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ISOLATION AND IDENTIFICATION OF SALMONELLAE AND *E .COLI* FROM MEAT AND POULTRY CUTS BY USING MULTIPLEX PCR

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A B S T R A C T

A total of 100 random samples of chicken (thigh and breast) and red meat cuts (mutton and beef shoulders) were collected from different poulterer's and butcher's shops at Cairo, El- Kalyobia and El-Gharbia governorates to detect level of *Salmonella* and *E.coli* contamination. The obtained results indicated that salmonella organisms were isolated from the examined samples of chicken thigh, chicken breast, mutton and beef with percentages of 16%, 16%, 8% and 8% respectively. Moreover, the isolated Salmonellae could be serologically identified as *S. Typhimurium* (28%), *S. Enteritidis* (16%) and *S. Haifa* (4%). On the other hand, the percentages of isolated *E. coli* from the examined samples of chicken thigh, chicken breast, mutton and beef were 16%, 12%, 28%and 12% respectively. Moreover, the results cleared that PCR is an ideal method for identification of Salmonella spp. as it was effective, less labor and more sensitive as well as reduces effort and time. Out of 10 strains of different serotypes of Salmonella isolated from chicken (thigh and breast), mutton and beef by traditional method, 4 strains were positive in m-PCR for Salmonella from which, one strain was identified as *S. Typhimurium.* As well as out of 10 strains of different serotypes of *E .coli* isolated from chicken (thigh and breast), mutton and beef shoulders, 2 strains were positive in m-PCR. *E.coli* O_{55}: K_{59} (B_5) and E.coli O_{119}: K_{69} (B_{14}) isolated from thigh and breast, respectively, which were positive for elt gene (labile toxin).

KEY WORDS: *E. coli*, Meat, Multiplex PCR, Salmonella.

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1. I N T R O D U C T I O N

Meat is considered as an important source of protein, essential amino acids, B complex vitamins and minerals. So, it offers a highly favorable environment for growth of pathogenic bacteria [2]. As well as, poultry meat is an excellent substrate for the growth of a wide variety of microorganisms including pathogens and spoilage microorganisms. On the other hand, chicken and turkey are the major types of poultry meat. Chicken meats comprise about the two-thirds of the total production in the world [31]. Meat and poultry carcasses and their parts are frequently contaminated with pathogens which reach the carcasses from intestinal tract or from fecal material on feet and feathers [14]. Salmonella is an important pathogen in the food industry and has been frequently identified as the etiological agent of food borne outbreaks [41]. *Escherichia coli* is commonly used as surrogate indicator, its presence in food generally indicate direct and indirect fecal contamination [12]. Conventional methods for bacterial pathogens detection in foods are generally based on identification of bacteria using selective culture media by their morphological, biochemical and immunological characteristics [45]. Polymerase Chain Reaction (PCR) based methods have been identified as a powerful diagnostic tool for the detection of pathogenic microorganisms [30].

Therefore, the objective of the current study was to determine the level of salmonella and *E.coli* contamination in meat and poultry meat cuts by convention method and PCR technique.

2. MATERIAL AND METHODS

2.1. *Collection of samples*:
A grand total of one hundred random samples of fresh meat cuts (beef shoulder and mutton shoulder) and poultry cuts (thigh and breast) were collected from different butcher's shops at Cario, El-Kalyobia and El-Gharbia governorates. The collected samples were fresh and transferred directly to the laboratory in an ice box under complete aseptic conditions without undue delay, to be examined bacteriologically for isolation of salmonella and *E.coli.*

2.2. *Preparation of samples*:
Twenty five grams of the both examined meat samples were transferred to a septic blender jar and 225 ml of 0.1 % sterile buffered peptone water were aseptically added to the content of jar. Each sample was then homogenized in the blender at 2000 rpm for 1-2 minutes to provide a food homogenate [8].

2.3. *Isolation and identification of salmonella*:
Previously prepared food homogenate incubated at 37 ºC for 18- 20 hours in case of isolation of salmonella (pre-enrichment). Then one ml of enriched sample was transferred to 10 ml Rappaport Vassilidis broth then incubated at 41.5±1.0ºC for 24±2 hours. A loopful from selective enriched broth was streaked onto the surface of previously prepared Xylose Lysine Desoxycholate (XLD) agar. Inoculated plates were incubated at 37±1ºC for 24 hours. From each positive plate, one typical salmonella colony was sub-cultured for biochemical characterization and serotyping according to the Kauffman-White scheme [23].

2.4. *Isolation and identification of E. coli*:
Isolation of *E.coli* was adopted by using MacConkey broth and Eosin Methylene Blue plates. The metallic green colonies were picked up and identified biochemically and serologically [22].

2.5. *DNA preparation from bacterial cultures*:
An overnight bacterial culture (200µl) was mixed with 800µl of distilled water and boiled for 10 min. The resulting solution was centrifuged and the supernatant was used as the DNA template [4].

2.6. *DNA amplification*:
2.6.1. *Amplification reaction of Salmonellae*:
A multiplex PCR was used for serotyping suspected Salmonella isolates [6]. The primers used in this study are listed in table (1). The bacterial genomic DNA samples were amplified by PCR in a reaction mixture(25µl) containing 13.25 sterile dH2O, 2.5ml 10 x buffer, 0.63ml 10mMNTPs, 1ml 25Mm Mgcl2 , 1.25 µl primer F(20pmol/ml) , 1.25 µl primer R(20pmol/ml) and fill up to 25 µl PCR grade water. The PCR protocol consisted of the following steps: An initial denaturation (2 min at 95°C) for 30 cycles, primer denaturation (1 min at 95°C) 1 cycle, primer annealing (1 min at 57°C), primer extension (2 min at 72°C) and a final elongation (5 min at 72°C). The PCR products were electrophoresed in 2.5% agarose gel and stained with ethidium bromide.

2.6.2. *Amplification reaction of E.coli*:
A multiplex PCR was used for serotyping suspected *E.coli* isolates [44]. The primers used in this study are listed in table (2). The bacterial genomic DNA samples were amplified by PCR in a reaction mixture (25µl) containing 13.25 sterile dH_2O, 2.5ml 10 x buffer, 0.63ml 10mMNTPs, 1ml 25Mm Mgcl2, 1.25 µl primer F(20pmol/ml), 1.25 µl primer

R(20pmol/ml) and fill up to 25 µl PCR grade water. The PCR protocol consisted of the following steps: primer denaturation (1 min at 95°C), primer annealing (1 min at 52°C), primer extension (1 min at 72°C) for 30 cycles, and a final elongation (10 min at 72°C). The PCR products were electrophoresed in 2.5% agarose gel and stained with ethidium bromide.

Table 1 Primer sequences of Salmonella used for PCR identification system

Primer	Sequence (5́……3́)	Target gene	Amplicon length (bp)	References
OMPCF	ATC GCT GAC TTA TGC AAT CG	Salmonella genus	204	[26]
OMPCR	CGG GTT GCG TTATAG GTC TG			
ENTF	TGT GTT TTA TCT GAT GCA AGA	*Salmonella Enteritidis*	304	[3]
ENTR	GGTGA ACT ACG TTC GTT CTT CTG G			
TYPHF	TTG TTC ACT TTT TAC CCC TGA A	*Salmonella*	401	[33]
TYPHR	CCC TGA CAG CCG TTA GAT ATT	*Typhimurium*		
HADF	ACC GAG CCA ACG ATT ATC AA	Salmonella serogroup	502	[29]
HADR	AAT AGG CCG AAA CAA CAT CG	C2		
4512F	CGC TGT GGT GTA GCT GTT TC	Salmonella serotype	705	[19]
4512R	TCT GCC ACT TCT TCA CGT TG	4,5,12:i:		

Table 2 Primer sequences of *E.coli* used for PCR identification system

Primer	Sequence (5́……3́)	Target Gene	Amplicon length (bp)	Reference
VTcom-u	GAGCGAAATAATTTATATGTG	stx	518	[46]
VTcom-d	TGATGATGGCAATTCAGTAT			
AL65	TTAATAGCACCCGGTACAAGCAGG	est	147	[21]
AL125	CCTGACTCTTCAAAAGAGAAAATTAC			
LTL	TCTCTATGTGCATACGGAGC	elt	322	[43]
LTR	CCATACTGATTGCCGCAAT			
ipaIII	GTTCCTTGACCGCCTTTCCGATACCGTC	ipaH	619	[38]
ipaIV	GCCGGTCAGCCACCCTCTGAGAGTAC			

3. RESULTS AND DISCUSSION

Results achieved in Table 3 indicated that Salmonella organisms were isolated from 16%, 16%, 8% and 8% of examined chicken thigh, chicken breast, mutton and beef shoulders, respectively. Salmonellae could be identified serologically as *Salmonella Typhimurium* (24%), *Salmonella Enteritidis* (16%) and *Salmonella Haifa* (4%). While, *salmonella* serotypes isolated from the examined samples of chicken thigh, chicken breast, mutton and beef were *S. Typhimurium* (12%, 8%, 0% and 4%) and *S. Enteritidis* (4%, 0%, 8% and 4%) respectively. But *S. Haifa* isolated only from 4% of the examined chicken meat samples. Salmonella organisms were previously isolated from chicken meat and mutton and beef shoulders [1, 18, 34, 35]. The leading source of contamination of carcasses by Salmonellae is the evisceration step at the slaughterhouse [10].

Table 3 Incidence and serotyping of isolated Salmonellae from the examined samples of chicken and meat cuts (n=25).

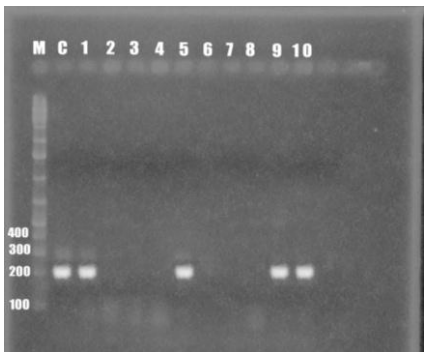
Isolated Bacteria	Chicken cuts				Red meat				Total	
	Thigh		Breast		Mutton shoulder		Beef shoulder			
	No	%	No	%	No	%	No	%	No	%
Salmonella Typhimurium	3	12%	2	8%	-	-	1	4%	6	24%
Salmonella Enteritidis	1	4%	-	-	2	8%	1	4%	4	16%
Salmonella Haifa	-	-	1	4%	-	-	-	-	1	4%
Total	4	16%	3	12%	2	8%	2	8%	11	44%

As well as poor hygiene conditions, regarding the temperature of storage, the equipment and the employees' personal hygiene. The cutting tables were seldom washed or disinfected before use. These benches could therefore be reservoirs from which Salmonellae could spread to other equipment through flies or direct contact [42]. The thigh muscle had a higher Salmonella contamination rate compared to that of breast muscle which might be due to during evisceration process the thigh / leg because of its proximity to point of evisceration are highly prone for contamination from the gut content in case of improper procedure [16]. On other hand, contamination of mutton and beef with Salmonella species may be attributed to surfaces of carcasses are easily contaminated with microorganisms, during skinning and evisceration, a variable percentage of which are potentially spoilage organisms and/or food borne pathogens including Salmonella organisms [40]. *S. Typhimurium* and *Salmonella Enteritidis* are the most frequently isolated serovar from food borne outbreaks throughout the world [20]. Results summarized in table 4 indicated that, *E.coli* was isolated from 16%, 12%, 28%and 12% of the examined samples of chicken thigh, chicken breast, mutton and beef shoulders, respectively. Moreover, the incidence of serologically identified *E. coli* as Enteropathogenic *E. coli (E. coli* $O_{86}:k_{61}$, *E. coli* $O_{119}:k_{69}$ and *E. coli* $O_{55}:k_{59}$*)* was 24%, Enterotoxogenic *E. coli (E. coli* $O_{125}:k_{70}$, *E. coli* $O_{127}:k_{63}$ and *E. coli* $O_{128}:k_{67}$*)* was 24%, Enterheamorrhagic *E. coli (E. coli* $O_{26}:k_{60}$ *and E. coli* $O_{111}:k_{58}$) was 12% and Enteroinvasive *E. coli (E. coli* $O_{124}:k_{72}$*)* was 4%. *E.coli* was previously isolated from chicken meat, mutton and beef shoulders samples [2, 5, 28, 36]. The presence of *E. coli* in high numbers indicates the presence of organisms originating from fecal population. This is due to improper slaughtering techniques, contaminated surfaces and/or handling of the meat by infected food handlers [32]. Also, the presence of these pathogens can be due to contamination taking place during the meat processing at slaughterhouse or to the retailers' poor handling of meat [25]. In the last decade, there has been a wide interest in the use of the multiplex PCR [mPCR) technique. mPCR approaches have been applied to detect different species of several bacteria, to differentiate closely related species and to recognize single species [39]. The use of primer pair specific to OMPC gene as a general primer to detect salmonellae out of 10 strains of different serotypes of Salmonella isolated from chicken (thigh and breast), mutton and beef shoulders, 4 strains were positive in m-PCR (2 strains from breast, one from mutton and other strain from beef).

Table 4 Incidence and serotyping of isolated *E.coli* from the examined samples of chicken and meat cuts (n=25):

Isolated bacteria	Chicken cuts				Red meat cuts				Types	Total	
	Thigh		Breast		Mutton shoulder		Beef shoulder			No	%
	No	%	No	%	No	%	No	%			
E coli $O_{86}:k_{61}$	-	-	-	-	1	4%	1	4%			
E coli $O_{119}:k_{69}$	1	4%	1	4%	-	-	-	-	EPEC	6	24%
E coli $O_{55}:k_{59}$	1	4%	-	-	1	4%	-	-			
E coli $O_{125}:k_{70}$	1	4%	1	4%	1	4%	-	-			
E coli $O_{127}:k_{63}$	-	-	-	-	2	8%	-	-	ETEC	6	24%
E coli $O_{128}:k_{67}$	-	-	-	-	-	-	1	4%			
E coli $O_{26}:k_{60}$	-	-	1	4%	1	4%	-	-			
E coli $O_{111}:k_{58}$	-	-	-	-	-	-	1	4%	EHEC	3	12%
E coli $O_{124}:k_{72}$	1	4%	-	-	-	-	-	-			
Total	4	16%	3	12%	6	24%	3	12%	EIEC	1	4%

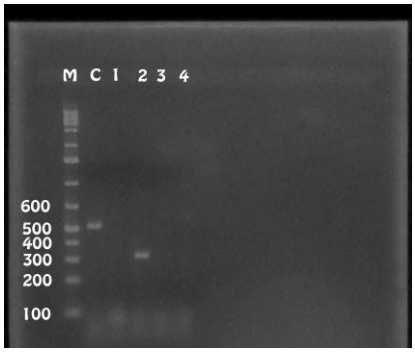
The results showed that the primer was able to amplify DNA fragments of about 204 bp in these four strains. Moreover, these positive 4 strains with OMPC gene were tested with different types of primers to know the species of isolated Salmonellae with m-PCR. Out of 4 strains, one strain (from 2 strains from breast) showed a band at 401 bp as shown in Photograph (1&2). These strains were identified as *Salmonella Typhimurium* in m-PCR which was similar to that isolated by conventional culture method. Nearly similar results were obtained in chicken meat, mutton and beef shoulders [11, 17].



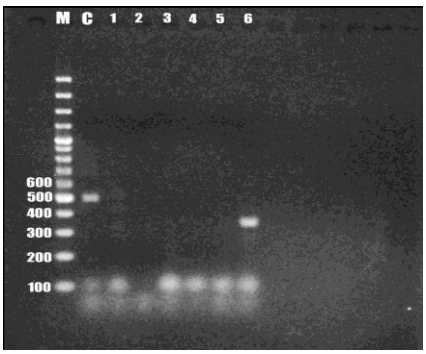
Photograph 1 Agarose gel electrophoresis of PCR amplification products using general primers of Salmonella organisms (OMPCR and OMPCF) .Lanes: M, molecular weight marker, C: control positive of *S. Typhimurium*,1-*S. Typhimurium*(breast),2-*S. Typhimurium*(thigh),3- *S. Enteritidis* (thigh),4- *S. Haifa (breast),*5- *S. Typhimurium*(breast),6- *S. Enteritidis* (beef),7- *S. Enteritidis*(mutton),8- *S. Typhimurium*(thigh),9-*S. Enteritidis*(mutton) and10- *S. Typhimurium*(beef).

Photograph 2 Agarose gel electrophoresis of PCR amplification products using specific primers of Salmonella (ENTR, ENTF, TYPHYR, TYPHYF, HADF, HADR, 4512F and 4512R). Lanes: M, molecular weight marker, C: control positive of *S. Typhimurium*, 1-*S. Typhimurium* (breast),2- *S. Typhimurium* (breast), 3-*S.Enteritidis*(mutton)and4-*S.Typhimurium*(beef).

As well as, out of 10 strains of different serotypes of *E.coli* isolated from chicken (thigh and breast), mutton and beef, 2 strains were positive in m-PCR. *E.coli* O_{55}*:* K_{59} *(*B_5*)* and *E.coli* O_{119}*:* K_{69} *(*B_{14}*)* isolated from thigh and breast respectively, which were positive for elt gene (labile toxin) and showed a band at 322 bp as shown in Photograph (3 and 4). These 2 strains were EPCE by conventional culture method but ETEC by m-PCR. Nearly similar results were obtained in chicken meat, mutton and beef shoulders [25, 27].
The negative results in PCR may be attributed to conventional method showed poor sensitivity and sometimes produced false-positives [13]. Moreover, PCR based detection mainly depends on the purity and amount of the template DNA used [15].



Photograph 3 Agarose gel electrophoresis of PCR amplification products using general primers of *E.coli* (VTcom-u, Vtcom-d, AL65, AL125, LTL, LTR, ipaIII and ipaIV). Lanes: M, molecular weight marker, C: control positive of *E.coli* O_{157}*:*H_7, 1- *E.coli* O_{127} (mutton), 2- *E.coli* O_{55} (thigh), 3- *E.coli* O_{128} (beef) and 4- *E.coli* O_{125} (breast).



Photograph 4 Agarose gelelectrophoresis of PCR amplification products using general primers of *E.coli* (VTcom-u, Vtcom-d, AL65, AL125, LTL, LTR, ipaIII and ipaIV). Lanes: M, molecular weight marker, C: control positive of *E.coli* O_{157}*:*H_7, 1- *E.coli* O_{125} (mutton), 2- *E.coli* O_{124} (thigh), 3- *E.coli* O_{55} (mutton), 4- *E.coli* O_{125} (thigh), 5- *E.coli* O_{111} (beef) and 6- *E.coli* O_{119} (breast).

The presence of PCR inhibitors in food samples and incomplete bacterial cell isolation lead to the production of false negative results in PCR based detection and the removal of PCR inhibitors, efficient bacterial cell isolation and efficient DNA extraction is important **[24]**. Therefore, the application of PCR-based methods is closely linked to the selection of suitable methods for DNA extraction **[7]** and efficient isolation of bacterial cells from food samples by immobilization. As well as, false negative results occur for various reasons, the presence of substances chelating divalent magnesium ions for PCR, degradation of nucleic acids targets or primers through nucleases (DNA and RNA) and direct inhibition of the Taq DNA polymerase **[37]**.These results highlight a disagreement between the genotype and phenotype. This indicates that the serotyping method originally used for identifying pathogenic *E. coli* such as EPEC, ETEC and EHEC, is not sufficient. The detection of pathogenic genes is necessary and more important than using the serotype method. Our results agree with those reported by researchers who have reported that the possession of specific O-antigens did not necessarily correspond with the pathogenic characteristics **[9]**. In conclusion, the m-PCR is rapid, effective and sensitive method than conventional culture method in detection of food born pathogens. So to reduce public health to consumer we must produce a safe meat to consumer by application of HACCP (Hazard Analysis and Critical Control Points) in meat and poultry slaughter houses and shops.

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عزل وتصنيف ميكروب السالمونيلا والايشيريشيا كولاى من قطعيات اللحوم والدواجن باستخدام تفاعل البلمرة المتسلسل

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الملخص العربى

أجريت هذه الدراسة على عدد مائه (100) عينة عشوائية من لحوم الدواجن (الصدور والأوراك) واللحوم الحمراء (لحوم الضانى ولحوم البقرى من منطقة الكتف) تم تجميعها من محلات مختلفة من محافظة القاهرة, القليوبية و الغربية (بمعدل 25عينة من نوع) حيث أجريت الفحوص البكتريولوجية لعزل السالمونيلا و الايشيريشيا كولاى بالطرق التقليدية و التقنيات الحديثة (تفاعل البلمرة المتسلسل) وقدأظهرت النتائج ما يلي: تم عزل ميكروبات السالمونيلا من عينات أوراك و صدور الدجاج, لحوم الضانى و البقرى بنسب 16%, 16%, 8% و 8% على التوالى.وبالفحص السيرولوجى تبين أن العترات المعزولة هى : .سالمونيلا تيفيميوريم, سالمونيلا انتريتيديس و سالمونيلا هايفى .حيث تم عزل ميكروب سالمونيلا تيفيميوريم من عينات أوراك و صدور الدجاج ولحوم البقرى بنسبة 12%، 12%، و4% على التوالى. كما تم عزل ميكروب سالمونيلا انتريتيديس من عينات أوراك الدجاج، لحوم الضانى و البقرى بنسبة 4%، 8%، و 4% على التوالى. وقد تم عزل سالمونيلا هايفى من صدور الدجاج فقط بنسبة 4%. وعلاوة على ذلك فقد تم عزل ميكروب الأيشريشيا كولاي من أوراك و صدور الدجاج, لحوم الضانى و البقرى بنسبة 16%، 12%، 24%، 12% على التوالى. وجد أن تفاعل البلمرة المتسلسل فقد وجد أن 4 عينات من اجمالى 10 عترات من السالمونيلا المعزولة من قطعيات اللحوم المختلفة كانت ايجابية لجين الخاص بالسلامونيلا وعينة واحدة فقط من الأخيرة كانت ايجابية لجين سالمونيلا تيفيميوريم. أيضا وجد أن عينتين من اجمالى 10 عترات من الايشيريشيا كولاى المعزولة كانت ايجابية لجين الايشيريشيا كولاى القادر على افراز توكسين القابل للتكسير بالحرارة. من هذه الدراسة نخلص الى أن أوراك الدجاج ولحوم الضانى هى الأكثر معدل للتلوث بالسالمونيلا والايشيريشيا كولاى والتى يوصى بمعاملتها حراريا قبل تناولها للحفاظ على سلامة المستهلك.

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