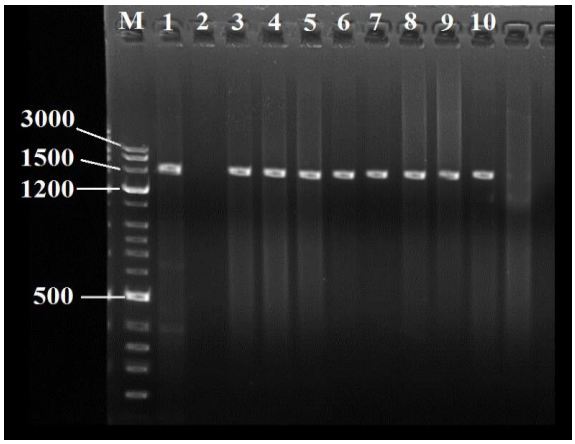

Figure 1: Amplified 16S rRNA gene for eight isolates on agarose gel stained with EB, Lane M: ladder; L1: Positive control; L2: negative control; L3: Qoqc65 gene; L4: Qoqc07 gene; L5: Qoqc29 gene; L6: Qoqc39 gene; L7: Qoqc03 gene; L8: Qoqc48 gene; L9: Qoqc13 gene; L10: Qoqc55 gene

The obtained DNA sequences were contrasted with gene bank sequences by numerical chain and maximum identification ratio after 16S rRNA sequencing using the Sanger technique. The similarity between the sequences varied from 99.92% to 93.31%. All 20 strains were grouped together and with GenBank data according to the

Al-Qadisiyah Journal of Veterinary Medicine Sciences
(P-ISSN 1818-5746/ E-ISSN 2313-4429)
www.qu.edu.iq/journalvm

16S rRNA sequences in reference demonstrating complete agreement with the biochemical test findings. NCBI has recorded 16S rRNA for these isolates with the accession numbers shown in Table (5).

Table 5. Sequences of the isolates and Gene Bank according to the similarity percentage by BLAST

NO.	Strain code	Species (GenBank)	Accession Number	Source	Similarity %
1	Qoqc07	*Staphylococcus aureus*	OQ346187	Cheese	96.71%
2	Qoqc65	*Staphylococcus aureus*	OQ346188	Cheese	96.78%
3	Qoqc29	*Staphylococcus aureus*	OQ346189	Cheese	97.34%
4	Qoqc39	*Staphylococcus aureus*	OQ346190	Cheese	97.50%
5	Qoqc03	*Staphylococcus epidermidis*	OQ346191	Cheese	97.59%
6	Qoqc48	*Staphylococcus epidermidis*	OQ346192	Cheese	97.88%
7	Qoqc13	*Staphylococcus intermedius*	OQ346193	Cheese	98.03%
8	Qoqc55	*Staphylococcus intermedius*	OQ346194	Cheese	98.57%

Phylogenetic analysis:

The link between our sequence and NCBI-published data (GenBank) is shown in figure (2) by a phylogenetic analysis of the isolates. Evolutionary links between taxa. The evolutionary history was inferred using the neighbor-joining method (18). The ideal tree is shown as having a total branch length of 1.99325332. The percentage of duplicate trees in which the related taxa gathered together is shown next to the branches in the bootstrap test (1000 repetitions) (19). The tree is shown to scale with branch lengths that generate the phylogenetic tree. The evolutionary distances, which are reported in terms of the number of base substitutions per site, were computed using the Kimura 2-parameter method (20). The analysis included 26 nucleotide sequences. All positions with gaps and missing data were eliminated. There were 1227 total sites in the final dataset. Evolutionary studies were carried out using MEGA6 software (21).

Al-Qadisiyah Journal of Veterinary Medicine Sciences
(P-ISSN 1818-5746/ E-ISSN 2313-4429)
www.qu.edu.iq/journalvm

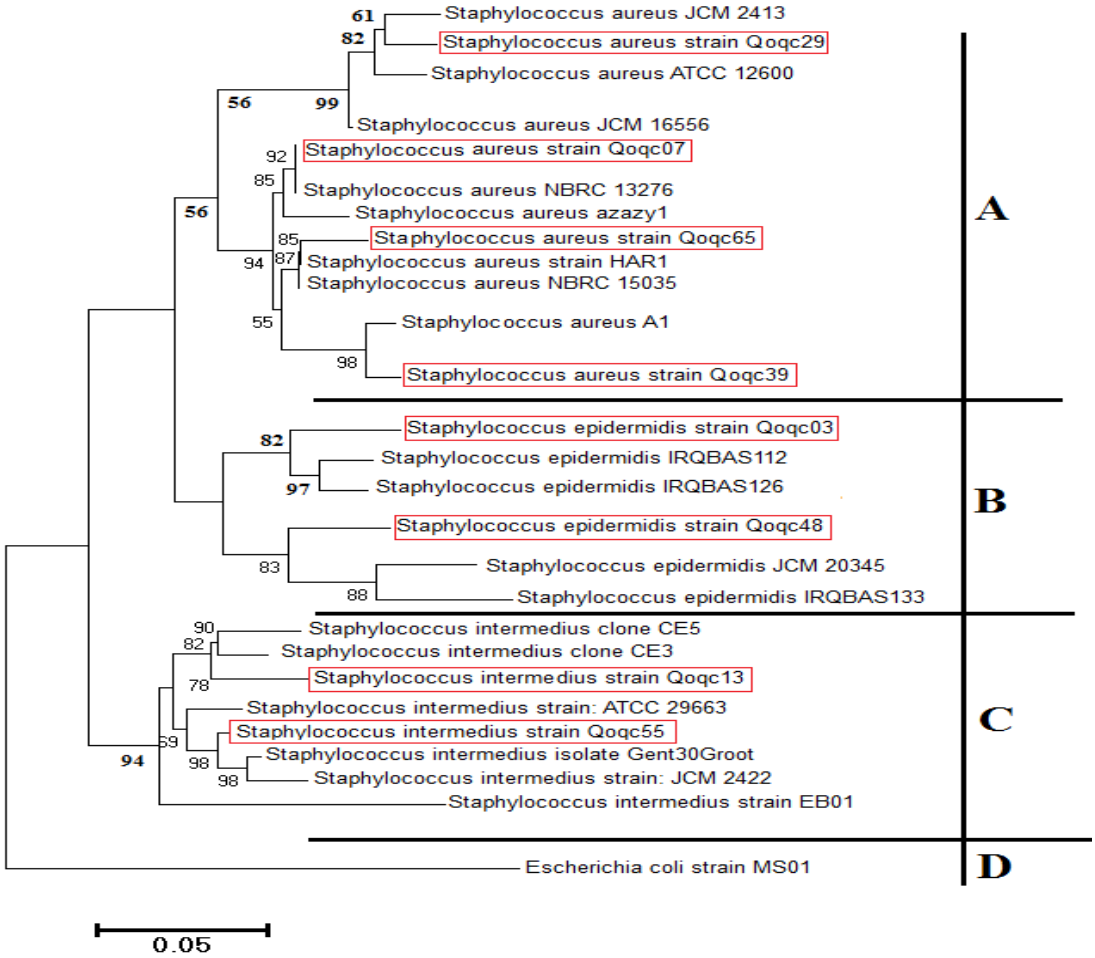

Figure 2: Phylogenetic analysis of 16S rRNA for eight staphylococcus spp

The phylogenetic tree showed the sequence groupings (Figure 2) that are more closely linked to one another than to the other sequences determined by similarity values. In this investigation, the studied bacteria were divided to A, B, C, and D clusters. The bootstrap value of 76%, the Staphylococcus aureus strains in Cluster A were particularly connected to one another. S. aureus strains within cluster A were shown to be linked, with bootstrap values ranging from 55% to 99%. The tested S. epidermidis strains that were especially connected to one another were grouped into cluster B (the main root of the bootstrap value was 81%), and the bootstrap values within cluster B varied from 82% to 97%. A unique relationship was shown by Cluster C of the S. intermedius strain (bootstrap value: 94%), while the bootstrap values within Cluster C varied from 59% to 98%. The tested isolates, which are gram positive bacteria, are represented by the gram-negative tree root sequence in cluster D. A and B Clusters were grouped with Cluster C at a measured bootstrap value of 69%, whereas the total bootstrap value of Cluster A and B was often measured at 56%.

Conclusion

According to the findings of this investigation, consumer cheese contains a significant number of species that cause contamination. As a result, the study's findings highlight to the need for additional care. The purpose of this study was to isolate and characterize bacteria present in cheese samples acquired from different Al-Diwaniyah City markets. From each isolating plate, a total of (36) isolates were successfully obtained. Staphylococcus aureus was found in three cheese samples from different sources. Eight Staphylococcus aureus isolates were amplified by PCR by using 16S rRNA. Define a terrifying event that necessitates extra care.

Acknowledgement

We would like to express our gratitude to FAO for providing logistical assistance.

Conflict of interest

Al-Qadisiyah Journal of Veterinary Medicine Sciences
(P-ISSN 1818-5746/ E-ISSN 2313-4429)
www.qu.edu.iq/journalvm

No conflict of interest is found for the present study.

References

1. Johnson ME. A 100-year review: cheese production and quality. J Dairy Sci. 2017;100(12):9952-65. https://doi.org/10.3168/jds.2017-12979

2. Górska-Warsewicz H, Rejman K, Laskowski W, Czeczotko M. Milk and dairy products and their nutritional contribution to the average polish diet. Nutrients. 2019;11(8):1771. https://doi.org/10.3390/nu11081771

3. Donkor ES, Aning KG, Quaye J. Bacterial contaminations of informally marketed raw milk in Ghana. Ghana Med J. 2007;41:58-61. https://doi.org/10.4314/gmj.v41i2.55302

4. Barukčić I, Ščetar M, Marasović I, Lisak Jakopović K, Galić K, Božanić R. Evaluation of quality parameters and shelf life of fresh cheese packed under modified atmosphere. J Food Sci Technol. 2020;57:2722-31. https://doi.org/10.1007/s13197-020-04308-6

5. Silva MP, Carvalho AF, Andretta M, Nero LA. Presence and growth prediction of Staphylococcus spp. and Staphylococcus aureus in Minas Frescal cheese, a soft fresh cheese produced in Brazil. J Dairy Sci. 2021;104(12):12312-20. https://doi.org/10.3168/jds.2021-20633

6. Saeed A. Detection of E. coli isolated from cheese by using virulence factors by PCR technique in AL-diwaniyia city. QJVMS. 2016;15(1):134-8.

7. Thiran E, Di Ciccio PA, Graber HU, Zanardi E, Ianieri A, Hummerjohann J. Biofilm formation of Staphylococcus aureus dairy isolates representing different genotypes. J Dairy Sci. 2018;101(2):1000-12. https://doi.org/10.3168/jds.2017-13696

8. Urmi MR, Ansari WK, Islam MS, Sobur MA, Rahman M, Rahman MT. Antibiotic resistance patterns of Staphylococcus spp. isolated from fast foods sold in different restaurants of Mymensingh, Bangladesh. J Adv Vet Anim Res. 2021;8(2):274. https://doi.org/10.5455/javar.2021.h512

9. Giovannucci E, Rimm EB, Wolk A, Ascherio A, Stampfer MJ, Colditz GA, et al. Calcium and fructose intake in relation to risk of prostate cancer. Cancer Res. 1998;58:442-7.

10. Pappa EC, Bontinis TG, Tasioula-Margari M, Samelis J. Microbial quality of and biochemical changes in fresh soft, acid-curd Xinotyri cheese made from raw or pasteurized goat's milk. Food Technol Biotechnol. 2017;55(4):496. https://doi.org/10.17113/ftb.55.04.17.5338

11. Center for Disease Control and Prevention (CDC). Food borne illness, January, vol. 10; 2018. p. 1-13.

12. Walker AW, Martin JC, Scott P, Parkhill J, Flint HJ, Scott KP. 16S rRNA gene-based profiling of the human infant gut microbiota is strongly influenced by sample processing and PCR primer choice. Microbiome. 2015;3(1):1-11. https://doi.org/10.1186/s40168-015-0087-4

13. Ektik N, Gökmen M, Çibik R. The Prevalence and Antibiotic Resistance of Methicillin-Resistant Staphylococcus aureus (MRSA) in Milk and Dairy Products in Balikesir, Turkey. J Hellenic Vet Med Soc. 2017;68(4). https://doi.org/10.12681/jhvms.16062

14. Haque ZF, Sabuj AAM, Mahmud MM, Pondit A, Islam MA, Saha S. Characterization of Staphylococcus aureus from milk and dairy products sold in some local markets of Mymensingh district of Bangladesh. J Nutr. 2018;8(6):1000743. https://doi.org/10.4172/2155-9600.1000743

15. Ismail ZB. Molecular characteristics, antibiogram and prevalence of multi-drug resistant Staphylococcus aureus (MDRSA) isolated from milk obtained from culled dairy cows and from cows with acute clinical mastitis. Asian Pac J Trop Biomed. 2017;7(8):694-7. https://doi.org/10.1016/j.apjtb.2017.07.005

16. Mphahlele MP, Oguttu JW, Petzer I-M, Qekwana DN. Prevalence and antimicrobial drug resistance of Staphylococcus aureus isolated from cow milk samples. Vet World. 2020;13(12):2736-42. https://doi.org/10.14202/vetworld.2020.2736-2742

17. Qolbaini EN, Khoeri MM, Salsabila K, Paramaiswari WT, Tafroji W, Artika IM, et al. Identification and antimicrobial susceptibility of methicillin-resistant Staphylococcus aureus-associated subclinical mastitis isolated from dairy cows in Bogor, Indonesia. Vet World. 2021;14(5):1180-4. https://doi.org/10.14202/vetworld.2021.1180-1184

18. Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987;4:406-25.

19. Felsenstein J. Phylogenies and the comparative method. Am Nat. 1985;125(1):1-15. https://doi.org/10.1086/284325

20. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 1980;16:111-20. https://doi.org/10.1007/BF01731581

21. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013;30:2725-9. https://doi.org/10.1093/molbev/mst197