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Molecular characterization of some food borne pathogens in soft cheese samples collected from Jeddah, Saudi Arabia

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Abstract: *The growing industrial attention toward rapid methods and the wide use of nucleic acid amplification techniques has led for developing and applying of PCR based methods for food-borne pathogens recognition. In the current study, 20 cheese samples, collected from a local supermarket of Jeddah, Saudi Arabia were examined for the presence of metals and some pathogenic bacteria. Cheese content of Na^+ and K^+ were found to be higher in fresh cheese than other elements. Cr^{2+}, Ni^{2+}, Zn^{2+} and Al^{3+} were detected at low concentrations in cheese. Additionally a multiplex PCR method was developed for detection of Salmonella spp., Listeria monocytogenes and Escherichia coli O157:H7 as the most common cheese borne pathogens. Bacterial enrichment was carried out and bacterial genomic DNA was extracted. A set of primers was designed based on specific genes for Salmonella spp. (invA), L. monocytogenes (prfA) and E. coli O157:H7(eaeA). Additionally, a universal –multiplex PCR based on the highly conserved sequences published on genbank database for the previous genes was used for detecting the previous cheese borne pathogenic bacteria. Three (15 %) out of 20 cheese samples, were contaminated with pathogenic bacteria. Finally, the used method is a promised method, simple, rapid and efficient for detecting pathogenic bacteria in contaminating cheese.*

Keywords*: Soft cheese, E. coli O157:H7, Listeria monocytogenes, Salmonella spp, Universal – Multiplex-primer*

I. Introduction

Food considered an important source for human diseases through harboring toxic metals or foodborne microorganisms. Heavy metals are extensively discreted in the environment. The toxicity introduced due to the presence of excessive levels of some elements, as chromium (Cr^{++}), cadmium (Cd^{++}), lead (Pb^{++}) and mercury (Hg^{++}) ions, are well documented. Several factors are responsible for contamination of milk and dairy products by heavy metals such as environmental conditions and the potential contamination during various stages of industrial processes (Llobet et al., 2003).

Foodborne diseases considered an extensive and increasingly problem of public health leaving its impact on both advanced and developing countries. every year the population percentage suffering from food borne diseases reaches to more than 30% in advanced and in developing countries. Contamination of food and water by microorganisms is the main reason for diarrhoeal diseases. Therefore, the safety of food production from microorganisms attracts a lot of interest of regulatory agencies owing to its possible impacts on human health, on the food industry which significantly involved in the risk of recalls of products from the market, and in the possible economic loss as well as the possible losses in the consumer confidence. The presence of food borne pathogens in milk can be due to direct contact with contaminated sources in the dairy farm environment and to excretion from the udder of an infected animal (Mead *et al*., 1999).

Classical microbiological methods for the detection of foodborne pathogens are dependent on plate culture and biochemical tests. These methodes are reliable and standardized procedures However, they are time consuming and laborious procedure. Recently, molecular events have featured as a promised technique for detecting microorganisms in food. the polymerase chain reaction (PCR) is widely used to amplify DNA fragments providing many advantages over the conventional microbiological procedures like shorter time of analysis, low limit of detection, specificity and possibility for interpretation (Mothershed and Whitney, 2006).

Food and water consumption can transmit many etiological factors, such as *S. enterica, L. monocytogenes* and *E. coli* O157:H7 which are considered among the most important food borne pathogens. These pathogenic agents have been detected by PCR techniques. The efficiency of various target genes and primer sets for each pathogen has been recorded (Alexandre and Prado, 2003; Churchill *et al*., 2006, Jofre *et al*., 2005; Maciorowski *et al*., 2005, Rahn *et al*., 1992).

In the current study, the content of Al^{+++}, Cr^{++}, K^{+}, Mn^{++}, Na^{+}, and Ni^{++} in cheese consumed by Saudi population was determined. Also a combination of overnight enrichment, DNA extraction and a multiplex PCR method was established, for the sensitive, rapid and reliable detection of *S. enterica, L. monocytogenes* and *E. coli* O157:H7. Sets of published primers and a universal primer were established for the recognition of the 3-foodborne pathogens in cheese samples. Also, all the tested cheese samples were investigated by classical microbiological methods to assess the efficiency of the multiplex PCR technique.

II. Materials and methods

2.1 The used bacterial isolates

The standard pathogenic bacterial strains of *L. monocytogenes*, *E. coli* O157:H7 (American) and *S. enterica* were obtained from the culture collection of Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia. Non-pathogenic strain of *E. coli* (BL21) was reserved in our laboratory.

2.2 Collection of cheese samples

Twenty cheese samples used in this study were bought from a local supermarket, of Jeddah, aseptically transported under refrigeration in ice-bucket to the Microbiology lab. and stored at 4 °C until analyzed. The type of cheese and location of manufacture were recorded.

2.3 Chemical analysis of cheese samples

The cheese samples were analyzed chemically for essential and heavy elements including Na^{+}, K^{+}, Al^{3+}, Cr^{2+}, Ni^{2+}, and Mn^{2+} by atomic absorption Spectrophotometer, Perkim Elmer 2380 according to Gajan and Lavry (1972) and El Sawi *et al.* (1994). Additionally, the cheese samples moisture content was directly determined using dry weight method (Aziz, 1987).

2.4 Microbiological analysis

All media used for isolation of different groups of microorganisms, incubation temperature and incubation period were recorded in Table 1. A sample 10 g were taken from the centre or inside portions of each cheese sample, homogenized in 90 ml of 0.1% sterile peptone water for 2 min in a Stomacher (Model 400, Seward Laboratory Systems, Bohemia, NY). Total counts of aerobic bacteria were achieved on soft nutrient agar at 37°C or 10°C for 24 hrs and 10 days, respectively and CFU/ml was calculated (Kosikowi and Mistry, 1997). Total coliforms were determined on Violet Red Bile Agar (Oxoid, CM 107) at 37°C for 24 h. For the detection of *E. coli*, coliform colonies were grown on Lactose Broth in sterile tubes with Durham's tubes at 44.5°C for 24 h and the cultures were observed for CO_2 formation within Durham's tubes and also examined for IMVIC tests.

Salmonella detection was carried out on tetrathionate (TT) broth (Oxoid, CM0029), xylose lysine desoxycholate (XLD) agar (Oxoid,) and MacConkey agar (Oxoid) after grown at 37°C overnight. Presumptive Salmonella colonies were initially screened using triple sugar iron agar, lysine iron agar (Merck), urea broth (Merck) and lysine decarboxylase broth (Oxoid). All biochemical tests were carried out at 37°C for 18–24 h (Andrews and Hammack, 2001). Durham tube, lauryl sulfate broth (Merck), brilliant green bile lactose broth (BGBLB, Merck) and the MPN table were performed to estimate total coliforms/g. *Shigella* detection of was carried out on Petri dishes of XLD agar (Merck), Salmonella–Shigella agar (SS, Merck) and TSI agar (Merck).

2.5 Molecular analysis

2.5.1 Isolation of bacterial DNA

Recovery of bacterial cells from the cheese matrix

Homogenized cheeses in sterile distilled water were centrifuged at 10,000 rpm/min. for 10 min at 4°C. The supernatant was removed and the pellet (bacteria and cheese particles) was resuspended in 5 ml sterile distilled water. About 2 ml of the homogenate was inoculated into 100 ml flasks containing 20 ml of Luria–Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl). After incubation at 37°C for 24 h, the bacterial cells were harvested, washed twice with 1 ml sterile distilled water. Bacterial pellets were resuspended in 200 µl of sterile distilled water and put in a boiling water bath for 20 min. After that, cooling on ice for 20 min, the samples were centrifuged at 10,000 rpm for 5 min and 5µl of the supernatant was used directly for the PCR (Kawasaki *et al*., 2005).

Furthermore, the overnight (18 h) grown culture of the standard bacterial strains: *E. coli* O157:H7, *L. monocytogenes*, and *S. enterica* containing approximately 10^8 and 10^9 viable cells/ml, respectively, were harvested by centrifugation 5min at 3000 rpm, then crude cell lysates were prepared as previously described and were taken as the positive controls to abolish the risk effect of PCR inhibitors which may be found in food materials.

2.5.2 PCR setting

The *invA* (invasion protein A) gene for *S. enterica*, the *prfA* (transcriptional activator of the virulence factor) gene for *L. monocytogenes* and the *eaeA* (attaching and effacing A) gene for *E. coli* O157:H7 are described as the most specific and consistent genetic targets for the considered microorganisms. Particular primers, ESC-F/R, LIS-F/R, and SAL -F/R described by Kawasaki *et al*., 2005, Germini *et al*., 2009, Rahn *et al*. 1992, were taken to identify the presence *E. coli* O157:H7, *L. monocytogenes*, and *S. enteric*, respectively. The primer sets selected for the multiplex PCR assay are revealed in Table 2. Universal primer and compound specific primer pairs of Ecoli706-F/R, LM440-F/R, and Sal320-F/R, designed based on the presence of highly conserved sequence in all previous genes were used for the specific detection of *E. coli* O157:H7, *L. monocytogenes*, and *S. enterica* (Yuan *et al*., 2009). Furthermore 16S rRNA gene was also targeted as an internal control of the existence of amplifiable bacterial DNA. The 16S rRNA gene was amplified as described in Tork *et al*., (2010).

All PCR reactions were carried out in a final volume of 25 µl using 5 µl of extracted DNA as template, hot star master mix from QIAGEN (2X) and 1µl of each primer (20 picomole/µl)). Thermal cycler conditions for multiplex and universal were: initial denaturation at 95 °C for 5 min.; 45 cycles composed of double strand DNA denaturation at 95 °C for 1 min., annealing of primers at 54-58 °C for 1 min., primer extension at 72 °C for 1 min.; final elongation at 72 °C for 7 min. PCR products were visualized on 1.5 % agarose gels using 0.5X TBE and ethidium bromide staining. The negative control was done using the hot star master mix with the primer only.

2.6 Statistical analysis

Each reading had three replicates. Means of variable and standard deviation were recorded. Data were subjected to statistical analysis and differences between mean values determined by the Student's *t*-test. Differences were considered significant when probability was less than 0.05.

III. Results

3.1 Chemical and microbiological analysis of cheese samples

This study was performed to estimate the chemical analysis and microbiological quality of some dairy products. About 20 samples of cheese locally or exported manufactured processed cheese were collected from the different supermarkets and the district bazaars of Jeddah, Saudi Arabia. The types of cheese samples were Feta, Eltaieb, Tomuate, Labina, Hongari and Karish. The origin of the cheese samples were Egypt, Hangria, Saudi Arabia, France, Syria, Turkey. The physical and chemical characters for all the tested cheeses were determined. % of TSS was in the range of 4.4-8.9%, pH was ranged from 5.2-7.7. The dry matter content was ranged from 49-79 %. Total soluble salts in dry matter ranged from 4-8.9%.

Aluminum is added as an emulsifying agent in many processed cheeses, especially those which are single sliced. The concentration ranges of mineral in the cheese samples were found to be 0.20–1.14 (0.65±0.41), 0.09–0.69 (0.248 ±0.17), 0.3–0.68(0.45±0.173), 2700–6718 (4442.4±147), 300–400 (352.0± 33.4) and 16-76 (41.75±16.4) mg/kg for Mn^{++}, Cr^{++}, Ni^{++}, Na^{+}, K^{+} and Al^{+++}, respectively (Table 3).

In the specimens collected, the counts of the Coliform group of bacteria were detected to be between 1.0 x 10^3 and 9.58 x10^8 cfu/g and the bacterial count of *E. coli* was found to be at the interval of $1.2x10^2$ - $3.6x10^8$ cfu/g. *Salmonella* spp. were isolated from one of the examined specimens (Table 4).

3.2 Molecular analysis

3.2.1 Verifying the existence of bacteria in the tested cheese samples

PCR assay targeting bacterial control gene (16S rRNA: 1500 bp) was used to confirm the presence of bacterial cells in all tested cheese samples (Figure 1). Single DNA PCR fragment of the expected size (1500 bp) was obtained (Figure 1).

3.2.2 Verifying the efficiency of UP-M-PCR for the three pathogens detection

The efficiency of UP-M-PCR for the three pathogens: *S. enterica*, *L. monocytogenes*, and *E. coli* O157:H7 detection was tested using crude lysate of the three standard bacterial strains as shown in figure 2. The obtained results demonstrated that after 18 h culture enrichment, the UP-M-PCR technique had the ability to correctly recognize the presence of the three pathogens. The expected DNA fragments for each pathogen were observed on 1.5 % agarose gels which remarked the reliable detection of pathogen specific gene (*S. enterica invA* gene: 320 bp; L. *monocytogenes prfA* gene: 440 bp; *E. coli* O157:H7 *eae*A gene: 706 bp) using the specific universal primer and the compound specific primer pairs. In contrast, no PCR amplification product was found on agarose gel for the non pathogenic *E. coli* (BL21).

3.2.3 Verifying the efficiency of universal primer for the three pathogens detection in all tested cheese samples

The viability of UP-M-PCR for the recognition of the three pathogenic bacteria in all the tested cheese samples was investigated. Efficient amplification of the three expected DNA fragments for each pathogen was seen on 1.5 % agarose gels. The obtained results were shown in figure 3. One band corresponding to *L. monocytogenes prfA* gene: 440 was detected in the crude lysate obtained from overnight culture enrichment and bacterial DNA extraction of Feta cheese sample (sample 2) and Feta cheese sample (sample 13). Whereas, one band corresponding to *E. coli* O157:H7 *eae*A gene: 706 was observed from El Taieb cheese sample (sample 4) and Feta cheese sample (sample 13). However the band corresponding to *S. enterica invA* gene: 320 bp; was only showed in El taieb cheese sample (sample 4). Therefore, the obtained results (Figure 3) confirmed that after 18 h bacterial enrichment, the multiplex PCR analysis had the ability to properly recognize the existence of the three pathogens at all the tested cheese samples.

3.2.4 The specificity and sensitivity of UP- M-PCR

To confirm the sensitivity and specificity of UP-M-PCR for the detection of the three targeting pathogens *S. enterica, L. monocytogenes,* and *E. coli* O157:H7, primer multiplex-PCR reactions were performed using specific primer pairs (ESC-F/R, LIS-F/R, and SAL -F/R). Our PCR results showed that reliable detection of pathogen specific gene (*S. enterica invA* gene: 278 bp; *L. monocytogenes prfA* gene: 217 bp; *E. coli* O157:H7 *eaeA* gene: 151 bp) could be obtained in three tested cheese samples (sample 2, 4, 13) as shown in figure 4.

IV. Discussion

Milk and milk products contain more than twenty different trace elements. Most of them are essential as copper, zinc, manganese and iron. These metals play an important role as cofactors for many enzymes implicated in many physiological functions of man and animals. The absence of these elements affects dramatically on animal metabolism. Other elements can contaminate milk and milk products and cause disturbances and pathological conditions. These metals were evaluated in the current study in twenty tested cheese samples. Results revealed that Na and K concentrations were higher in fresh cheese than other elements. Cr, Ni, Zn and Al were detected at low concentrations in cheese (000 – 000 mg/kg, respectively). The major technological steps and heat treatment may affect the concentration of some heavy metals in cheese.

In the conventional white cheese industry, the curd is processed extensively by the cheese maker (Hayaloglu *et al*., 2002) and at every stage of cheese production, many risk factors cause microbial contamination. Therefore, using non pasteurizing milk for cheese formation and consumption of cheese without maturation will be not complying with hygienic rules and affect microbiological quality (Temelli *et al*., 2006). Concerning the examined samples, the bacterial count in the specimens of fresh white cheeses was found to be at the interval of 0.2 -1.2x105 cfu/g and the mean value was 0.7 x 105 cfu/g. This result was lower than that of Temelli *et al*., (2006). Bacteria count decrease depends upon an increase in the cheeses maturation period (Manolopoulou *et al*., 2003). Many bacteria affect the cheese hygienic quality, such as *Staphylococci, E. coli, Enterococci* and coliforms. The high bacteria count in this study may be due to sale before the completion of their maturation period. As it is known, coliform group of bacteria is quite high in human and animal faeces. Its invasion to foods demonstrates that food is exposed to a faecal borne infection. The differences between the analysis results of fresh white cheese specimens in this research and the values reported by other researchers may be either due to the variations in productions, storage conditions and durations of the examined specimens or due to the difference in the methods used.

Various type of microorganisms can penetrate cheese during its manufacture process (Turantas *et al*., 1989) and the coliform bacterial count was detected to be between 1.0 x 10^3 and 9.58 x 10^8 cfu/g and the *E. coli* bacterial count was found to be at the interval of 1.2 x 102 – 3.6 x 108 cfu /g. According to the Turkish Food Codex, maximum possible coliform bacterial count in cheese should be 95 cfu/ g. In addition, there should be no *E. coli* or *Salmonella* spp in cheese (Turkish Food Codex, 2011). According to our knowledge there wasn't any study in the literature that showed *Salmonella* spp. in cheese sold at markets. In one cheese samples *Salmonella* spp. was isolated (1 %).

Milk and milk product-borne outbreaks correspond to 2–6% of the bacterial food-borne outbreaks. *E. coli* O157:H7, *L. monocytogenes*, and *S. enteric* are 3 kinds of the most important food-borne human pathogens. PCR technique considers one of the most important techniques used in detection and identification of microbial agents due to its high sensitivity, specificity and speed. However, many improvements are still needed to increase its efficiency and applicability.

In the last few years, many studies were carried out using PCR techniques with different pre-enrichment methods for detecting pathogenic microorganisms. Also, different target genes and set of primers were validated for each pathogen (Alexandre and Prado 2003; Churchil *et al*., 2006; Jofre *et al*., 2005; Maciorowski *et al*., 2005, Rahn *et al*., 1992). In our study, overnight culture enrichment and DNA extraction

method was used for preparing sufficient amount of bacterial DNA template. This protocol was considered a suitable method to carry on further PCR experiments and can be used as a simple, cheap, easy and quick method. Previous studies illustrated the importance of pre-enrichment procedures for ensuring the detection of low numbers of viable *L. monocytogenes* in foods (Norton, 2002; O'Grady *et al*., 2008).

High PCR amplification yield of the internal control gene (16S rRNA) confirmed the efficiency of the above DNA extraction protocol and the presence of bacteria in the entire tested cheese samples. This finding was correlated with results previously reported by Germini *et al*., (2009) and in accordance with the general guideline for PCR technique proposed by the European Standardization Committee and International Standard Organization (Anonymous, 2002). Also the efficiency of Up-M-PCR in multiple pathogen detection was evaluated using three standard pathogenic bacteria: *S. enterica, L. monocytogenes, E. coli* O157:H7 and Non-pathogenic strain of *E. coli* (BL21). A significantly PCR amplification of target DNA by electrophoresis assay was observed for the three pathogens whereas no amplification fragment was found with non pathogenic strain. This result referred the high specificity and sensitivity of universal primer-Multiplex -PCR in multiple pathogen detection which also confirmed that compound specific primer pairs had a high sensitivity in a single PCR. Yuan *et al*., (2009) confirmed the efficiency of this multiplex in detection of the three pathogens in artificial contaminates food. The presence of the three pathogenic bacteria in the twenty tested cheese samples was evaluated using Up-M-PCR. Three out of twenty samples (15%) were found to be contaminated with the three pathogens. The intensity of PCR amplification fragment decreased dramatically when the amount of DNA extract was decreased from 5 µl to 2 µl in PCR reaction (data not shown). It is obvious that sample DNA concentration had a critical role in detection of the three pathogenic bacteria using multiplex PCR. This finding was in agreement with that reported by Germini *et al*., (2009). So, the optimization of Up-M-PCR condition favorised the accurate recognition of pathogenic microorganisms in the tested cheese samples. Our results also showed that the DNA templates from the three contaminated cheese samples (sample 2, 4, 13) can be efficiently amplified using specific primer sets of ESC-F/R, LIS-F/R, and SAL -F/R This result validates the specificity and efficiency of UP-PCR amplification for the detection of the three expected DNA fragments for each pathogen on agarose gels. This result was in accordance with the conclusion of universal multiplex PCR (Yuan *et al*., 2009). These results confirm that *invA, prfA, eaeA* gene sequences targeted are specific for *S. enterica* , *L. monocytogenes* , *E. coli* O157:H7 and the combination of 16 h enrichment and PCR assay is sufficiently discriminatory to enable detection of these three pathogens in food.

Finally, we can conclude that the DNA concentration, selection of a suitable primer and optimization of multiplex PCR might be useful for setting a robust method with high performances for the pathogens detection in the contaminated food samples. Furthermore, the finding of an amplifiable bacterial DNA as an internal control added an additional specificity on the test developed. So, the overall strategy proposed, depend on an overnight enrichment stage pursued by DNA isolation and multiplex PCR, was adequately examined for its specificity and sensitivity for the bacterial DNA detection and the target pathogens identification. This high sensitivity, specificity and strength of the technique used and its ability to detect pathogens in food samples, make it a suitable method for the recognition of the target pathogens in food samples.

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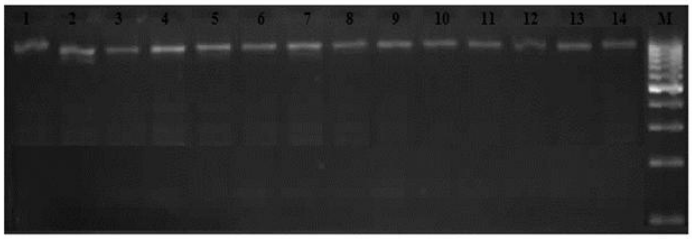

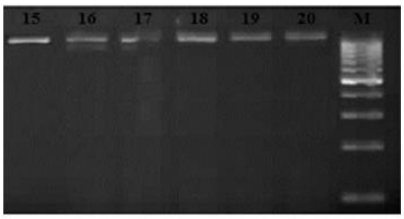

Figure (1): Detection of bacterial control gene (16S rRNA 1500 bp) as an internal control of the presence of amplifiable bacterial DNA in all tested cheese samples. M: 100 bp DNA ladder.

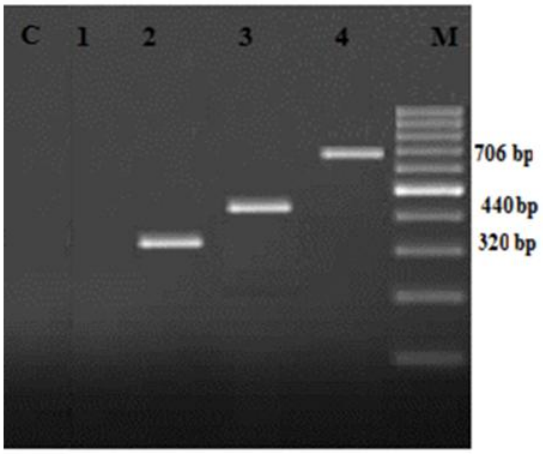

Figure (2): Verifying the efficiency of UP-M-PCR for detection of *E. coli* O157:H7 (706 bp), *L. monocytogenes* (440 bp), and *S. enteric* (320 bp). Each lane represented the amplification result of a single PCR with universal primers, M, 100 bp DNA Marker; 1, negative control without template, 2: *S. enterica*, 3: *L. monocytogenes*, 4: *E. coli* O157:H7, C, *E. coli* (BL21)

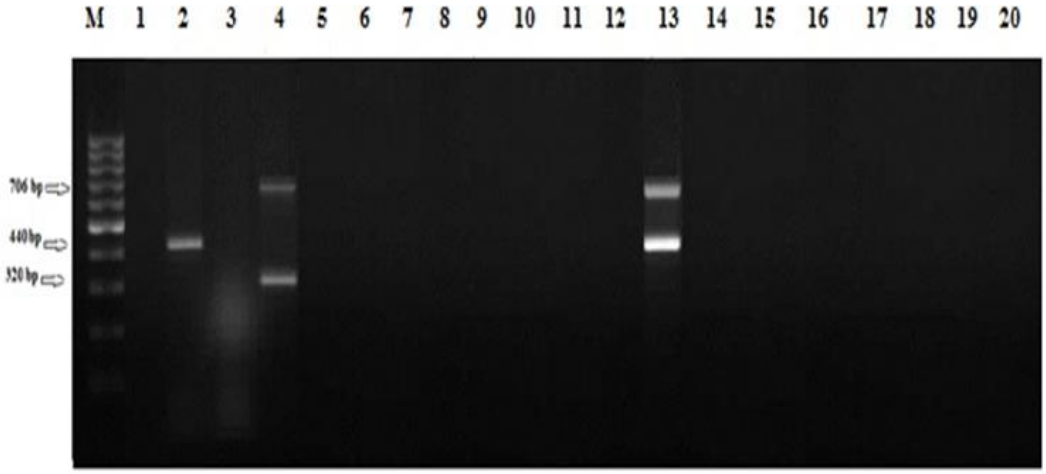

Figure (3): Sensitivity detection of UP-M-PCR PCR of all tested cheese samples, Bands 706, 440, and 320 bp represented the amplification of target gene from *E. coli* O157:H7, *L. monocytogenes*, and *S. enterica*, respectively. M, 100 bp DNA ladder;

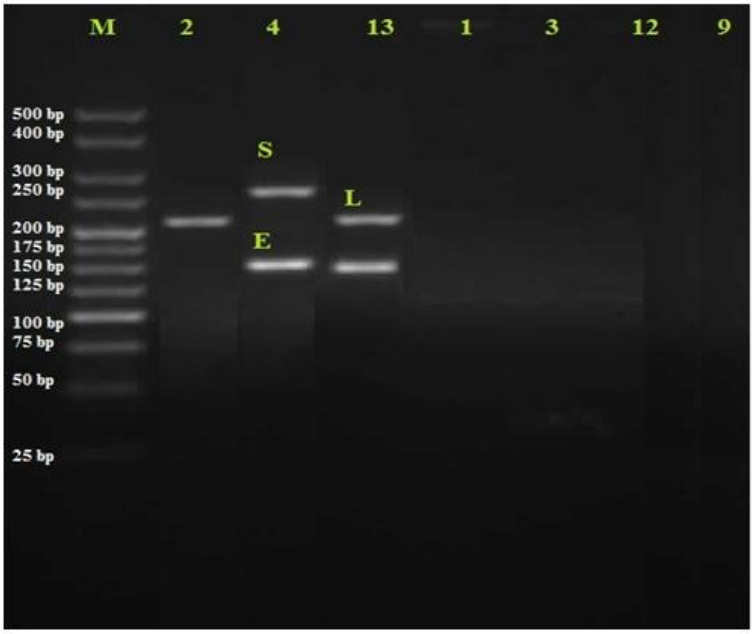

Figure (4): Feasibility of the multipex PCR applied to overnight LB enriched bacterial culture of some cheese samples. Bands 278, 217, and 151 bp represented the amplification of target gene from *S. enteric*(S), *L monocytogenes* (L), and *E. coli* O157:H7 (E) respectively. M: Marker HyperLadder V 25-500 bp

Table (1): The media used for isolation of different groups of microorganisms and incubation temperature in addition to incubation period.

Type of microorganism	Media used	Incubation temperature (°C)	Incubation period (days)
Mesophylic bacteria	Nutrient agar	30, 10	2
Psychrophylic bacteria	Nutrient agar	12	4
Coliform	Brilliant Green Bile Lactose Broth	37	2
Salmomella	Salmonella Chromogen Agar	37	2
Listeria	Tryptone soy broth	37	2
Enterococci	Vancomycin selective agar	37	1

Table (2): Primer pairs selected for the multiplex PCR

Microorganisms	Primer sequence (5'-3')	PCR product size (bp)	Reference
S. Enteric	SAL-F: AAT TAT CGC CAC GTT CGG GCA A SAL-R: TCG CAC CGT CAA AGG AAC C	278 bp	Rahn *et al*. (1992)
E. coli O157:H7	ESC-F: GGC GGA TAA GAC TTC GGC TA ESC-R: CGT TTT GGC ACT ATT TGC CC	151 bp	Kawasaki *et al*. (2005)
L. monocytogenes	LIS-F: TCA TCG ACG GCA ACC TCG G LIS-R: TGA GCA ACG TAT CCTCCA GAG T	217 bp	Germini *et al*., (2009)
Compound specific primer pairs	Ecoli706-F CCTTCCTTCCTTCCCCCCACCTGCGTTGCGTAAATA Ecoli706-R CCTTCCTTCCTTCCCCCCGGGCGGGAGAAGTTGATG LM440-F CCTTCCTTCCTTCCCCCCATCATCGACGGCAACCTCGGAGAC LM440-R CCTTCCTTCCTTCCCCCCCACCATTCCCAAGCTAAACCAGTGC Sal320-F CCTTCCTTCCTTCCCCCCGTGAAATTATCGCCACGTTCGGGCAA Sal320-R CCTTCCTTCCTTCCCCCCTCATCGCACCGTCAAAGGAACC	706 bp 440 bp	Yuan *et al* (2009)
Universal primer(UP)	CCTTCCTTCCTTCCCCCC	320 bp	

Table (3): Chromium, Nickel, Potassium, Sodium, Manganese and Aluminum (mg/kg) in some samples of white cheese collected from Jeddah, Saudi Arabia

Tested metal	Essential and heavy metal ranges					
	Chromium (Cr^{++})	Nickel (Ni^{++})	Potassium (K^{+})	Sodium (Na^{+})	Aluminum (Al^{+++})	Manganese (Mn^{++})
Range (mg/kg)	0.09-0.49	0.3 -0.48	305-369	2009-4003	33-40	0.29-0.82

Table (4): The counts of different groups of bacteria using different media and incubation temperature

Tested bacteria	T.C./g mesophilic bacteria $x10^5$	T.C. /g (psychrophylic bacteria $x10^2$	Coliforms $x10^2$/g	*Salmonella* $x10^2$/g	*Shigella* $x10^2$
Range (cfu/g)	0.020-1.2	0.11-0.19	0.12±0.29	0.0-0.11	0.010±0.12