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Prevalence of both *Streptococcus agalactiae* and *Staphylococcus aureus* isolated from Raw milk and Soft cheese

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ABSTRACT: A total of 125 random samples of raw milk and soft cheese (25 each) of cow's milk, sheep's milk, goat's milk, kareish cheese and Domiati cheese samples were collected from different markets and shops in Alexandria city, Egypt and examined for the presence of *Staphylococcus aureus* and *Streptococcus agalactiae* as food poisoning and mastitis causing organisms. The incidence of *Staphylococcus aureus* in examined samples were 28, 36, 40, 20 and 16% in the examined cow's milk, sheep's milk, goat's milk, kareish cheese and Domiati cheese, respectively. *Streptococcus agalactiae* was detected in 16, 20, 24, 12 and 4% of the examined samples, respectively. *Streptococcus agalactiae* was identified using primers V1 and V2, specific to rRNA as an early diagnosis of subclinical mastitis using, PCR technique. The sanitary and public health importance of these organisms as well as control measures to improve the quality of dairy products and to safeguard the consumers from infection were discussed.

Key words: Raw milk, Soft cheese, Staph. aureus, Strept. agalactiae, Public health hazard

INTRODUCTION

Milk and milk products rank high among other foods and are considered as the most perfect food for human from birth to senility. They are not only having good sensory properties, but also containing all nutrients required for the body which can prevent or reduce risks of many nutritional deficiency diseases. Although salt content in cheese, at which it is produced, stored and served, yet various microorganisms may gain access to these products during production, processing and storage, then

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grow and affect the quality and safety of such products.[1- 3]

Kareish cheese is a kind of soft cheese which is manufactured from raw buffaloe's and cow's skimmed milk in farmer's houses. The increasing demand for it by the Egyptian consumers is mainly attributed to its high protein content and low price. Raw milk is considered as a good medium for growth of different pathogenic microorganisms.[4] Hence, the main source of pathogenic bacteria in cheese is raw milk contaminated with microorganisms discharged from either the diseased udder of unhealthy animal or from contaminated environment as food handlers, dust, utensils and insects.These microorganisms may be responsible for different diseases including food poisoning or render the product of inferior quality and unfit for human consumption.[5]

Domiati cheese can be considered the most popular type of cheese that is craved for by all socio-economic classes in Egypt. When fully ripened, it has a strong sharp flavor and a smooth creamy body and texture. It is commonly made from whole or partially skimmed raw, pasteurized or sub pasteurized milk. The tendency to reduce the heat treatment of milk is due to the belief of many cheese manufacturer that this enhances the rate of ripening and produces cheese with a full ripened flavor at a much shorter time and at a higher intensity. The cheese is usually held at least 60 days at room temperature to allow time for the inactivation of pathogens during ripening process.[6]

Dairy animals are probably the main source of contamination of raw milk with Staphylococci.[7] In particular, dairy animals with subclinical Staphylococcus mastitis may shed large numbers of Staphylococci into the milk. However, contamination of raw milk and raw milk products from human handling or from the environment during manufacture is also possible. Environmental conditions such as

temperature, pH, water activity, salt concentration, and competing micro flora influence Staphylococci growth and enterotoxins production.[8] Milk and milk products were the vehicle in 8% of 359 outbreaks and sporadic cases of Staphylococcal food poisoning in the United Kingdom between 1969 and 1990.[9]

Staphy lololllus aureus (staph. Aureus) that contaminate the milk is mostly isolated from the udder and teat apices. It is also present commensally (normally) in the nose and throat of about 40% of healthy personnel.[10,11] The human reservoir of *Staph. aureus* does not play a major role as a source of bovine intramammary infections.[12] Among the predominant bacteria involved in food-borne diseases, *Staph aureus* is a leading cause of gastroenteritis resulting from the consumption of contaminated food.[13] Also *Staph aureus* is the most predominant contagious pathogen responsible for clinical and subclinical infections in lactating cows.[14] and small ruminant.[15] *Staph aureus* strains produce heat-resistant enterotoxins, which cause nausea, vomiting and abdominal cramps when ingested by human and are responsible for Staphylococcal food poisoning outbreaks.[16]

Streptococci cause a variety of diseases; streptococcal sore throat (fever, exaudative tonsillitis, and pharyngitis), streptococcal skin infections (impetigo or pyoderma- usually superfacial), scarlet fever (skin rash, fever, and Nausea, with case fatality rate of 3%),[17] and streptococcal food poisoning with symptoms including diarrhea, nausea and abdominal pain appearing with in an incubation period of 3-18 hours. The organism can tolerate asodium chloride concentration of up to 10% as well as pasteurization processes.[18]

Streptococcus agalactiae (strept. Agalactiae) is of particular importance because it is highly infectious, unless care

is taken and causes mainly subclinical infections, which are not identified by the herd man.[19] *Str. agalactiae* can spread widely within a herd, causing immediate loss due to reduced milk yield and large losses, when it is finally recognized. For this reason, it is important to identify the presence of *Str. streptococcus Tolouns agalactiae* in a herd with the appearance of the first infected animal. Because of its subclinical nature, such identification must rely upon laboratory diagnosis. Outbreaks of *Str. agalactiae* in a herd could be detected before the infection spreads. Because *Str. agalactiae* is not normal constituent of udder flora, aggressive monitoring and treatment may be able to completely eradicate this pathogen from national herds.[20, 21]

Recently, a number of PCR-based methods for diagnosis of group B Streptococci have been presented.[22] Of particular interest in our contex are studies based on rRNA sequences.[23,24] Polymerase chain reaction (PCR) assays is considered less time- consuming, rapid, aspecific identification method and can discriminate between closely related organisms.[25]

It is well established that food borne diseases cause significant economic and social losses. And that the consumption of raw milk remains a well-identified risk factor food borne diseases. It was reported that milk, and cheese have been identified as the vehicle for less than 1.5% of all food borne disease outbreaks investigated by the Centers for Disease Control.[26]

The potential threats to human health related to milk and dairy products include errors in pasteurization, consumption of raw milk products, contamination of milk products by heat-resistant pathogens and emergence of antimicrobial resistance. Therefore the objectives of this study was to allow qualitative checking of hygienic conditions of examined raw cow, sheep, goat milk and soft cheese (kareish and

domiati) for the prevalence of *Staph aureus and Str. agalactiae* in Alexandria city and to develop a PCR-based system for a highly sensitive, low cost, rapid and specific identification of *Str. agalactiae* through the following scheme.

MATERIAL AND METHODS

Collection of samples: A total of 125 random milk and cheese samples were aseptically collected from dairy shops, street vendors and farmers' houses in Alexandria city. These samples included raw marketable cow's milk, raw sheep's milk, raw goat's milk, street vendors' kariesh cheese and domiati cheese (25 of each) were transferred to the laboratory with a minimum delay of examination for the concerned microorganisms.

Samples preparation: Milk samples were examined by starch test according to Lampert,[27] to detect heat treated samples. Soft cheese samples were thoroughly mashed in a sterile mortar for homogenization.

Isolation and identification of *Staphylococcus aureus*:

Enrichment procedure by adding one ml of each milk samples or one gm of homogenized soft cheese samples to 10 ml of selective enrichment broth [brain heart infusion broth (BHI) HiMedia Laboratories Pvt. Ltd. 23, vadhani Ind. Est., LBs Marg, Mumbai-400 086, India]. The inoculated broth was incubated at 37°C for 48 hours . A loopful of the incubated broth was streaked into plates of selective media Baird-Parker agar[28]. Inoculated plates were incubated at 37°C for 2days. The suspected colonies were inoculated into slope of nutrient agar for morphological and biochemical tests. The identification was carried out using the following tests: Gram staining, production of coagulase, catalase and fermentation of mannitol.[29,30]

Isolation and identification of *Streptococcus agalactiae*

In a sterile test tube 9.5 ml of milk samples or homogenized soft cheese

samples were added to 0.5% sterile aqueous solution of bromocresol purple. The contents were mixed by shaking followed by incubation at 37 °C for 24 hours. Positive results was indicated by changes of color into yellow one or appearance of yellow balls or yellow flakes adhering to the wall of the tube. Negative results were indicated by no change in color (light purple). A loopful was taken from the positive tube and streaked onto Edward's medium which was prepared according to Quinn *et al.,*[31] then incubated at 37 °C for 24 hours. Violet colonies indicates the presence of *Str. agalactiae.* The separate colonies were picked up on slope agar then incubated at 37 °C for 24 hours. Pure culture was subjected to confirmatory tests (Sodium hippurate hydrolysis test, Sugar fermentation, Gelatin liquefaction, Blood haemolysis and Catalase activity test).

Polymerase chain reaction (PCR)

DNA Extraction: DNA was extracted from bacterial cultures by incubating a loopful of over night bacterial colony with lysozyme and proteinase K, followed by extraction with phenol then chloroform, isoamyl alcohol and ethanol precipitation according to Jersek *et al.,* and Greisen *et al.*[32,33]

PCR Primers: Primers amplifying a 120 bp product of the transposase gene of *Str. agalactiae* (AB023574). The sets of primer pairs are shown below:

V_1: 5'- TTTGGTGTTTACACTAGACTG-3'

V_2: 5'- TGTGTTAATTACTCTTATGCG-3'

PCR Methods.[34] The PCR technique was performed in a thermo cycler (BECO Omni Gene, Germany) in a total reaction volume of 50 µl with 25 µl 2xPCR Master Mix (Bioron, Germany), 0.5 µM of each primer, 2µl of total DNA. Thermal cycling involved: Initial denaturation at 94°C for 4 min ; five cycles of 94°C, Tm °C and 72°C for 45s each step ; 20 cycles of 94°C (denaturation), (Tm-4) °C (annealing) and 45s extension at 72°C each step and followed by 72°C extension for 5 min, at

the end of the reaction, then hold at 4°C.

Detection of the amplification product: Five micro liters of PCR product was electrophoresed on 1.8% to 2.0% agarose gel stained with 0.005% of Ethedium Bromide (Et. Br.) /ml to determine and visualize the size of the product. Negative control, positive control (Kindly supplied from Microbiology Department, High Institute of public Health Alex.,andria University) and 1000 bp molecular DNA marker (Promega, Madison, WIUSA) were included in each PCR run at a constant current of 40 V for one hour. The negative control consisted of all PCR components except the template DNA. If negative control became positive, the entire PCR was repeated. The gels were visualized under UV illumination (Eagle Eye II, Start agene, Germany) and thereafter photographed using digital Camera. The sizes of the amplified product were determined by comparison to DNA marker.

RESULTS AND DISCUSSION

Staphylococcus aureus

Staph. aureus is found in a wide variety of habitats, including human skin, where many strains are commensals that might be clinically significant or contaminants of food. In the present study, *Staph. aureus* was identified in 48 samples from 125 samples obtained from raw cow's, sheep's, and goat's milk and soft cheese (kareish and domiati) samples (Table 1). In other words *Staphylococcus aureus* was isolated from all types of examined samples.

It was isolated from 32% of cow's milk samples. Higher estimates were detected by Chye *et al.,*[34] and Ekici *et al.,*[35] who showed that *Staph. aureus* was isolated from more than 60% and 75% of the raw cow's milk samples,respectively. However, lower percentage was detected by Abdel hameed.[36] and El-Bassiony *et al.,*[37], who isolated *Staph. aureus* from cow's milk samples at percentages of 9.28% and 15.57%, respectively.

Regarding raw sheep's milk samples, *Staph. aureus* was detected in 44%. Higher result (78.9%) was reported by Ariznabarreta *et al.,*[38]. On the contrary, lower records (4.04%) and (20%) were obtained by El-Bassiony *et al.,*[37] and Abdel-Hameed and El-Malt[39].

The baiterivm was also isolated from goat's milk samples (48%) which is higher than that obtained by El-Bassiony *et al.,*[37] (13.5%). However extremely higher incidences were recorded by Hassanain and Zaabal.[40] (58.33%).

The main sources of contamination with *staphylococcus aureus* are humans (handlers contaminate food via manual contact or via the respiratory tract by coughing and sneezing), and after heat treatment of the food. Nevertheless, in foods such as raw meat, sausage, raw milk, and raw milk cheese, contaminations from animal origins are more frequent due to animal carriage or infections (e.g., mastitis)[13].

Morove *staph. aureus* was detected in 36% of the examined kareish cheese samples. A nearly similar finding was reported by Zaki.[41] (37.5%) A higher incidence (44%) was reported by Tawfik *et al.,*[42]. whereas lower incidences were reported by Hassan and Afify,[43] Abd El-Goad.[44] and Al-Hawary *et al.,*[3] (24% , 30% and 26%, respectively).

Concerning Domiati cheese samples, the incidence of *Staph. aureus* was 32%. This finding is almost in agreement with Zaki.[41] who isolated *Staph. aureus* at a rate of 32.5% of brined cheese. A higher incidence of *Staph. aureus* was reported by Sabreen.[45] (49%) and Ahmed Abd El-Aal.[46] (64%). while lower findings were reported by Sheliah *et al.,*[47] (12%) and El-Gamal, et al.,[48] (28%).

Concerning Coagulase Negative Staphylococci (CNS), it was recorded in 4% of cow's milk samples. This was in accordance with the results detected by Abd El-Hameed et al.,[39] A higher result

(5.4%) was obtained by Abdel hameed.[36] CNS was also isolated from 8% of sheep's milk samples. Lower results (4.5%) and (6.6%) were obtained by El-Bassiony *et al.,*[37] and Abd El-Hameed and El-Malt,[39]. respechively Additionally, 16% of kareish cheese samples were contaminated by CNS. A lower result (6%) was obtained by Al-Hawary*et al.,*[3] similarly, was found that CNS were isolated from Domiati cheese (16%). A higher result (20%) was reported by Zaki.[41]

Table 2 indicated that the lowest frequency distribution for *Staph. aureus* was reported for Domiati cheese samples (8.4%). Whereas the highest frequency was calculated for Goat's and sheep's milk samples which showed afrequency of 20.8% and 18.8%. respectivety. Additionally, similar frequency For CNS were estimated for sheep's and goat's milk samples (4.2%). While, the highest frequency distribution For CNS was recorded for kareish and Domiati cheese samples (8.4%). The high incidence of staph. auseus might be attributed to either that the kareish cheese produced by farmer is not heat treated, or that there is no starter added to the cheese which lowers the pH before manufacturing.[49] as well as contamination from different sources. The results obtained in this experiment disagree with the Egyptian standard of kareish cheese which states that the product should be free from pathogenic microorganisms (Egyptian Standard).[50]

It is proved that *Staph. aureus* growth is not suppressed by the salt present in the cheese.[41]

Any type of produced food with low number of staphylococci will remain free of enterotoxins if it is kept either below 40°F or above 140°F until it is consumed. Al-Hawary *et al.,*[3] summarized the factors that contributes to food poisoning outbreaks as inadequate refrigeration, poor personal hygiene, inadequate processing

and availability of bacterial growth environment.

Streptococcus agalactiae

Table 3 shows that *Strept. Agalactia* was detected in raw cow's milk samples [4(16%)]. Mohmade (2001).[51] reported a higher result (22.6%). Lower result was reported by El-Bassiony *et al.,*[37] (4.49%). While, *Strept. agalactiae* in raw sheep's milk samples was isolated with an incidence of 5(20%), this was higher than the results obtained by Ariznabarreta *et al.,*[38] and El-Bassiony *et al.,*[52] who recorded incidences of 7.2% and 1.01%, respectively. In addition, the present study found that *Strept. agalactiae* was detected in 6 (24%) of raw goat's milk samples. This results agreed with that of Salem *et al.,*[53] who reported an incidence of *Strept. agalactiae* of 24%. Higher result was reported by Salem (2003).[54] (50%). While, lower results were obtained by Ariznabarreta et al.,[38] (7.4%). Moreover *Strept. agalactiae* was detected in 3 (12%) of the examined kareish cheese samples. Higher incidence was reported by Hassan et al.,[43] (16%).

Although, *Strept. agalactiae* is a well known bacterial pathogen in animal infection little information is available at present about the occurrence of this bacterial species in Domiati cheese. In the present study one Domiati cheese sample out of a total of 25 samples showed the present of this pathogen (4%).

Strept. agalactiae is a highly infectious bovine mastitis pathogen that can rapidly spread through a herd from a single infected animal. Consequently. Early diagnosis of the presence of the infection in a herd is important for effective control. Good farming management, with high level of veterinary monitoring and treatment, may allow eradication of this udder pathogen from the herd. Diagnosis is difficult, however, because of the normally subclinical expression of the pathogen. Current methods for identifying *Strept.*

agalactiae are based on bacteriological examination of blood agar plates including the hemolysis caused by an exocellular product such as Christie, Atkins and Munch-Peterson(CAMP) test or the lack of ability to hydrolyze esculin, and on the production of colored colonies when grown anaerobically on starch. Serological methods based on surface polysaccharide antigens are often used to confirm the biochemical identification.[55,23]

The aim of this study was to develop PCR-based system for a highly sensitive, rapid and specific identification of *Strept. agalactiae*. Our results indeed, showed high sensitivity and specificity of *Strept. agalactiae* identification using primers V1and V2 specific to 16s rRNA. All *Strept. agalactiae* isolates and all *Strept. agalactiae* sequences in the Genbank had identical V1-V2 primer sequences. All *Strept. agalactiae* isolates tested produced an amplification product with the V1- V2 specific primers. Thus, the results of PCR method were completely specific and consistent with those of the classical bacteriological methods. The PCR procedure did not give any false-positive or false-negative reactions.

From our conducted study we concluded that contamination of milk and dairy products by pathogenic microorganisms can be of endogenous origin, following extraction from the udder of an infected animals or may be also of exogenous origin, through direct contact with infected herd or through environment contamination (water and personnel). Heat treatment and processing of milk can inhibit or encourage the multiplication of microorganisms. Deficiencies in the hygienic measures of milk and dairy products storage, particularly refrigeration and in the HACCP plan that was not properly implemented should be corrected. It is important to inspect the manufacturing plant than to examine the single dairy product on the market.

Table 1 : Incidence of *Staph. Ylococcus aureus* in Raw milk and Soft cheese

Examined samples	Type of examined samples	No. of examined samples	Positive samples		Isolated strains			
					Staph.aureus		CNS*	
			No	%	No	%	No	%
Raw milk	Cow's milk	25	8	32%	7	28%	1	4%
	Sheep's milk	25	11	44%	9	36%	2	8%
	Goat's milk	25	12	48%	10	40%	2	8%
Soft cheese	Kareish	25	9	36%	5	20%	4	16%
	Domiati	25	8	32%	4	16%	4	16%
Total.	_	125	48	192%	35	140%	13	52%

*CNS = Coagulase Negative Staphylococci

Table 2: Frequency distribution of Staph. *Ylococcus* aureus and CNS in the examined samples

Examined samples	Type of examined samples	Isolated strains		*Staph. aureus*		CNS*	
		No / 48	%	No / 48	%	No / 48	%
Raw milk	Cow's milk	8	16.7	7	14.6	1	2.1
	Sheep's milk	11	22.9	9	18.8	2	4.2
Soft cheese	Goat's milk	12	25	10	20.8	2	4.2
	Kareish	9	18.8	5	10.4	4	8.4
	Domiati	8	16.7	4	8.4	4	8.4
Total	-	48	100.1	35	73	13	27.3

*CNS = Coagulase Negative Staphylococci

Table 3: Incidence of Strept. *Yloccocus* agalactiae in Raw milk and Soft cheese

Examined samples	Type of examined samples	No. of examined samples	Positive strept. agal.	
			No.	%
Raw milk	Cow's milk	25	4	16%
	Sheep's milk	25	5	20%
	Goat's milk	25	6	24%
Soft cheese	Kareish	25	3	12%
	Domiati	25	1	4%
Total.	-	125	19	76%

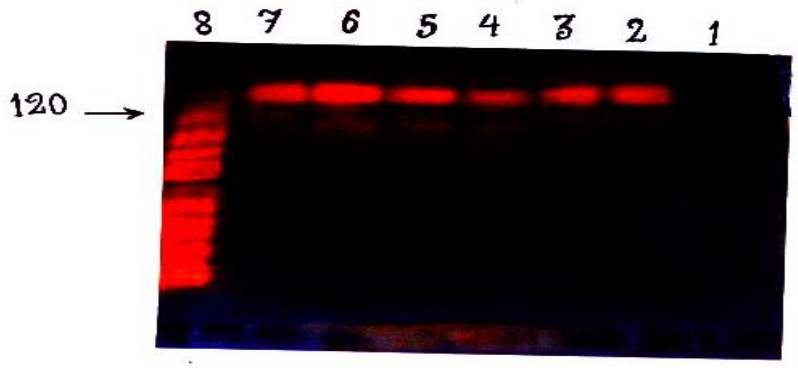

Figure 1: Agarose gel showing amplification products with *Streptococcus agalactiae*, using the V1 and V2 primer pairs (lanes 2 to 7). Lane 8, negative control and Lane 1, DNA molecular marker. Size of PCR products: V1- V2, 120 bp, showen by arrows.

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