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Detection of mastitis pathogens by multiplex PCR in Raw milk and some dairy products from Menoufia Governorate, Egypt

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ABSTRACT

Streptococcus agalactiae, *Escherichia coli* and *Staphylococcus aureus* are considered the main bacterial mastitis in Egypt which initiate many severe pathogenic potential for public health. In this study, the three mastitis pathogens were isolated from milk and some homemade dairy products namely: Karish cheese, Mish cheese and Buffalo butter. Bacterial isolates from studied samples were identified through the biochemical multiple-steps culture method and be compared to the results of their existence in the raw dairy product samples using multiplex PCR (m-PCR) analysis. PCR accession primers for studied strains were established upon the conserved region of species-specific regions of DNA coding for 16S and 23S rRNA. PCR analysis of standard bacterial strains yielded 190 bp, 286 bp, and 374 bp product length bands for *Str. agalactiae, E. coli* and *Staph. aureus* respectively. Culture method revealed 36.6 % mastitis pathogen existence in milk, and 64, 84 and 5% in Karish cheese, Mish cheese and buffalo butter respectively. M-PCR succeeded to detect *Staph. aureus* in raw milk and *E. coli* in buffalo butter where both were culturally negative. The study provides a rapid and highly specific method for detection of bacterial mastitis pathogens in milk and milk products directly, with cost and time reduction, compared to the conventional plate method.

Keywords: *Streptococcus agalactiae*, *Escherichia coli* and *Staphylococcus aureus,* mastitis pathogens, Karish cheese, Mish cheese and Buffalo butter

Introduction

Mastitis is continuously implicated as one of the serious tribulations of the dairy bovines and buffaloes inducing severe economic losses during the lactation season. These losses are primarily due to lower milk yield, reduced milk quality, and higher costs of treatment and control (Nicholas *et al*., 2009). Microorganisms causing mastitis of dairy animals are numerous, but mastitis pathogens of dairy ruminants may be categorized as environmental (*Escherichia coli* with other Coliforms) or contagious (such as *Str. agalactiae* and *Staph. aureus*) depending on their primary reservoir; environment or infected mammary gland (Arefaine & Kashwa, 2015). However, raw milk load of microbial and somatic cell counts determine the quantity of heat-stable proteases and lipases to cause spoilage of raw milk or its products (Todaro *et al.,* 2013). In Egypt, milk and milk products are crucial economic assets in Egyptian villages while dairy products are mainly homemade with no observation or control from health authorities. Mycotic mastitis in Menoufia governorate-Egypt was diagnosed in milk samples using m-PCR protocol, where the study proved the increment of bacterial mastitis due to antibiotic treatment misuse (Abd El-Razik *et al.,* 2011). For almost 80 % of all diagnoses in Egypt, The most frequent isolated bacterial strains of mastitis causatives are *Str. agalactiae*, *E. coli*, and *Staph. aureus* (Mohamed *et al.,* 2013). Diagnosis of mastitis depends mainly on the clinical inspection of the grossly affected mammary quarter (s) and usually be treated with the available antibiotic drug chosen based on the experience of villagers without detection of mastitis pathogen (Zaitoun, 2008). However, the common detection method for routine diagnosis of bacterial mastitis is time consuming and non-accurate (Mafra *et al.,* 2017). Therefore, the aim of the present study was to evaluate m-PCR protocol for detection of bacterial mastitis directly in milk and some milk products to verify its

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applicability in milk quality control programs. The chosen of the studied genera was due their abundance as pathogenic potential for public health in Egypt.

Materials and Methods

Collection of Samples

During summer 2014, 100 random samples of raw milk and some traditional homemade dairy products (Karish cheese, Mish cheese and Buffalo butter) were collected from five different sites in Menoufia governorate-Egypt. The samples were taken under possible aseptic condition in sterile screw-capped vessels and transferred in an ice box to the laboratory.

Bacteriological examination

The collected samples were individually vortexed and aerobically incubated overnight at 37 ^{o}C, and thereafter centrifuged and the sediment was streaked onto sheep blood agar (Oxoid, UK) and MacConkey agar (Oxoid) plates. The cultured plates were aerobically incubated for 24 – 48 h at 37 ^{o}C. The suspected growing colonies were picked-up, purified and morphologically identified.

Identification of bacterial isolates

For isolation of *Str. agalactiae,* all isolate clones morphologically proved to be gram-positive were streaked individually on sheep blood agar slants containing 1% esculin (Oxoid, UK). Grown cultures were subjected to hydrogen peroxide drops. Catalase-negative streptococci isolates were further cultivated on kanamycin esculin azide agar (Oxoid). Identification of *Str. agalactiae* was confirmed by API strips (API 20 Strep identification system, Ref No. 20600, bio-Mérieux, France). Baird–Parker agar was used to isolate *Staph. aureus* where representative gram-positive cocci colonies with typical black appearance and surrounded by clear zone were picked up, and subjected to catalase test (Staphylex, Oxoid). Colonies that showed hemolysis and catalase-negative were identified as *Staph. aureus*. For isolation of *E. coli*, gram-negative bacilli colonies were further streaked on MacConkey agar plates where the pink color of the bacterial colonies indicates *E. coli* growth. For confirmation of *E. coli* isolates, a three-tube Most Probable Number (MPN) technique was employed. Positive tube from MPN was streaked onto eosin methylene blue (EMB) agar and incubated overnight at 35ºC, where green-metallic sheen dye was developed around bacterial growth within 24 – 36 h.

M-PCR protocol

For comparison, standard bacterial reference strains of *Staph. aureus*; ATCC 25923, *E. coli;* ATCC 11229 and *Str. agalactiae;* ATCC 13813 were obtained from Egyptian Vaccines & Veterinary products, VACSERA, Abbasia branch, Cairo, Egypt.

DNA extraction

The protocol of Ogier *et al*. (2004) for phenol-chloroform extraction was used with slight modifications as follows: 1 ml raw milk or prepared dairy product samples was centrifuged separately for 2 min at 6500 ×g, the upper fatty layer and the supernatant was pipetted off and discarded. Five-hundred microliters of 0.9% NaCl solution was added, with the samples mixed for 30 s, and centrifuged for 3 min at 6800 ×g. Then, 500 µl TENS buffer made up of 10 mmol·l-1 Tris-HCl pH 8.1 (Promega, UK), 1 mmol·l^{-1} EDTA, 100 nmol·l^{-1} NaCl, 0.7% SDS and 25 mg·ml^{-1} proteinase K was added to the pellet, the samples were re-mixed for 30 s, and incubated in a water bath for 2 h at 50 °C. After digestion of proteins, 500 µl of a mixture of phenol: chloroform 1:1 was added, and the samples were mixed for 40 s and then centrifuged for 3 min at 10000 ×g. The clear upper water phase was transferred to a new microtube and 500 µl; chloroform was added. The samples were mixed, centrifuged for 3 min at 10000 ×g, and the upper water phase was transferred to a new microtube.

Then, 50 µl 3 mol·l^{-1} Na-acetate and 400 µl isopropanol were added, the samples were mixed and incubated for 30 min at –20 °C. Following a 10-min centrifugation at 13000 ×g, the supernatant was carefully pipetted off and discarded. The remaining pellet was dried, and re-suspended in 25 µl sterile distilled water. For DNA extraction of standard bacterial strains, Promega kits (UK) were used following the manufacture instructions.

PCR conditions

Reactions were performed using Delbes *et al.* (2007) method with slight modifications as follows: in a final volume of 20 µl. The reaction mixture contained 0.5 U DyNAzymeTM DNA polymerase, 0.4 µl 10 mmol/L dNTP Mix, 2 µl Optimized DyNAzymeTM 10× reaction buffer (Promega, UK), and 1 µl of each primer (conc. 25 µmol/l). The amplification program was denaturation; 5 min, 96 °C, then 30 cycles of denaturation (1 min, 96 °C), annealing (1 min, 55 °C) and extension (2 min, 72 °C), followed by a final extension (8 min, 72 °C). Ten µl of PCR-amplified product was analyzed by electrophoresis on 0.9 % ethidium bromide-stained agarose gel where DNA ladder (0.3 mg/L) or bacterial suspension (2 µl) was used as a template.

PCR primers

Primers were designed from species-specific regions of the DNA coding for 16S and 23S rRNA based on the sequence of the entries available in GeneBank database (Duarte *et al.,* 2004). Primers, pathogen organisms, accession numbers and product lengths are exhibited in Table (1).

Table 1: Primers used for detection of mastitis pathogens in the study samples.

Organism	Sequence	Accession no.	Amplicon size
Str. agalactiae	5′-CGT TGG TAG GAG TGG AAA AT-3′	NC 004368	190 bp
E. coli	5′-GCT TGA CAC TGA ACA TTG AG-3′	AB035925	286 bp
Staph. aureus	5′-GGA CGA CAT TAG ACG AAT CA-3′	AY688035	374 bp

M-PCR Conditions

For m-PCR, the same conditions of PCR were performed. While mixture of the three standard bacterial strains primers were used for the detection of those pathogens in raw milk and dairy products DNA extraction. For comparison, each strain was run as positive marker in addition to DNA ladder marker. For negative control, water was added to the same reaction mixture instead of DNA extract.

Visualization of amplified products

The amplified material was visualized by electrophoresis in agarose gel (2%) added with 1.0µl/ml of SYBR Safe DNA gel stain (Invitrogen - USA). Electrophoresis was carried out in horizontal cube containing TBE 1X (89 nMTris-HCl, 89mM boric acid and 20mM EDTA) solution at 65V. Products of mPCR were visualized in an image analyzer (GelDoc-IT™ Imaging System - UVP, USA) by using VisonWorks®LS. Amplified DNA (8µl) was added to 2µl of stained buffer before loading on gel while 4µl of 100 bp ladder was used as marker (Invitrogen, USA).

Results and Discussion

The hygienic quality of both milk and dairy products is important for insuring consumer safety and for optimum economic value. Therefore implementing good hygienic practice during milk production at farm level and during dairy products processing is crucial, while accurate and rapid detection for pathogens in the products would decline the possibilities of public health hazards, so quality control measure is highly recommended (Mayer, 2005). In the present study, mastitis

pathogens in raw milk and some traditional dairy product samples were isolated and identified by biochemical conventional methods which are most widely used and depend mainly on availability of the growth of pathogens on the utilized media. Percentage of abundance for each bacterial strain was calculated as their existence in the total number of samples for each tested food material (milk and traditional dairy products). The percentage of pathogens existence using the culturing plating method is displayed in Table (2). The data showed that mastitis pathogen were represented in 30 samples of milk with percentage 36.6 %, while their percentage in traditional dairy products were 64, 84 and 5 % for Karish cheese, Mish cheese and Buffalo butter respectively. Separately, mish liquor of most positive mish cheese sample was tested to investigate the difference in pathogens abundance where the pathogen isolates were observed in both mish cheese and mish liquor, but with little count in the latter, and that was further confirmed in m-PCR results Figure (1; lanes 8 & 9). However the existence of bacteria in mish cheese is influenced by the presence of nutritive materials while the liquor is hardly impacted by secondary products of all metabolic process occurs in mish development, and this result is in accordance with that of Mabrouk *et al.* (2014).

Table 2: Pathogen existence in milk and some dairy products by culturing method.

Organism	Milk (30)		Karish cheese (25)		Mish cheese (25)		Buffalo butter (20)	
	+ ve	%	+ ve	%	+ ve	%	+ ve	%
Str. agalactiae	2	6.6	3	12	5	20	1	5
E. coli	9	30	11	44	13	52	0	0
Staph. aureus	0	0	2	8	3	12	0	0
Total	11	36.6	16	64	21	84	1	5

However, this method would be influenced by the conditions of either media or pathogen abundance (El-Baradei *et al.,* 2007). Therefore, it would be necessary to investigate the pathogens existence in food especially in dairy products directly with no plating and hence to prepare a rapid detectable method depends on a specific sequence unique for each investigated pathogen. However, in the present study, the designed primers for the studied pathogens were able to amplify and produce the specific fragments of 190 bp, 286 bp and 374 bp for *Str. agalactiae*, *E. coli*, and *Staph. aureus* respectively directly from the extracted DNA from milk and utilized traditional dairy products. Previous work of Di Pinto *et al.* (2004) had determined the possibilities of PCR assay for Mozzarella cheese from buffalo milk. Also, Bottero *et al.* (2003) had employed the m-PCR assay for cows and sheep's milk identification in dairy products.

In the present study, traces of main bovine mastitis pathogens was detected in DNA extract of raw milk and some traditional dairy products and be compared with the DNA extract of the standard strains. The m-PCR results (Figure 1) showed that there is a homologous between pathogen DNA-standard bands and that of milk and utilized dairy products which indicate specifically that the bacterial strains are adequately represented in the products (Forsman *et al.,* 1997, Abd El-Razik *et al.,* 2011). The variance in intensity of isolate bands reflects the pathogen existence in the dairy products (De-Buyser *et al.,* 2001). Thus in Figure (1), existence of pathogen bands were similar in both lanes of mish cheese and mish liquor (lanes no. 8 & 9) with noticeable difference in intensity. However, m-PCR protocol was capable to detect the presence of *Staph. aureus* in raw milk and *E. coli* in Buffalo butter and both were culturally negative as shown in Table (2). Development of m-PCR technology has introduced a new detection platform for direct identification of bacteria in the dairy products which replaced the previous assays as immunocromatographic assay (Stanciuc & Rapeanu, 2010). Detected primers were designed from species-specific regions of the DNA coding for 16S and 23S rRNA and successfully used for mastitis pathogens in raw dairy food materials. The described method may be simply implemented into veterinary practice, laboratories of food microbiology and all milk quality control programs.

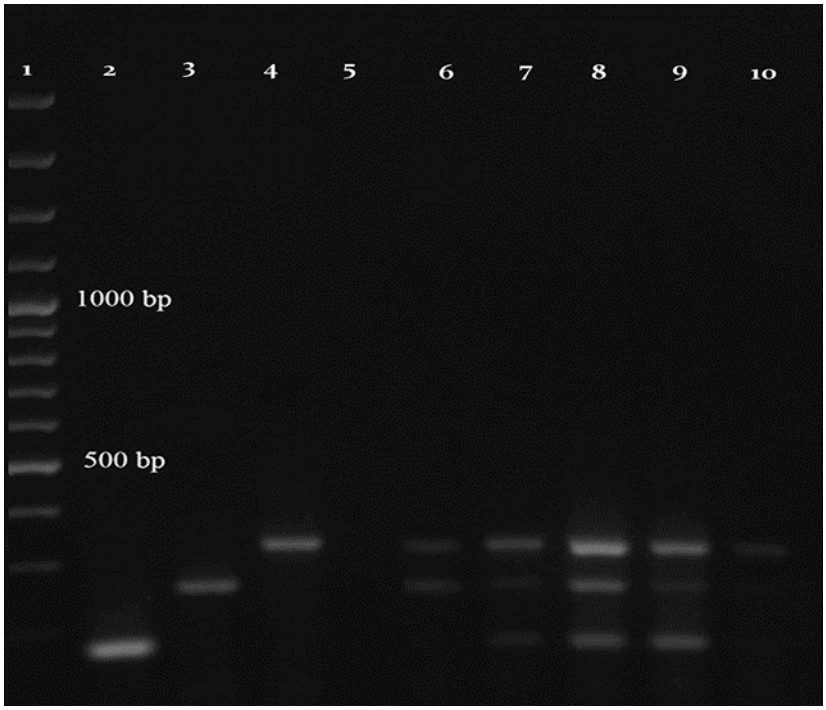

Fig. 1. M-PCR assay, amplifying fragments of 190 bp, 286 bp and 374 bp were amplified for *Str. agalactiae*, *E. coli*, and *Staph. aureus* respectively. Lane1: 100bp marker, Lanes 2, 3, and 4: positive control of *Str. agalactiae*, *E. coli*, and *Staph. aureus* standard strains, Lane 5: negative control with no DNA, Lanes 6-10: raw milk, Karish cheese, Mish cheese, Mish liquor and Buffalo butter samples respectively.

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