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Detection of Shiga toxins producing *E.coli* in meat products by multiplex PCR

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ABSTRACT

The current investigation was carried out for 100 random samples of meat products represented by frozen beef burger, frozen kofta, minced meat and oriental sausage (25 each) gathered from various shops and retail stores in Monufia governorate. The gathered samples were subjected to bacteriological examination for the isolation and identification of non O157 *E.coli and E.coli* O157 by using Conventional and recent techniques as multiplex PCR. By the conventional method the incidence of *E.coli* in the tested samples of beef burger, kofta, minced meat and oriental sausage was 6(24%), 9(36%), 5(20%) and 11(44%) respectively. Multiplex-PCR technique was applied on 10 random meat product samples (3 negative and 7 positive for the isolation of *E.coli* by conventional method). M-PCR technique was applied in order to detect *stx*1 and *stx*2. In this study, the M-PCR gives negative result with all tested food samples. This study clarified that multiplex PCR may give negative results due to inhibitors that can be found in microbial DNA solutions extracted from meat sample or due to inhibitors which added during processing of meat products

1. INTRODUCTION

There is no doubt that the life style of these days differs from the past days. The modern life style ,the way it runs quickly, working women led to variable changes in the food preparation and consumption habits. So there is a great trend towards ready-to-eat foods and meat products in Egyptian food market as these meat meals prepared quickly and get rid of the problem of fresh meat shortage and high price of fresh meat (EUFIC, 2006).

Currently microbiological food safety is considered the mystery of developed societies. Current food safety issues are harmfully reshaping the life style of the population in the developing world. The prevalence of food borne illness in developing world is the most ignored area to control disease. E.coli infection is extensively prevalent and has a major threat to human health in underdeveloped communities (Akhtar et al., 2014).

Microbiological evaluation is serious to detect food safety and quality. In the past, cultural methods were essential in detection and identification of food borne microorganisms. These methods are the most dependable and accurate in the identification of food borne pathogen. Although, they are labour intensive and time consuming since it can acquire two-three days for any results to point up and need up to seven – ten days for confirmation (Jasson et al., 2010). Therefore, It was necessary to develop more creative methods to detect food borne pathogens. Development of biotechnology drive to the creation of more rapid and developed methods which reduce manipulation and obtain the results in less time. (Naravaneni and Jamil, 2005).

PCR is an excellent and effective technique which make revolution in researches of molecular biology because PCR is considered rapid diagnostic test for genetic diseases and microbial infections , also in finding of pathogen in food samples. PCR become the most tremendously employed technique for amplifying of DNA. The specificity of detection relies on the selection of DNA region to be amplified. In the previous ten years, several authors have suggested the use of PCR for the revealing of food borne pathogen to substitute the consuming of the time of culture based classical methods (Elmerdahl Olsen, 2000)

Multiplex PCR posses an advantage comparing with the culturing methods as plentiful amounts of selective DNA can be used in one PCR reaction. New reports have revealed that m-PCR extremely develop sensitivity and specificity for the identification of pathogen because m-PCR can detect several different target genes in one PCR reaction tube concurrently (Huang et al., 2009)

2. MATERIAL AND METHODS

2.1. Animals:

00 random samples of meat products represented by frozen beef burger, frozen kofta, minced meat and oriental sausage (25 each) were gathered from different supermarkets and retail stores in Monufia Governorate.

The gathered samples was labeled, transferred directly to the laboratory in an ice box under complete aseptic condition thawed at room-temperature

2.1. Isolation and identification of E.coli from meat product samples

The samples were prepared according to the technique recommended by (APHA 1992) as follows: ten grams of the examined meat products samples was transported to a sterilized polyethylene bag, & 90 ml of 0.1 % sterile

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buffred pepton water were aseptically added to the content of the bag. Each sample was then homogenized on a blender in 2000 rpm for 1-2 minutes to provide a homogenate

2.1.1. Enrichment (ICMSF, 1996)

One ml from prepared sample was inoculated into MaConkey broth tubes which have inverted Durhams tubs. The inoculated tubes are incubated in 37°C / 24 hours. The development of acid and gas indicate positive result.

A loopfull from each positive MacConkey broth tubes are inoculated into another MacConkey broth tube Previously heated to 44ºC and incubated at 44 ± 0.5ºC for 48 hours(Eijkman's test).

2.1.2. Selective Platting media

The loopfuls of positive MacConkey broth tubes were individually streaked on the surface of Eosine Methylne Blue agar media (EMB) and incubated at 37°C for 24 hours. The obtained colonies were purified and inoculated into nutrient agar slope tubs for further identification.

2.1.3. Isolation and Identification of Enterohaemorrhgic E.coli O157:H7 according to Chapman et al. (1991).

2.1.3.1. Enrichement technique:

The samples were prepared according to the technique recommended by USDA (2010) Twenty five grams from each sample were blended on a blender at 2000 rpm / 1-2 minutes to provide a homogenate then this homogenate added to 225 ml of mTSB modified tryptic soya broth which supplemented by novobiocin (20 mg/l). The inoculated broth was incubated at 37°C for 24 hrs.

2.1.3.2. Selective plating media:

A loopful of the incubated enrichment broth is streaked onto sorbitol MacConkey agar plate(CT-SMAC) with Cefixime- Telurite supplement and incubated at 37°C for 24hrs.

Non sorbitol fermenting colonies which gave neutral / grey with smoky center or pale (colourless) were picked up and purified into nutrient agar slants and incubated at 37°C overnight to be subjected for further examinations.

2.2. Identification of suspected E.coli isolates

2.2.1. Morphological Identification (Qunin et al., 2002*)*

2.2.2. Biochemical identification

Biochemical testes are Methyl red test, Indole test, Citrate utilization test Voges-Proskauer test (VP), Triple sugar iron tst (H2S production test), Triple sugar iron tst (H2S production test), Urease test, Sugar fermentation test, Catalase test and Oxidase test

2. Serological identification of the isolates

Serological identification of the *E.coli* non O157 from the isolates by Kok et al., (1996)

Serological identification of enterohaemorrhagic *E.coli* O157:H7

2.4 Detection of some virulence genes using multiplex polymerase chain reaction(m-PCR):

The used primers have specific sequence and amplify specific products as shown in Table (1).

24.1. Extraction of DNA:

As indicated by QIA amp DNA mini kit instructions

2.4.2. Preparation of PCR Master Mix according to 2X Dream Taq Green master mix kit

2.4.3. Cycling conditions of the primers during m-PCR

The amplification was performed on a Thermal Cycler. Amplification conditions were: denaturation for 5 min at 94°C, followed by 35cycles of 94°C for 1min, 58°C for 1 min and 72°C for 1min, with final extension at 72°C for 5 min. Amplified products were analyzed by 1.5% of agarose gel electrophoresis stained with ethidium bromide and visualized and captured on UV transilluminator.

2.4.4. DNA Molecular weight marker

2.4.5. Agarose gel electrophoreses (Sambrooket al., 1989)

Table (1): Oligonucleotide primers encoding for 16SrRNA and *clfA* genes.

Target gene	Primer sequence (5'-3')	Length of amplified product	Reference
Stx1	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG	614 bp	Dipineto *et al.*, 2006
Stx2	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	779 bp	

3. RESULTS

By the conventional method, the incidence of *E. coli* in the examined frozen samples of beef burger, kofta, minced meat and oriental sausage was 6(24%), 9(36%), 5(20%) and 11(44%) respectively as shown in table (2)

Table (2): Incidence of Enteropathogenic *E.coli* isolated from the examined meat product samples (n=25).

Meat products	No .of positive samples	%
Minced meat	5	20
Beef burger	6	24
Kofta	9	36
Sausage	11	44

The serotypes of *E. coli* isolated in this study were *E.*coli O26: H11, O44: H18, O55: H7, O86, O91: H21, O103: H2, O111: H2, O119: H6, O121: H7, O124, O127:H6, O128: H2, O157:H7 as shown in table (3) and fig (1).

The incidence of EHEC O157:H7 in minced meat was (0%), prevalence rate of EHEC O157: H7 in beef burger was (1%), prevalence rate of EHEC O157:H7 in kofta was (0%) and prevalence rate of EHEC O157: H7 in sausage was (1%) as shown in table (4) fig(2) .

The multiplex PCR of 10 samples give negative result *stx*1 and *stx*2 fig(3).

4. DISUCSSION

4.1. Incidence of isolated E. coli:

The incidence of ***E.coli*** in the examined samples of beef burger, kofta, minced meat and oriental sausage was 6(24%), 9(36%) , 5(20%) and 11(44%) respectively, by using conventional method while the incidence of E. coli) such result was nearly similar to results obtained by Sameh_Sama (2016) which were 6(24%) , 7(28%),4(16%) and 11(44%) respectively .

4.2 Serotyping of isolated E. coli

The serological identification of E.coli isolated from all examined meat samples were E.coli O26 : H11, O44 : H18, O55 : H7, O86, O91 : H21, O103: H2, O111 : H2, O119 : H6, O121 : H7, O124, O127:H6, O128 : H2, O157:H7 such result was not similar to results obtained by Sameh_Sama (2016) which were O_{26},O_{55} ,O_{103},O_{111},O_{114},O_{119},O_{124},O_{125} and O_{128}

4.3 E.coli O157:H7

The prevalence rate of EHEC O_{157}:H_7 in minced meat, beef burger, kofta and sausage were (0%), (1%), (0%) and (1%) respectively.

4.4 Shiga toxin-producing E. coli STEC

Multiplex PCR of 10 samples give negative result *stx*1*and stx*2.

In this study multiplex PCR carried on food sample so that multiplex PCR used as method for detection of STEC not for confirmation of the isolated E.coli.

Classical technology and uniplex PCR approaches are not suitable for such studies in which Sxt genes can be identified. The current study manifested that the m-PCR technique was very suitable to gather DNA templates directly from the meat products samples after extraction of DNA and there is no need to take from the culture as it is time consuming, labor intensive and very costly such as Kim et al. (2014) and Al Jobori et al. (2015) who investigated directly from the food samples devoid of the use of bacterial cultures dissimilar Nadugala and Rakshit (2007); Kawasaki et al. (2009); Latha et al., (2014) and Adhikari et al. (2015) who investigated their multiplex PCR technique by the use of bacterial culture.

Table (3): *E.coli* serotypes isolated from the examined samples of meat products (n=25).

Meat Products / *E.coli* strains	Minced meat		Beef burger		Kofta		Sausage		Total (100)	
	No.	%	No.	%	No.	No.	No.	%	No.	%
O26 : H11	1	4	1	4	2	8	3	12	7	7
O44 : H18	-	-	-	-	-	-	1	4	1	1
O55 : H7	1	4	-	-	1	4	1	4	3	3
O86	-	-	1	4	-	-	-	-	1	1
O91 : H21	-	-	-	-	1	4	1	4	2	2
O103: H2	-	-	-	-	1	4	-	-	1	1
O111 : H2	1	4	2	8	1	4	1	4	5	5
O119 : H6	-	-	-	-	2	8	-		2	2
O121 : H7	-	-	-	-	-	-	1	4	1	1
O124	-	-	-	-	1	4	-	-	1	1
O127:H6	-	-	1	4	-	-	-	-	1	1
O128 : H2	2	8	-	-	-	-	2	8	4	4
O157:H7	0	0	1	4	0	0	1	4	2	2
Total	5	20	6	24	9	36	11	44	31	31

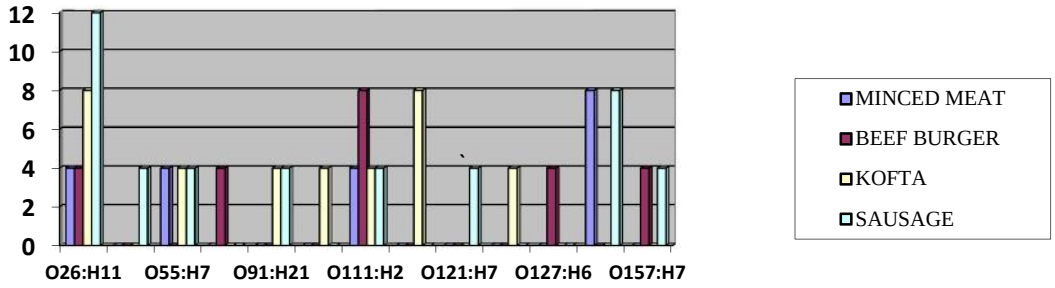

Fig (1): *E.coli* serotypes isolated from the examined samples of meat products (n=25).

Table (4): Summarized results for the examined samples of meat products (n=25).

Meat Products / *E.coli* strains	Minced meat		Beef burger		Kofta		Sausage		Total (100)	
	No.	%	No.	%	No.	No.	No.	%	No.	%
O26 : H11	1	4	1	4	2	8	3	12	7	7
O91 : H21	0	0	0	0	1	4	1	4	2	2
O103: H2	0	0	0	0	1	4	0	0	1	1
O111 : H2	1	4	2	8	1	4	1	4	5	5
O121 : H7	0	0	0	0	0	0	1	4	1	1
O157:H7	0	0	1	4	0	0	1	4	2	2
Total	2	8	4	16	5	20	7	28	18	18

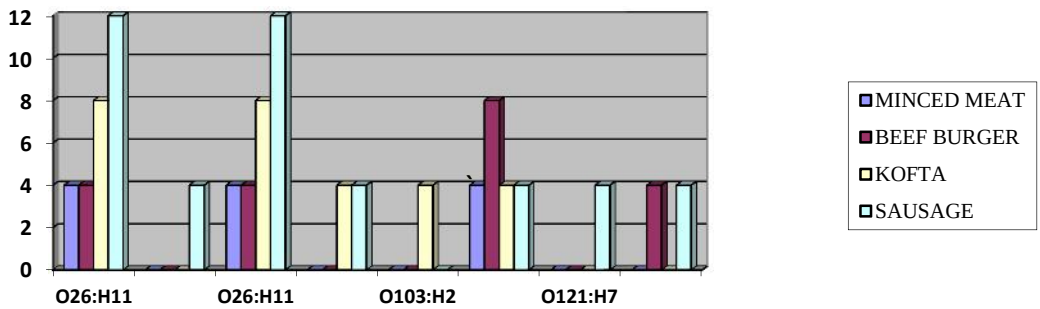

Fig (2): Enterohaemorrhagic *E. coli* isolated from the examined samples of meat products .

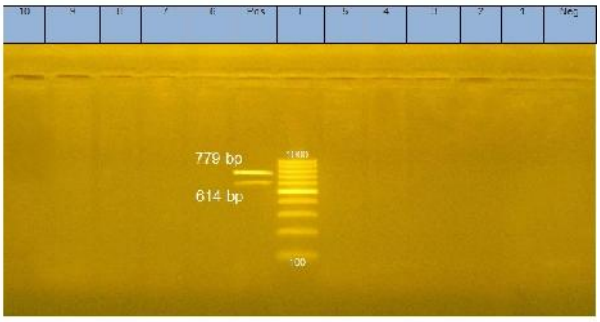

Fig (3): PCR for detection of virulence genes *stx*1 and *stx*2 of *E. coli* strains (n= 10). Agarose gel electrophoresis of multiplex PCR of *stx1*(614bp)and *stx2* (779bp) and for characterization of *E.coli.* Lane L: 100 bp ladder as molecular size DNA marker. Pos: Control positive for *stx*1 and *stx*2 genes. Neg : Control negative. Lane 1 &2, koffta sample give negative for *stx*1 and *stx*2 genes. Lane3, 4 & 5 burger sample give negative for for *stx*1 and *stx*2 genes. Lane 6,7 & 8 sausage sample give negative for for *stx*1 and *stx*2genes. Lane 9 & 10 minced meat sample give negative for *stx*1 and *stx*2genes.

The prevalence of *EHEC* O157 in all examined meat products samples was 2% such result was nearly similar to results obtained by Aly (2006) which was 2.85%.

M-PCR used to identify the presence of genes encoding Shiga toxin 1 and 2 (stx1 and stx2), H7 flagella (flicC), enterohemolysin (hly) and intimin (eaeA) in 400 E.coli isolates (Murinda et al., 2004). Therefore, (MPCR) in this study may give negative results owing to E.coli in meat sample don't have stx1 and stx2 and possess enterohemolysin (hly) and /or intimin (eaeA).

M- in this study may give negative results due to inhibitors that can be found in microbial DNA solutions extracted from meat sample also (m-PCR) in this study may give negative results due to m-PCR in this study carried on processed meat sample and this processed meat sample may have inhibitors to DNA of E.coli and this inhibitors may be added during processing of meat products .the same causes were illusterated also by Jamil et al. (1993).

M-PCR in this study may give negative results due to any minute error in relative concentrations of primers, PCR buffer concentration, cycling temperatures, amounts of template DNA and amount of TaqDNA polymerase. The same causes were illusterated also by Markoulatos et al. (2002).

MPCR in this study may give negative results due to E.coli in meat sample do not possess stx1 and stx2 and possess universal stress protein (uspA) gene. The same causes were illusterated also by Chen and Griffiths (1998).

In this study, two primers of the stx1 and stx2 were added to master mix of multiplex PCR together and two primers could make inhibition to each others.

In this study Five random positive meat products samples by conventional method were reexamined by m-PCR, this 5 samples showed negative results with m-PCR (false negative) .The false negative may be due to addition of spices, curing techniques which inhibit the effect of the PCR. The same causes were illusterated also by Jeníkova et al. (2000)

The false negative result in this study may be due to presence of substances chelating divalent magnesiumions for PCR, degradation of nucleic acids targets or primers through nucleases (DNA) and direct inhibition of the Taq DNA polymerase. The same causes were illusterated also by Scheu et al. (1998)

The false negative result in this study may be due to low number of bacterial load which can't be detected by microbiological assay. The same causes were illusterated also by Estrada et al. (2007)

The false negative result in this study may be due to minute error in the concentration of selective enrichment that used to suppress the natural background microorganisms. The same causes were illusterated also by Suo and Wang (2013).

5. CONCULOSIONS

DNA sample preparation, the PCR process and reaction mixture assemblage, in addition to the subsequent reaction product analysis, must be done in separate areas and also we must utilize high quality DNA templates to improves the success of m-PCR. The length of PCR primers is usually 15-30 nucleotides because Longer primers provide higher specificity.

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