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Isolation and Identification of Enteric Bacteria as Index Organisms for Milk Hygiene

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ABSTRACT

The present study was designed to isolate enteric bacteria from Egyptian raw milk. Twenty two samples were assembled randomly from local stores in Mansoura city and surrounding hamlets. Those samples are including Rayeb milk, buffalo farm milk, cow farm milk, bulk tank farm milk and bulk tank milk. A total of 136 of enteric bacterial isolates could be cultured from these samples by selective media and subjected to morphological and biochemical examinations. Out of these isolates, 2 cultures were isolated from cow farm milk and bulk tank farm milk and could be identified as *Salmonella enterica* and *Staphylococcus aureus* using 16S rRNA. Obtained results show that Egyptian raw milk can contain harmful bacteria such as *Salmonella* sp, *E. coli* and *Staphylococcus* sp, responsibility for causing many foodborne diseases.

Keywords: Enteric bacteria, *Salmonella enterica, Staphylococcus aureus*, Raw milk, PCR technique.

INTRODUCTION

Milk and dairy products provide wealth of nutritional benefits they are the most important food products from animal origin and milk is considered as a complete food because it contains protein, sugar, fat and contains a large amount of water, including a wide range of nutrients such as vitamins, proteins, fats and carbohydrates are suspended (Godefay and Molla 2000).

It is common knowledge that raw milk is the vector of pathogenic agents to humans that contain dangerous microorganisms that can cause serious health risks to humans (Donkor *et al.,* 2007).

Raw milk is the product of cows, sheep, buffalo, or goats that have not been pasteurized to kill harmful bacteria. This unpasteurized raw milk can contain dangerous bacteria such as *Salmonella* sp, *E. coli*, *Staphylococcus* sp and *Listeria* sp, which cause many foodborne illnesses. Where a dangerous effect can affect the health of anyone who drinks this raw milk or eats the foods which made from it. These bacteria can pose a risk to people with impaired immune systems, pregnant women, children, and older adults (Garedew *et al.,* 2012).

The bacteria can reach the milk and contaminate it through various stages such as procurement, processing and distribution. Pollution can also be caused by animal feed, hanger, milk collection materials, as well as various ingredients added to dairy products and dairy farm workers (Teka 1997 and Claeys *et al.,* 2013).

Salmonella sp., Staphylococcus sp., Listeria sp., Escherichia coli, and coliforms have been detected with milk borne diseases, and these are often isolated from fresh cow milk (Fadaei 2014). The presence of intestinal bacteria is a strong indicator of contamination of faeces in general, whether in water, food, milk or other dairy products. The presence of *E. coli* particularly in food is a sign of the possibility of intestinal microorganisms and/or toxins that can pose a public health hazard (Bouazza *et al*., 2012).

The present work was therefore designed to isolate and identify enteric bacteria associated with Egyptian milk samples; the identification of obtained isolates was done using traditional methods and molecular one of PCR technique based on 16S rRNA.

MATERIALS AND METHODS

Milk samples

22 Samples are the total samples collected from Rayeb milk and raw milk of buffalo farms, cow's farms and bulk tank milk were randomly collected from different farmer's houses and local markets in Mansoura city and villages. These samples include 6 samples of Rayeb milk, 4 samples of buffalo farm milk, 4 samples of cow farm milk, 4 samples of bulk tank farm milk and 4 samples of bulk tank milk. Samples were directly transferred to the laboratory under special conditions for microbiological examinations and all these samples were microbiologically examined for enteric bacteria.

Cultivation media

The following general and specific cultivation media were used for different purposes. MacConkey agar medium (MacCA) (Oxoid, 2012) was used for isolation of coliform bacteria. The composition of this medium was as mentioned in Oxoid (2012). About 51.5 g of powder was suspended in 1 L of distilled water to the boil (pH 7.2 ± 0.2) and sterilized in the autoclave at 121ºC for 15 minutes.

Salmonella-Shigella agar medium (SS agar).

This selective medium was used for isolation of *Salmonella spp.* and *Shigella spp.* The formation of this medium was as mentioned in Oxoid (2012). About 60.1 g of the powder was suspended in 1 L of distilled water to the boil with frequent agitation and allow it to simmer gently dissolving the agar at pH 6.90 ± 0.2, then it was cooled to 50°C, mixed well and poured into sterile Petri dishes.

Baird Parker agar medium (BP agar).

This selective medium was used for isolation of *Staphylococcus spp.* The formation of this medium was as mentioned in Oxoid (2012). About 60 g of the powder was suspended in 950 mL of distilled water, then allowed to soak and brought to the boil stirring constantly (pH 7.0 ± 0.2), sterilized in the autoclave at 121°C for 15 minutes, it was cooled to 50ºC and added 50 mL of egg yolk tellurite sterile emulsion (Art. No.06-026), homogenized and distributed into plates. Once prepared, the medium must neither reheated nor sterilized again.

Listeria selective agar medium and *Listeria* selective supplement (oxford formulation) was used for isolation of *Listeria monocytogenes*. The formation of this medium was as mentioned in Oxoid (2012). About 27.75g of the *Listeria* selective agar base (Oxford formulation) was suspended in 500ml of distilled water. Then it brought gently to the boil to dissolve (pH 7.0 ± 0.2) and sterilized by autoclaving at 121°C for 15 minutes after cooled to 50°C and aseptically added the contents of one vial of *Listeria* selective supplement (code SR 0140) reconstituted

with 5ml of 70% ethanol, mixed well and poured into sterile Petri dishes.

Tryptic Soy agar medium (TSA) was used as general purpose solid medium. The composition of this medium was as mentioned in Oxoid (2012). About 40 g of powder was suspended in 1 L of distilled water. It was soaked and brought to the boil to dissolve the agar (pH 7.3 ± 0.2) then sterilized in the autoclave at 121°C for 15 minutes.

Tryptic Soy broth medium (TSB) (Oxoid, 2012) was used for a highly nutritious general purpose for the growth of bacteria and fungi. The composition of this medium was as mentioned in Oxoid (2012). About 30g of powder was suspended in 1 L of water, mixed well and distributed into final containers (pH 7.3 ± 0.2), then sterilized by autoclaving at 121°C for 15 minutes.

Isolation of enteric bacteria from milk samples

Milk samples were serially diluted in sterilized saline solution (0.85% NaCl). Resultant dilutions were plated onto the surface of the different cultivation media, followed by incubation at 37°C for 48 h. Obtained single colonies were picked up and maintained for identification tests.

Identification of selected bacteria

Obtained bacterial isolates were subjected to morphological and biochemical examinations including Gram-staining, catalase test, milk coagulation test, and lactose fermentation test. Prior to each test, suspected isolates were grown overnight at 37°C in Tryptic Soy agar (TSA) medium.

Bacterial staining

Overnight bacterial cultures were experimented for Gram staining and cell morphology and endospore reactions as explained by Pollack *et al.* (2005).

Catalase activity

Suitable amount of growth from one discrete colony of each bacterial isolate was transferred into a clean glass slide, followed by addition of 1 drop of H_2O_2 (30%). Immediate bubbling (gas formation) was taken as a positive result (MacFaddin, 2000).

Milk coagulation

Overnight bacterial cultures were inoculated with 1% (v/v) into sterilized reconstituted skim milk (10% total solids), followed by incubation at 37°C. Cultures were observed for milk coagulation every 30 minutes up to 6 h (MacFaddin, 2000).

Lactose fermentation test

Overnight bacterial cultures grown in TSB were inoculated into phenol red lactose broth (the medium is a nutrient broth to which 1.0% lactose is added) containing phenol red (0.5% w/v). The pH indicator phenol red is red at neutral pH but turns yellow at pH <6.8, the inoculated tube is incubated at 37°C for 24 h. The fermentation of lactose by isolates was indicated by a change of the indicator color from red to yellow, indicating a pH change to acidic. (MacFaddin, 2000).

Polymerase Chain Reaction (PCR) Identification

The polymerase chain reaction (PCR) method based on 16S rRNA gene for more identification. DNA extraction was done by using protocol of Gene Jet genomic DNA purification Kit of Thermo K0721 (Sigma Company). A single colony of each bacterial isolates was grown on a suitable medium in Erlenmeyer flask and incubated at 37°C for 24 h. Culture was harvested by centrifugation at 4°C at 5000 rpm for 10 min, DNA was extracted from pellets using Thermo K0721 (Sigma company).

RESULTS AND DISCUSSION

Isolation of enteric bacteria from Egyptian milk samples

Twenty two samples of Egyptian milk were assembled randomly from local stores in Mansoura city and surrounding hamlets. The samples were divided into 6 of Rayeb milk, 4 samples of buffalo farm milk, 4 samples of cow farm milk, 4 of bulk tank farm milk and 4 samples of bulk tank milk (Table1).

Table 1. Examined milk samples and the potential enteric bacterial isolates found in each sample

Samples	No. of Samples	No. of enteric bacterial isolates cells	No. of enteric bacterial isolates depending on the shape of cells	
			Coccoid	Rods
Cow milk	4	38	-	38
Buffalo milk	4	38	10	28
Market milk	4	34	4	30
Mixed farm milk	4	26	7	19
Rayeb milk	6	-	-	-
Total	22	136	21	115

Samples were serially diluted and plated onto four selective cultivation media such as MacConky agar, *Salmonella-Shigella* agar, *Listeria* selective agar and Baird parker agar. Formed enteric bacterial colonies were picked up from agar plates and subjected to preliminary identification tests including Gram-staining, endospore-staining, catalase test, milk coagulation test and lactose fermentation test.

The three selective media namely MacConky agar, *Salmonella-Shigella* agar and Baird parker agar gave positive results with different milk samples except *Listeria* selective agar medium which gave negative result with different milk samples. Among 136 bacterial isolates, 19 were coccoid shaped cells, 2 oval coccoid shaped cells, 104 short rods and 11 long rods isolates. Seventy six isolates were Gram positive while 60 isolates were Gram negative. Following the completion of microscopic examination, some identification tests were performed according to Brenner *et al.,* (2005) as shown in Table (2).

Results in Table (2) showing that Gram-negative and catalase-positive isolates which also coagulate milk-negative and negative for lactose fermentation was isolated from *Salmonella Shigella* agar medium considered as potential *Salmonella* sp. or *Shigella* sp. (Brenner *et al.,* 2005).

Colonies appeared on the middle of SS agar medium with a black color picked up and striped onto TSA slant. After growth, the assay tests proved that all cells were short rods, non-spore formers, Gram negative, catalase positive, negative for coagulation and lactose fermentation. Isolates No. B_1, $B`_2$ , $B`_3$, $B`_8$ , $B`_9$, $J`_1$ , $E_2$ , $Q`_1$, M_7 , M_8 , M_9 , $L`_1$ , $L`_3$, $L*_1$ and $L*_2$ considered as *Salmonella* sp. according to Bergey's Manual of Systematic Bacteriology (Brenner *et al.,* 2005).

Pink color colonies appeared on the middle of SS agar medium were picked up and striped onto TSA slant. After growth, the exploration tests presented that all cells were Gram negative, short rods, non-spore formers, catalase positive, lactose fermentation positive and 50% of isolates were coagulase negative and other 50% were coagulase positive. Isolates no. $B`_5$, $B`_7$, E_1 and M_4 examined as *Escherichia* sp. (Fig.1) conferring to Bergey's Manual of Systematic Bacteriology (Brenner *et al.,* 2005).

Table 2. Some physiological characteristics of obtained bacterial isolates from *Salmonella-Shigella* agar and *Listeria* selective agar

Isolate No.	Source of isolates	Cultivation medium	Gram stain reaction	Spore staining reaction	Catalase test	Milk Coagulation	Lactose fermentation
B_1	CM	SSA	G^-	-	+	-	-
$B`_2$				-	+	-	-
$B`_3$				-	+	-	-
$B`_5$				-	+	-	+
$B`_7$				-	+	+	+
$B`_8$				-	+	-	-
$B`_9$				-	+	-	-
$J`_1$	BM	SSA	G^-	-	+	-	-
E_1				-	+	+	+
E_2				-	+	-	-
$Q`_1$	MFM	SSA	G^-	-	+	-	-
M_4				-	+	-	+
M_7				-	+	-	-
M_8				-	+	-	-
M_9				-	+	-	-
$L`_1$	MM	SSA	G^-	-	+	-	-
$L`_3$				-	+	-	-
$L*_1$				-	+	-	-
$L*_2$				-	+	-	-
None isolates	CM,BM, MFM,MM	*Listeria* agar	-----	-----	-----	-----	-

CM: Cow milk BM: Buffalo milk MFM: Mixed farm milk MM: Market milk SSA: *Salmonella Shigella* agar

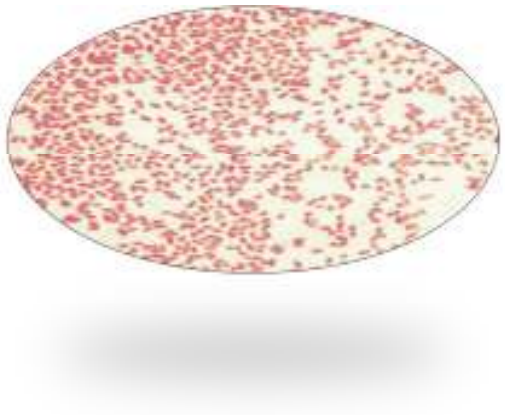

Fig. 1. Microscopic photograph showing short rods bacterial shape obtained from SS agar medium inoculated by some milk samples

In Table 3 Bacterial isolates appeared on MacConkey agar medium with dark violet or pink color were captured and plotted on TSA slant. After growth, the assay tests showed that all cells were non-spore formers, Gram negative, short rods, catalase positive, coagulation negative and lactose fermentation positive. Isolates no. A_1, A_2, A_5, $A`_2$, $A`_3$, $A`_4$, $A`_5$, $A`_6$, $A`_7$, $A`_8$, $A`_{10}$, C_1, C_2, C_3, C_4, C_5, C_7, C_8, C_9, C_{10}, C_{15}, C_{16}, D_1, K_4, L_2, $P`_1$, $N`_5$, $N*_4$ supposed as *Escherichia* sp. in accordance with Bergey's Manual of Systematic Bacteriology (Brenner *et al.,* 2005).

Table 3. Some physiological characteristics of obtained bacterial isolates obtained from MacConky agar and Baird parker agar

Isolate No.	Source of isolates	Cultivation medium	Gram stain reaction	Spore staining reaction	Catalase test	Milk Coagulation test	Lactose fermentation
A_1	CM	MacCA	G^-	-	+	-	+
A_2				-	+	-	+
A_5				-	+	-	+
$A`_2$				-	+	-	+
$A`_3$				-	+	-	+
$A`_4$				-	+	-	+
$A`_5$				-	+	-	+
$A`_6$				-	+	-	+
$A`_7$				-	+	-	+
$A`_8$				-	+	-	+
$A`_{10}$				-	+	-	+
C_1	BM			-	+	-	+
C_2				-	+	-	+
C_3				-	+	-	+
C_4				-	+	-	+
C_5				-	+	-	+
C_7				-	+	-	+
C_8				-	+	-	+
C_9				-	+	-	+
C_{10}				-	+	-	+
C_{15}				-	+	-	+
C_{16}				-	+	-	+
D_1				-	+	-	+
K_4	MFM	B.P	G^+	-	+	-	+
L_2				-	+	-	+
$P`_1$				-	+	-	+
$N`_5$				-	+	-	+
$N*_4$	MM			-	+	-	+
S_1	BM			-	+	+	+
S_2				-	+	+	+
S_5				-	+	+	+
S_7				-	+	+	+
S_8				-	+	+	+
$S`_1$				-	+	+	+
R_1	MFM			-	+	+	+
R_2				-	+	+	+
R_3				-	+	+	+
R_4				-	+	+	+
R_5				-	+	+	+
$R`_1$				-	+	+	+
$R`_2$				-	+	+	+
$Y`_1$	MM			-	+	+	+
Y_2				-	+	+	+
Y_4				-	+	-	-
Y_6				-	+ -	-	-

CM: Cow milk BM: Buffalo milk MFM: Mixed farm milk MM: Market milk MacCA: MacConkey agar B.P: Baird-Parker Agar

Two White color colonies and fifteen black color typical colonies of *Staphylococcus* spp on Baird Parker agar medium were captured and plotted on TSA slant. After growth, the microscopic test confirmed that the shape of cells of two white color colonies was big oval and the fifteen black color colonies were coccoid. All of them were Gram positive, non-spore formers and arranged in clusters and one isolate arranged in tetrads. All isolates were catalase positive and only two isolates were coagulase negative and not fermented lactose, the other fifteen isolates were coagulase positive and fermented lactose. These results suggested that isolates no. Y_4 and Y_6 belong to yeasts and isolates no. S_1, S_2, S_5, S_7, S_8, $S`_1$, $R_1$, $R_2$, $R_3$, $R_4$, $R_5$, $R`_1$, $R`_2$, $Y`_1$ and Y_2 belong to *Staphylococcus* sp. (Fig.2) Based on Bergey's Manual of Systematic Bacteriology (Brenner *et al.,* 2005). Previous results indicated also that possibility of yeast appearance on Baird Parker agar medium (Oxoid 2012).

These results are in approval with both of Garedew *et al*., (2012), Minj and Behera (2012) who isolated *E. coli* and *Salmonella* sp. from raw milk by using MacConky agar and SS agar medium. These results consisted with those of Pathak and Verma (2013) who isolated *E. coli* and *Salmonella* sp. from raw milk, El Nahas *et al.,* (2015) who isolated *E. coli* from raw milk from El Menofiya governorate, Reta *et al.,* (2016) who isolated 70 isolates of *E. coli* ,4 isolates of *Salmonella* sp. and 29 isolates of *S. aureus* from raw cow's milk and finally obtained results compatible with Yohannes (2018) who isolated and identified *Escherichia coli* from raw cow's milk.

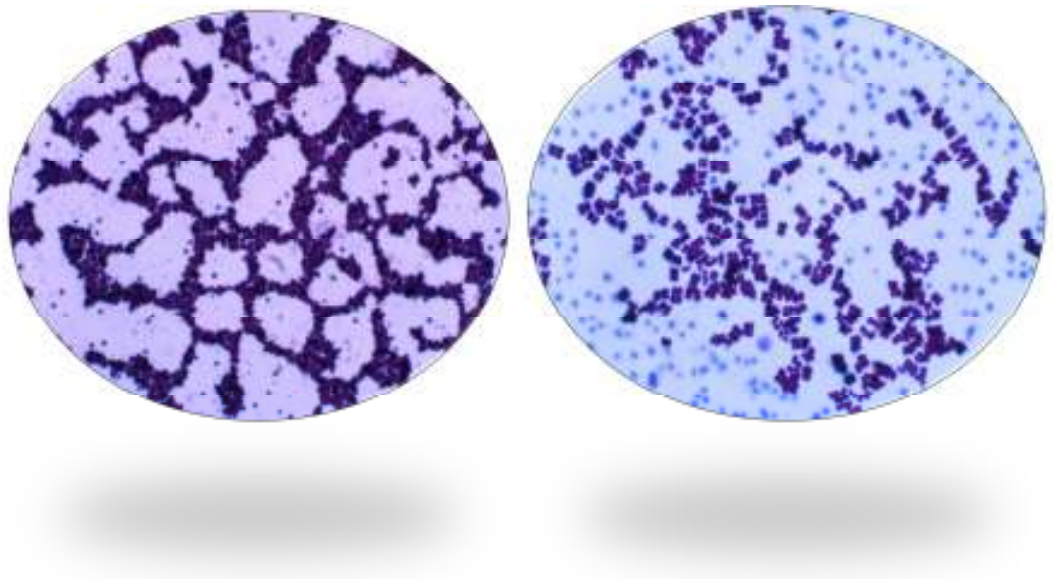

Fig. 2. Microscopic photograph showing coccoid bacterial shape obtained from Baird-Parker agar medium arranged in clusters and tetrads

Obtained results also consistent with previous findings reported by Thaker *et al*., (2013) who isolated *S. aureus* from samples of milk using Baird-Parker agar medium and the characteristic of the appearance of black colonies which considered being *S. aureus* and it was tested for catalase test and coagulase test and it was positive for both tests. The Obtained results are in compatible with Chen *et al.,* (2010) and Spanamberg *et al.,* (2014) who isolated yeast from raw milk and this result consistent with Zhu *et al.,* (2016) who isolated 8 isolates of suspected typical *Staph. aureus* out of 30 samples of dairy farm milk which were Gram-positive coccoid that arranged as clusters using light microscope also catalase and coagulase test were positive and confirmed the isolates by multiple Multiplex PCR. Finally, obtained results are compatible with Duguma *et al.,* (2018) who identified *S. aureus* by using positive catalase test and coagulase test which isolated from milk samples.

Identification by Using Polymerase Chain Reaction (PCR)

Two isolates coded B_1 and $R`_1$ that have been isolated from different milk sources and from different selective media (Table 2 & Table 3). They identified according to PCR method by Sigma Company (Cairo, Egypt). The identification of isolate B_1 was based on 16S ribosomal RNA, partial sequence. Sequences of the isolate was accessed through the National Center for Biotechnology Information (NCBI) database using the accession number and this isolate are belonging to *Salmonella enterica* subsp. *enterica* strain Ty2 with similarity 99% (Fig.3).

The identification of isolate $R`_1$was based on 16S ribosomal RNA, partial sequence. Sequences of the isolate was accessed through the National Center for Biotechnology Information (NCBI) database using the accession number and this isolate are belonging to *Staphylococcus aureus* strain S33 R with similarity 99% (Fig.4).

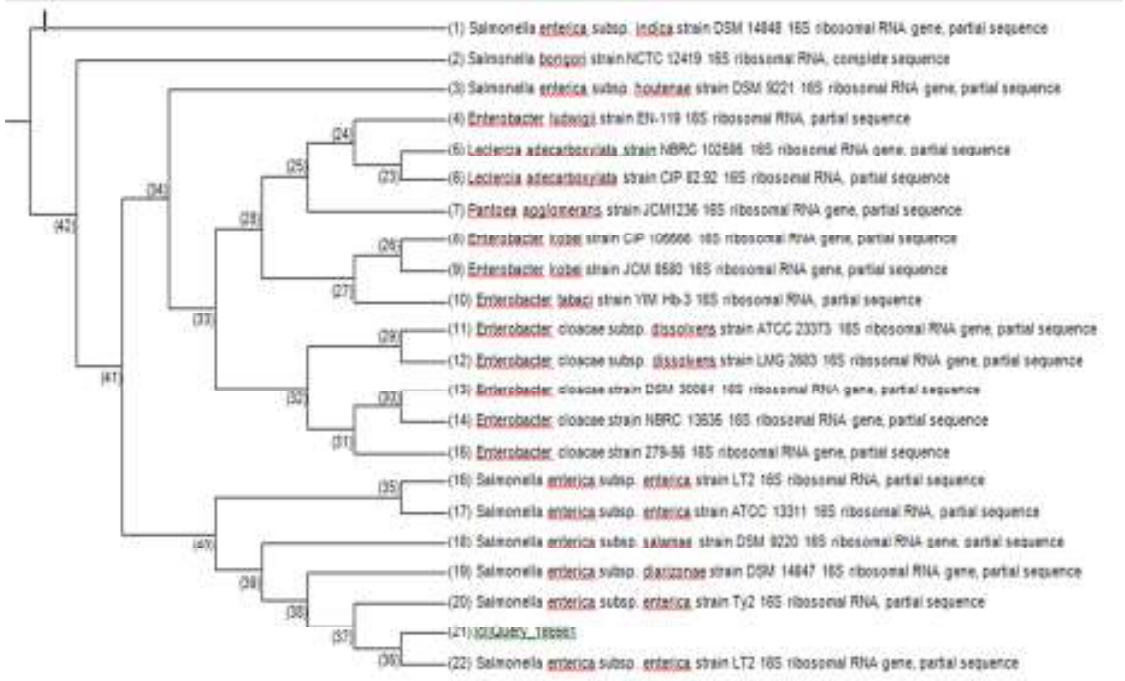

Fig. 3. Genotype tree of *Salmonella enterica* subsp. *enterica* strain Ty2 (20)

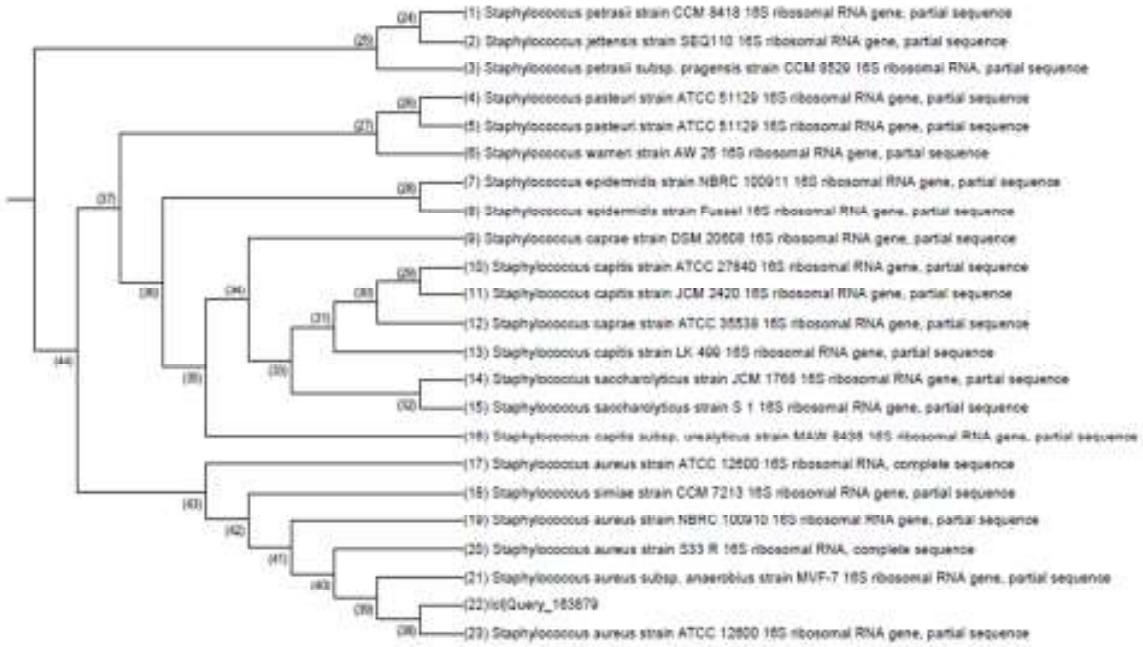

Fig. 4. Genotype tree of *Staphylococcus aureus* strain S33 R (20)

CONCLUSION

Finally, it can be concluded that, the majority of examined raw milk collected from local markets in Mansoura city and villages were contaminated with pathogenic bacteria that gives an indication of poor health measures adopted during milking, manufacturing, handling and distribution of milk products. The following proposal should be taken into consideration to improve milk quality; raw milk must be produced from healthy animals, proper cleaning of animal's udder before milking, washing and disinfection of all tools and dairy equipment and pasteurization/boiling milk required and refrigeration of milk, to about 4°C and milk products.

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عزل و تعريف بكتيريا معويه كمؤشر ميكروبي علي جودة اللبن

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يعد اللبن و منتجاته من الاغذيه التي لها فائده عظيمه لصحه الانسان. وكذلك يعد اللبن غذاء متكامل لاحتوائه علي البروتينات و السكريات و الدهون و العديد من الفيتامينات و المعادن. و قد استهدفت هذه الدراسة عزل عدد من البكتريا المعويه من الألبان الخام المصرية مع دراسة الصفات المورفولوجيه و الفسيولوجية لهذه العزلات كمؤشر ميكروبي علي مدي جودة اللبن. لذلك تم تجميع ٢٢ عينة من اللبن الرايب واللبن الخام الجاموسي واللبن الخام البقري و اللبن الخام المجمع من مزارع و متاجر مختلفه بمدينه المنصورة و القري المجاورة . أمكن عزل ١٤٣ مزرعة من البكتريا المعويه بإستخدام البيئات المتخصصه و تم اجراء الاختبارات التعريفيه عليها و دراسه صفاتها المورفولوجيه و الفسيولوجيه ، وقد تم تعريف مزرعتين منها - مصدرها اللبن الخام البقري و اللبن المجمع الفلاحي - علي أنها تنتمي إلي بكتريا سالمونيلا انتريكا و استافيلوكوكس اوريس وذلك بإستخدام تحليل "تفاعل السلسلة المتبلمر" (PCR). تشير هذه النتائج إلي أن اللبن الخام المصري يحتوى علي نسبه عاليه من البكتريا المعويه الممرضه و هذا يدل علي عدم اتباع الشروط الصحيه اللازمه في انتاج و نقل و توزيع اللبن الخام مما يشكل خطر كبير علي الصحه العامه للمستهلك. وكذلك بينت النتائج ظهور الخميرة علي بيئه B-P المتخصصه لعزل *Staphylococcus* sp. .