Phylogenetic analysis and molecular identification of bacteria isolated from fresh cow milk

Suha Najem Abed[1], Orooba Meteab Faja[2]
[1, 2] Department of public health, Veterinary College, Al-Qadisiya University, Iraq.
Email: vet20.post30@qu.edu.iq

Abstract
The study's purpose was to identify bacteria in raw cow's milk using molecular methods. 90 milk samples were gathered from three distinct sources in Al- Diwaniya city: Taj Al-Nahrain Company for Agricultural Production and Livestock, breeders, and super markets. Milk samples were cultured on various culture media to isolate microorganisms. Staining features, cultural properties on various selective medium, biochemical tests, catalase and coagulase tests, and ultimately PCR were used to identify the isolated bacteria. Ten (11.1%) of the 90 milk samples tested positive for E. coli, whereas the other eight were negative (8.8 percent) 16 Proteus spp., 16 (17.7%) Klebsiella spp., 3 (3.3%) Salmonella spp., and 10 (11.1%) Staphylococcus spp. was found. A 16S rRNA gene-based PCR was used to amplify 20 isolates. The polymerase chain reaction (PCR) and DNA sequencing were used to detect and identify every suspect isolate. The PCR was directed toward the 16S rRNA gene. The sequencing results revealed that the strains belonged to Citrobacter spp., E. coli spp., Staphylococcus spp., Klebsiella spp., Proteus spp., Enterobacter spp., and Streptococcus spp., and that all of the tested isolates had a similarity of 93 percent to 99 percent to those in the Gen Bank of The National Centre for Biotechnology.
KEYWORDS: RAW MILK, GENE 16S RRNA, PHYLOGENETIC ANALYSIS.

Introduction

Milk is among the foods that have the highest value in terms of its nutritional content. Cow's milk is used to produce whole milk, which typically has around 3.5 percent milk fat in it. Milk and milk products are even being studied for their potential health benefits. Evidence supports its effects on immunity, moderate hypertension, tumor prevention (7), suspending system standard precautions, and enhancing dieter satiety, among other favorable effects. It is, however, an ideal medium for bacterial pathogen growth and transmission to people (4). In both industrialized and developing countries, foodborne illnesses constitute a major public health concern. There are around 250 different types of foodborne illnesses. The majority of these foodborne illnesses are caused by a common microorganism [3]. Public health is very worried about bacterial infections that come from food [6]. Bacteria may be discovered in many different environments, including water, soil, plants, animals, and goods derived from animals, such as meat, milk, cheese, and yoghurt [3]. Because of its high-water activity and nutritional value, cow's milk is a perfect substrate for the development of germs. This can lead to a variety of health problems [11]. There is a significant risk to the general population's health posed by bacteria that are routinely identified and discovered in milk and items made from milk. Milk often harbors harmful bacteria such as E. coli, Staphylococcus aureus, Salmonella species, and Listeria monocytogenes [1]. One of the pathogens that cause the majority of foodborne diseases is Salmonella [3]. It's a disease-causing opportunistic infection that can affect humans, animals, and birds (2). Temperature has a considerable impact on the diversity of bacteria and the deterioration of milk during shipping, processing, and storage. Since the 1970s, researchers have known that the storage and distribution of fluid milk and other perishable dairy products in refrigerators poses a persistent risk of contamination with psychotropic bacteria (14).As a result, investigators have paid more attention to psychographs in recent years, as modern advances in milk processing and shipping have resulted in milk being stored for longer periods of time prior to processing, production, or consumption, a time of refrigeration is required (10).

Materials and Methods

Sample collection

From the several marketplaces in Al-local Diwaniya, a total of ninety samples of raw cow's milk were collected. The samples were gathered between September and December 2021, and an investigation was conducted afterward. The milk samples that were collected were transferred on ice to the Microbiology Laboratory in the Department of Microbiology and Hygiene at the Veterinary College so that bacteriological analysis could be performed on them.

Bacterial isolation and identification

The milk samples were diluted ten times in 0.1 percent peptone water before even being inoculated onto NA, BA, MA, SS A, BHI A, and MacConkey (MC) agar using the pour plate method and incubated at 370C for 24 hours. The cells were then streaked over eosin methylene blue (EMB) agar

doi.org/10.31838/hiv23.01.20 Received: 26.09.22, Revised: 05.10.22, Accepted: 19.11.22

and MC agar to get pure culture. These isolates were stored away in the event that it was necessary to identify the bacterium. Gram staining, colony morphology on MC and EMB agars, biochemical characterization (using sugar fermentation, indole, and MR-VP assays), and catalase and coagulase tests were utilized in order to select the strains. Amplification of the 16S rRNA gene, which is unique to bacteria, was used to confirm each of the isolates.

Molecular Identification

1. Genomic DNA extraction

The Wizard genomic DNA purification kit was used to extract the whole genomic DNA of the total isolated bacterium (Promega, USA). The culture was cultured overnight on Nutrient Broth (NB) and extracted after 1 minute of centrifugation at 13000 rpm. The pellet was then suspended in 480l of 50 mm EDTA solution. The supernatant was decanted after a second centrifugation at 13000 rpm for 2 minutes. A 600l nuclei lysis solution was progressively added and thoroughly mixed using pipetting. The mixture was allowed to cool to room temperature after a 5-minute incubation period at 80°C. The mixture was then incubated at 37 C for 15-60 minutes before being allowed to cool to room temperature, with (3μL) of RNase solution added. This mixture was given 200 L of protein precipitation solution, which was vortexed before being incubated for 5 minutes on ice. After that, the mixture was centrifuged for three minutes at a speed of 13,000 revolutions per minute. The supernatant was placed into a clean tube that contained 600 L of isopropanol at room temperature, and the contents of the tube were vigorously mixed. The supernatant was thrown away after the mixture was centrifuged for two minutes at a speed of 13,000 revolutions per minute. The solution was mixed before being centrifuged at 13,000 rpm for 2 minutes with 600 microliters of 70 percent ethanol at room temperature. The pellet was air dried for 10-15 minutes after aspirating the ethanol. A 100L rehydration solution was used to rehydrate the DNA pellet for 1 hour at 65 C.

1. DNA Amplification:

The amplification results were validated by electrophoresis analysis on DNA isolated from all of the isolates tested. According to the findings of this research, the strands of DNA were produced as a result of the effective binding of certain primers to isolates of recovered DNA. Using ethidium bromide as a particular DNA stain, these successful bonds appeared as separate bands under UV light. Based on the DNA marker 2000 bp DNA Ladder, electrophoresis was also utilized to measure DNA m.w.

2. PCR Thermos cycler Conditions:

The following table shows the PCR heat cycler settings used with a standard PCR thermos cycler:

PCR Thermos cycler Conditions:

Initial denaturation	1	95	5 min
Denaturation	30	95	5 min
Annealing		55	30 sec
Extension		72	45 sec
Final extension	1	72	7 min
Hold	-	4	forever

3. Gel electrophoresis

The 16s rRNA gene PCR products were evaluated by putting them into 1.5% agarose as follows:
1.5 % Agarose gel was made by dissolving 1X TBE in a water bath at 100°C for 15 minutes and then allowing to cool to 50°C. After that, the ethidium bromide stain was added three times to the agarose gel solution. After positioning the comb correctly, an agarose gel solution was poured into the tray and allowed to harden at room temperature for 15 minutes. This procedure involved carefully removing each comb from the tray, and then filling each comb well with 10ml of PCR product and 5ml of (2000bp Ladder). It was necessary to fix the gel tray in the electrophoresis chamber and then add 1X TBE and buffer to start the process. At 80 volts and 60 hertz, the electricity was administered for 1.5 hours. Gene-specific PCR products(406bp) were detected using the gel documentation method (Rahn et al., 1992). Sanger's technique was used using Mega Base 1000 (GE HealthCare, UK) for 16S rDNA sequencing, according to Reysenbach et al (2000). The data from Gen Bank, which may be accessible on the NCBI website, were compared using the BLAST method.

RESULT &DISSCTION:

Isolation of bacteria:

A total of 90 bacterium strains were recovered from cow's milk. 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. A total of 90 isolates were shown to have Gram-stain and biochemical qualities, as well as phenotypic characteristics such as cocci or rods in shape. To evaluate the rates of bacterium isolation, the isolates were collected and bacteriologically tested. Out of the total 90 raw milk samples examined, Klebsiella spp. 16 (17.7%), E. coli and Staphylococcus spp. 10 (11.1%), Pseudomonas spp. and Proteus spp. 8 (8.8%), Streptococcus spp. 5 (5.5%), Citrobacter spp. 4 (4.4%), Salmonella spp. and Enterobacter spp. 3 (3.3%), and 10 samples exhibited no growth, respectively. (See Table 1). The

number and percentage of different bacterium species. Microbiological tests for Staphylococcus aureus and Escherichia coli revealed varied levels of contamination (Table 1). Staphylococcus aureus was found in raw milk at a rate of 60% for an average of the samples (n= 23), while E. coli was found in 94 % of the samples by (15). In dairy cattle farms, this germ is thought to be the principal cause of clinical and subclinical mastitis (9). These bacteria, even at low concentrations in the milk, suggest faecal contamination and point to worsening sanitary conditions during milking or shipment. More than 60% of the isolates were found to be Salmonella spp., according to the sequencing results (12). The sampling procedure could be to blame for the variation in prevalence with the previously stated research. Instead of taking samples directly from the udder, those studies used bulk tanks, which might lead to dilution or a temperature impact, slowing down the isolation procedure.

For these strains, the PCR method was used (Figure 1). The average length of each sequence was 1500 bp, which was compared to those in Gen Bank employing the BLAST tool (http://www.ncbi.nlm.nih). On the one hand, the outcomes of the investigation appeared to suggest that the 20 isolates had correctly identified the bacterium (Table 2). MEGA 10 software was used to investigate the evolutionary link between the trial sequence and its near relatives. A phylogenetic tree was generated, and the same programmed was utilized to reduce any gaps and missing data in the trail sequence (Figure 2).

Table (1): the number and proportion of bacterial species isolated from 90 milk samples

samplesOrganisms	Taj-AlnahreenCompany(30) sample	Supermarket(30) sample	AL – Hamza(10) sample	AL-Daghara(10) sample	AL-Sannia (10) sample	Total strain	percentages
E.coli	3	1	2	1	3	10	11.1%
Proteus spp.	3	3	1	1	-	8	8.8%
Klebsiella spp.	5	3	3	2	3	16	17.7%
Salmonella spp.	-	-	-	2	1	3	3.3%
Citrobacter spp.	-	-	1	1	2	4	4.4%
Enterobacter spp.	2	-	1	-	-	3	3.3%
Pseudomonas spp.	2	5	-	1	-	8	8.8%
Staphylococcus spp.	3	4	1	1	1	10	11.1%
Streptococcus spp.	2	1	1	1	-	5	5.5%
No growth	10	-	-	-	-	10	11.1%
X2							21.31
P value							0.011*

* Significant difference at P< 0.05

Genotyping of most common isolates.

Twenty isolates that had previously been identified by phenotypic and biochemical testing were chosen for confirmation using the 16S rRNA gene sequencing approach, which included PCR amplification of the referenced gene. The 16S rRNA gene was amplified using PCR products obtained from total genomic DNA to identify the selected isolates. The PCR products, which corresponded to this group of bacteria, were around 1500 kb in size (Figure 1).

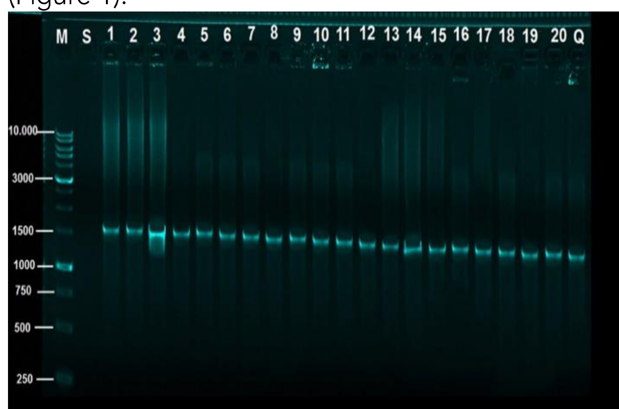

Figure (1): PCR product the band 1500 bp. Results of the amplification of 16S rRNA of 20 bacterial isolates were fractionated on 1% agarose gel electrophoresis stained with Ethidium bromide, Lane M: 1kb ladder marker; Lane S: Control Negative; Lane Q: Control positive (E. coli ATCC 25922); Lanes 1-20 positive PCR results

Following 16S rRNA sequencing using Sanger method, the obtained DNA sequences were compared with other sequences in Gen Bank also with numerical chain and maximum identification ratio. The sequences gave a similarity ranged between 99.92 % to 93.31 % (Table: 2). According to the sequences of the 16S rRNA gene in Gen Bank, all 20 strains were grouped with each other and with Gen Bank data. Revealing the complete concordance with the biochemical test results. These isolates' 16S rRNA genes have been registered with the National Center for Biotechnology Information (NCBI), with accession numbers as stated in Table (2).

Table: (2) Comparison between isolates sequences with Gen Bank sequences based on percentage of similarity by using BLAST software					
NO.	Strain code	Species namefrom Gene Bank	Accession Number	Source	Similarity %
1	MS01	Escherichia coli	OM907716	Milk	97.95%
2	MS02	Salmonella enterica subsp. enterica serovar Enteritidis	OM907717	Milk	93.31%
3	MS03	Streptococcus equines strain (bovos)	OM907718	Milk	96.69%
4	MS04	Proteus mirabilis	OM915407	Milk	95.96%
5	MS05	Streptococcus uberis	OM915408	Milk	96.56%
6	MS06	Streptococcus agalactiae	OM915409	Milk	95.58%
7	MS07	Streptococcus dysgalactiae	OM915410	Milk	96.13%
8	MS08	Proteus mirabilis	OM915411	Milk	95.55%
9	MS09	Salmonella enterica subsp. enterica serovar Typhimurium	OM915412	milk	95.27%
	MS10	Klebsiella pneumoniae	OM915413	Milk	94.26%
10	MS11	Escherichia coli	OM918267	milk	95.11%
11	SAVQ1	Citrobacter freundii	OM877349	Milk	98.46%
12	SAVQ2	Citrobacter braakii	OM877350	milk	97.04%
13	SAVQ3	Klebsiella pneumoniae	OM877351	milk	99.90%
14	SAVQ4	Klebsiella pneumoniae	OM877352	milk	97.41%
15	SAVQ5	Klebsiella pneumoniae	OM877353	milk	97.59%
16	SAVQ6	Klebsiella pneumoniae	OM877354	milk	99.92%
17	SAVQ7	Enterobacter cloacae	OM877355	milk	99.78%
18	SAVQ8	Pseudomonas aeruginosa	OM877356	milk	99.72%
19	SAVQ9	Klebsiella pneumoniae	OM877357	milk	99.74%
20	SAVQ10	Staphylococcus aureus	OM877358	milk	99.77%

Phylogenetic tree of the isolated:

A phylogenetic tree of the examined isolates (Figure 2) was generated to highlight the relationship and comparison between our sequence data and previously released NCBI data (Gen Bank). In evolution, there are taxonomic relationships. The evolutionary history was inferred using the Neighbor-Joining method (13). With a branch length sum of 1.98323341, the optimal tree is depicted. Next to each branch is the proportion of duplicate trees in which related taxa grouped together in the bootstrap test (1000 repetitions) (5). The phylogenetic tree is presented proportionally, with branch lengths in the same units as the evolutionary distances used to build the tree. The evolutionary distances were estimated in terms of base substitutions per site using the 2-parameter Kimura method (8). The researchers looked at 26 different sets of nucleotides. Gaps and missing data were taken out of all positions. The final dataset had a total of 1239 places in it. MEGA6 was used to do the analysis of evolution (16).

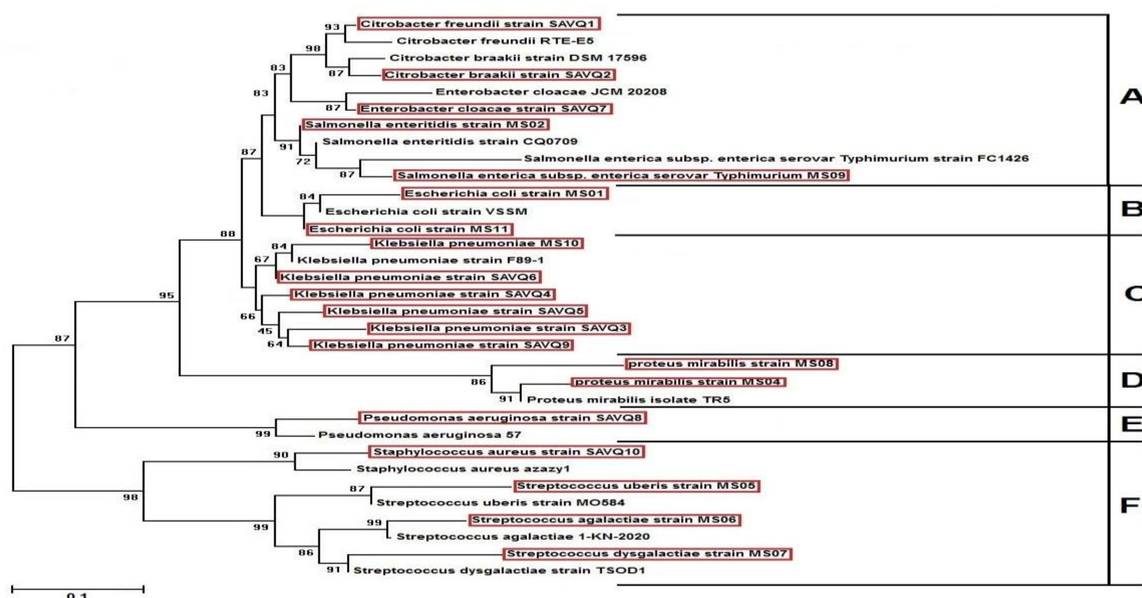

Figure (2): Phylogenetic tree showing the evolutionary relationships of the 16S rRNA gene

The phylogenetic tree revealed sequence groups that are more closely linked to each other than to

other sequences based on similarity values, as shown in Figure (2). In this study, negative gram stain bacteria were divided into five clusters: A, B, C, D, and E, while gram positive bacteria were divided into Cluster F. Citrobacter freundii strain SAVQ1 and Citrobacter braakii strain SAVQ2 strain MS02 were shown to be closely related (bootstrap value 98 %). A specific association (bootstrap value 91%) was found between Salmonella enteritidis strain MS02 and Salmonella enterica subsp. enterica serovar Typhimurium MS09, as well as a cluster B of Escherichia coli strain MS01 and Escherichia coli strain MS11 (bootstrap value 84 %) and a bootstrap value of 87% for Cluster A and B. Cluster C of Klebsiella pneumonia constituted a monophyletic group that was recovered in 88 percent of bootstrapped trees containing cluster A and B. Proteus mirabilis strain MS04 and Proteus mirabilis strain MS08 were bootstrapped at 86 percent with each other, and its cluster (cluster D) was bootstrapped at 95 % when cluster A,B, and C were integrated. Cluster E of Pseudomonas aeruginosa strain SAVQ8 and Pseudomonas aeruginosa strain 57 were specifically related to each other at bootstrap value of 99% and it showed 87% bootstrap value with cluster A, B, C and D combined. Cluster F includes Staphylococcus and Streptococcus species were bootstrapped at 98% with each other and were shown bootstrap value at 87% with all other clusters combined.

Conclusion

This study found that raw milk supplied to customers has a substantial number of organisms that cause contamination. As a result, the findings of this study point to the need for additional care. The goal of this study was to isolate and characterize bacteria found in raw milk samples obtained from several local markets in Al-Diwaniya city. 15 (75%) of the 90 milk samples were positive for E. coli, while 10 (15.5%) of the milk samples tested negative. A 16S rRNA gene-based PCR was used to amplify 15 Escherichia coli isolates. Define a frightening scenario that requires special attention.

Acknowledgements

We would like to extend our thanks to the United Nations' Food and Agriculture Organization (FAO) for providing logistical help.

References

[1] Abeer A, Azza SA, Dardir HA, Ibrahim AK. Prevalence of some milk borne bacterial pathogens threatening camel milk consumers in Egypt. Glob Vet. 2012; 8(1):76–82

[2] 2- Amany, I.E.; Ebtehal, A.E. and Osman, A.O. (2006): Plasmid mediated carbapenems resistance in clinical and environmental isolates of Pseudomonas aeruginosa recovered from sheep farm.S.C.V.M.J. X (1)7-16.

[3] 3- Center for Disease Control and Prevention (CDC). Food borne illness, January, vol. 10; 2018. p. 1–13.

[4] 4 -Donkor ES, Aning KG and Quaye J (2007). Bacterial contaminations of informally marketed raw milk in Ghana. Ghana Medical Journal 41: 58-61.

[5] 5-Felsenstein, J. (1985) Phylogenetic and Comparative Method. The American Naturalist, vol.125.no 1, p 1-15.

[6] Forshell PL, Wierup M. Salmonella contamination: a significant challenge to the global marketing of animal food products. Revue ScientifqueEt Technique-Ofce International Des Epizooties. 2006; 25(2):541–54.

[7] 7-Giovannucci E, Rimm EB, Wolk A, Ascherio A, Stampfer MJ, Colditz GA and Willett WC (1998). Calcium and fructose intake in relation to risk of prostate cancer. Cancer Research 58: 442-447.

[8] 8-Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of molecular evolution, 16, p 111–120.

[9] 9-Leonard FC, Markey BK. Meticillin-resistant Staphylococcus aureus in animals. Vet J. 2008; 175: 27-36.

[10] 10- Olfa, S.B.; Imène, F.; Rouaa, L.; Hamadi, A. and Mohamed, A.A. (2013): Study of Proteolytic and Lipolytic Activities of Pseudomonas spp. Isolated From Pasteurized Milk in Tunisia. Journal of Agricultural Science; Vol. 5, No. 7; 2013.

[11] 11. Parekh TS, Sub hash R. Molecular and bacteriological examination of milk from different milk animals with special reference to Coliforms. Curr Res Bacteriol. 2008; 1(2):56–63.

[12] 12-Rubiela, C.; Adriana,P. ; Geraldine,L. ; Camila ,A. ; Olimpo ,O. (2021) : Isolation and identification of Salmonella spp. in raw milk from dairy herds in Colombia. J Vet Res Anim Sci. 58: e172805.

[13] 13- Saitou, N., Nei, M. (1987): The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4(4):406-425.

[14] 14- Singh, P.; Wani, A.A; Karim, A.A. and Langowski, A.A. (2012): The use of carbon dioxide in the processing and packaging of milk and dairy products: A review. International Journal of Dairy Technology, 65, 161-177.

[15] 15-Soumeya, K.; Nesrine, B.; Soumeya, M. (2014): Prevalence of some pathogenic bacteria of raw milk in Algeria. Journal of Archives of Biomedical Sciences, 2 (2): 30-33.

[16] 16-Tamura,O. Stecher ,G. Peterson, D., Filipski, A., and Kumar, S.(2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Journal of molecular evolution, 30(12): 2725–2729.