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Molecular Characterization of Multidrug-Resistant *Salmonella species* from Meat in Delta Area, Egypt

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ABSTRACT

A total of 180 samples from meat (30 fresh slaughtered poultry, 30 frozen poultry, 30 poultry organs, 30 fresh beef meat, 30 frozen beef meat and 30 beef organs) were obtained randomly from slaughterhouses, restaurants and supermarkets at Delta area, Egypt. Collected samples were examined for the presence of *Salmonella spp*. Biochemical and serological identification were applied, molecular characterization using multiplex PCR technique was used for confirming the serotyping of *Salmonella spp*. and identification of antimicrobial resistance genes. Results revealed that incidence of *Salmonella spp*. was 8.3% (15 isolates), of these 15 isolates, *Salmonella typhimurium* was the highly isolated serovar represented in (9) isolates, *Salmonella paratyphi* A (3), *Salmonella enteritidis* (2) and *Salmonella kentucky* (1). Antibiogram resistance phenotype showed that ciprofloxacin and chloramphenicol were most effective against strains while ampicillin and cefotaxime were the lowest effective against strains. Then, molecular characterization of β-lactamases resistance genes showed presence of bla_{TEM} gene in 6 isolates, bla_{CMY} in 2 isolates, bla_{CTX-M} in 12 isolates where bla_{OXA} and bla_{SHV} genes could not be identified.

Keywords: β-lactamases, Egypt, meat, *Salmonella.*

INTRODUCTION

Over the last 25 years, the global incidence of foodborne infections has markedly increased, with nearly a quarter of the population at a high risk of illness (Oliver et al., 2005). The World Health Organization (WHO, 2010) estimates that foodborne and waterborne diarrheal diseases together kill around 2.2 million people annually. Foodborne pathogens are a major threat to food safety, especially in developing countries where hygiene and sanitation facilities are often poor. *Salmonella enterica* are among the major causes of outbreaks of foodborne diseases (Ahmed and Shimamoto, 2014), it is a significant cause of foodborne illness in humans with 108,614 reported cases in the European Union in 2009 (EFSA, 2011). *Salmonella* spp. are recognized as major food-borne pathogens in humans worldwide. In the United States, there are an estimated 800,000 to 4 million *Salmonella* infections annually, and approximately 500 of these are fatal and the number of cases of Salmonellosis was estimated at between 696,000 and 3,840,000 with 3,840 deaths and at an estimated cost between $600 million and $3.5 billion (Busby and Roberts, 1995). In fact, contaminated meat and dairy products are probably the most common cause of human Salmonellosis worldwide (Herikstad et al., 2002**)**. Little is known about the incidence of foodborne pathogens on a large scale in developing countries (Sehgal et al., 2008, Tassew et al., 2010 and Favier et al., 2013). Therefore, this study was conducted to investigate the incidence of *Salmonella spp*. in meat from Delta area in Egypt, to use PCR assays for molecular characterization of *Salmonella spp*. In recent years, the prevalence of antimicrobial-resistant bacterial pathogens has become a major public health concern, and increasing antimicrobial resistance in *Salmonella enterica* is a serious clinical problem worldwide. During the last decade, a single clone of multiresistant *S. enterica* serovar *Typhimurium* of phage type DT104 has spread worldwide. This *Salmonella* type has emerged within a few years as one of the most common causes of human salmonellosis in several countries (Ahmed et al., 2005).

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MATERIALS AND METHODS

Sampling

A total of 180 samples were collected from poultry and beef meat and organs as following: 30 from freshly slaughtered poultry (15 breast and 15 thigh muscles), 30 from frozen poultry meat (15 breast and 15 thigh muscles), 30 from poultry organs (15 from liver and 15 from gizzard), 30 from fresh beef meat, 30 from frozen beef meat and 30 from beef organs (10 from heart, 10 from liver and 10 from spleen). All samples were aseptically collected and sent to the laboratory.

Microbiological and molecular analysis: Isolation and identification of Salmonella spp.

The standard cultivation method for Salmonella isolation was carried out as recommended by ISO 6579 (ISO, 2002), samples (25 g or ml) were placed in stomacher bags containing 225 ml of Buffered Peptone Water. After stomacher homogenization for 2 min at 320 rpm and overnight incubation at 37°C, 0.1 ml aliquots were inoculated into tubes containing 10 ml Rappaport Vassiliadis (RV) broth and incubated for 48 h at 42 °C. Then, Xylose Lysine Deoxycholate agar plates were inoculated from each of the RV broths and incubated for 18–24 h at 37°C. Suspect colonies with typical Salmonella morphology were confirmed biochemically by TSI, Urease test, MR, VP, Catalase and Oxidase test.Typical Salmonella isolates were further serotyped by using specific Salmonella O and H agglutinating antisera (Difco) following the Kauffman-White serotyping scheme (Grimont and Weill, 2007).

Bacterial DNA preparation and molecular characterization of Salmonella spp.

A smooth single colony was inoculated in 5ml nutrient broth and incubated at 37◦C for 18 hours, then 200 µl from bacterial culture was mixed with 800 µl of distilled water then made vortex for good mixing then heating at 96◦C for 5 minutes in heat block. The resulting solution was centrifuged at 10,000 rpm for 5 minutes and the 200µl from supernatant was used as the DNA template (Ahmed et al. 2007). One primer pair specific for genus Salmonella with a target PCR amplicon size of 204 bp and two other primer pairs specific for the most common *S. enterica* serovars *enteritidis* (target size 304 bp) and *typhimurium* (target size 401 bp) were used. The primer sequences and expected PCR product sizes are shown in table (1).

Bacterial DNA preparation, PCR of the class 1 and 2 integrons

Preparation of bacterial DNA templates and the PCR conditions used for detection of class 1 and class 2 integrons were carried out. Both DNA strands of the entire class 1integron segments were sequenced using an ABI automatic DNA sequencer (Model 373; Perkin-Elmer). Two other primers were designed according to the preliminary DNA sequencing results of class 2 integrons. These primers were located within the PCR fragment and were used for complete sequencing of the whole class 2 integron segment (Ahmed et al., 2005). The class 1 integron primers 5'-CS and 3'-CS (Table 1) which amplify the region between the 5'-conserved segment (5'-CS) and 3'-CS of class 1 integrons, were used. For the detection of class 2 integrons, PCR was performed with the primer pair hep74 and hep51, specific to the conserved regions of class 2 integrons.

Screening for ß-lactamase-encoding genes

Salmonella strains were tested for TEM, CTX-M, OXA, SHV and CMY ß-lactamase-encoding genes by PCR using universal primers for the bla_{TEM}, bla_{OXA}, $bla_{CTX-M,}$ bla_{SHV} and bla_{CMY} families (Table 1).

Antimicrobial sensitivity discs

Ampicillin (AMP 10µg), amoxycillin–clavulanic acid (AMC 30µg), cefotaxime (CTX 30µg), chloramphenicol (C 30µg), ciprofloxacin (CIP 5µg), streptomycin (S 10µg), nalidixic acid (NA 30µg), enrofloxacin (ENR 5µg), ceftriaxone (CRO 30µg), ceftazidime (CAZ 30µg),

cefpodoxime (Cpd 10μg) and tetracycline (TE 30μg).

RESULTS

A total of 15 *Salmonella spp*. isolates were recovered from 180 samples, *Salmonella typhimurium* represented 60% (9 isolates), *Salmonella paratyphi* A represented 20% (3 isolates), *Salmonella enteritidis* represented 13.3% (2 isolates), and finally *Salmonella kentucky* represented 6.7% (1 isolate), serotyping of isolated *Salmonella spp*. from examined samples is shown in Table (3). Identification of serotypes by using multiplex PCR are shown in Figure (1) and (2). Antimicrobial susceptibility tests for the 15 *Salmonella* isolates in Table (4) showed that most effective antimicrobials were ciprofloxacin and chloramphenicol (0% resistance), while ampicillin and cefotaxime were the lowest effective antimicrobials (80% resistance). Molecular characterization of the ß-lactamase resistance genes were shown in figure (3), (4) and (5) and finally, resistance phenotype and incidence of resistance genes in *Salmonella spp* shown in Table (5).

DISCUSSION

From 180 meat and organs samples, incidence of *Salmonella* species isolates was 8.3%. From 90 poultry samples (meat and organs), *Salmonella* spp. were 7 isolates representing 7.8%, this result was lower than the one detected by Capita et al. (2003) who examined the incidence of *Salmonella* in Spanish poultry products samples included chicken carcasses and chicken parts (wings, legs, giblets, livers and hearts). The average detection rate was 49% and also lower than result of Tibaijuka et al. (2003) who determined the presence and prevalence of *Salmonella* in 301 retail raw chicken meat and giblets (gizzard and liver) in supermarkets of Addis Ababa (Ethiopia). They detected *Salmonella* in 54 (17.9%) of the 301 samples examined, while these result was higher than result of Ahmed and Shimamoto (2014) who isolated *Salmonella enterica* in a incidence of 4.3% from retail markets and slaughterhouses in Egypt. From meat and meat products, *Salmonella* serovars represented 8.9% (8 isolates), and this result was found to be higher than result of Bosilevac et al. (2009) who found that Overall prevalence of *Salmonella* strains was 4.2% from meat samples in 7 regions of the united states.

In this study, *Salmonella t*yphimurium was most detected serovar representing 60%, *Salmonella paratyphi* A 20%, *Salmonella enteritidis* 13.3% and *Salmonella kentucky* 6.7%. Our results have some differences from result of Hernandez et al. (2005) who found that most isolated serovar was *Salmonella enteritidis* followed by *S. heidelberg* and then *S. typhimurium* that was this result was lower than result of Capita et al. (2003) which was 34.3% *Salmonella enteritidis*. On the other hand, this result was higher than result of Tibaijuka et al. (2003) which was 3.7% for *Salmonella typhimurium*.

Our results of PCR identification for *Salmonella* serovars were identical (100%) of those obtained with biochemical and serological methods, as same as the results of Carl et al. (2001) and differ from results of Whyte et al. (2002) who recovered *Salmonella* from 32 (16%) of 198 samples obtained from 40 poultry flocks, using traditional culture methods. In contrast, the PCR assay proved to be more sensitive and detected *Salmonella* DNA in 38 (19%).

Results of antibiotic sensitivity test revealed that most effective antibiotics against *Salmonella* were Ciprofloxacin and Chloramphenicol, while isolates were mostly resistant to Ampicillin and Cefotaxime. Geornaras and VonHoly (2001) approved that most effective antibiotic was danofloxacin. This result was similar to result of Randall et al. (2003) who examined 397 strains of *Salmonella* of human and animal origin and found that most serovars were resistant to ampicillin. Also our results agree with results of Larkin et al. (2004) who found that none of 390 *Salmonella* isolates was resistant to Ciprofloxacin.

In our study, the β-lactamase encoding genes, bla_{CMY} could be identified in 2 of 15 isolates, which agree with Hanson et al. (2002) who identified *bla_{CMY}* from *Salmonella typhimurium*. *bla_{CTXM}* resistance gene could be identified in 12 of 15 *Salmonella* isolates, that result was so higher than result of Taguchi et al. (2006) who reported that there have been only two reports of extended spectrum Cephalosporines ESC-resistant *Salmonella spp*. in Japan. One was regarding CMY-2 β-lactamase-producing S. Newport isolates, and the other a CTX-M-14β-lactamase-producing *S. enteritidis* isolate.

The β-lactamase encoding genes, *bla_{TEM}*, was identified in 6 of 15 *Salmonella* isolates, which was higher than result of Ahmed et al. (2009) who identified the genes in 14 of 48 isolates. We couldn't identify *bla_{SHV}* in our samples while Hanson et al. (2002) could identify the gene in *Salmonella enterica* serotype *typhimurium* isolate, also β-lactamase encoding genes, *bla_{OXA}* couldn't be identified as all samples were negative, this result disagrees with result of Hanson et al. (2002) who could identify the same gene in *Salmonella typhimurium* isolated from a diarrheatic girl.

Table (1): Primers used in this study

Primer / Salmonella Serotyping	Sequence (5' to 3')	Amplicon size (bp)	Target gene	Reference or Gene Bank accession no.
OMPCF OMPCR	ATCGCTGACTTATGCAATCG CGGGTTGCGTTATAGGTCTG	204	*Salmonella*	Alvarez et al. (2004)
ENTF ENTR	TGTGTTTTATCTGATGCAAGAGG TGAACTACGTTCGTTCTTCTGG	304	*Eneteritidis*	Alvarez et al. (2004)
TYPHF TYPHR	TTGTTCACTTTTTACCCCTGA A CCCTGACAGCCGTTAGATATT	401	*Typhimurium*	Alvarez et al. (2004)
β-Lactamases				
TEM-F TEM-R	ATAAAATTCTTGAAGACGAAA GACAGTTACCAATGCTTAATC	1080	*bla_{TEM}*	Ahmed et al. (2007)
OXA-F OXA-R	TCAACTTTCAAGATCGCA GTGTGTTTAGAATGGTGA		*bla_{OXA}*	Ahmed et al. (2007)
CTX-M-F CTX-M-R	CGCTTTGCGATGTGCAG ACCGCGATATCGTTGGT		*bla_{CTX-M}*	Ahmed et al. (2007)
CMY-F CMY-R	GACAGCCTCTTTCTCCACA TGGAACGAAGGCTACGTA		*bla_{CMY}*	Ahmed et al. (2007)
SHV-F SHV-R	TT ATCTCCCTGTTAGCCACC GATTTGCTGATTTCGCTCGG		*bla_{SHV}*	Ahmed et al. (2007)
Integron/resistance genes				
5 -'CS 3 -'CS	GGCATCCAAGCAGCAAG AAGCAGACTTGACCTGA		*Class 1 integron*	Levesque et al. (1995)
hep74 hep51	CGGGATCCCGGACGGCATGCACGATTTGTA GATGCCATCGCAAGTACGAG		Class 2 integron	Ahmed et al. (2007)
Plasmid mediated quinolone				
qnrA-F qnrA-R	ATTTCTCACGCCAGGATTTG GATCGGCAAAGGTTAGGTCA		*qnrA*	Robicsek et al. (2006)
qnrB-F qnrB-R	GATCGTGAAAGCCAGAAAGG ACGATGCCTGGTAGTTGTCC		*qnrB*	Robicsek et al. (2006)
qnrS-F qnrS-R	ACGACATTCGTCAACTGCAA TAAATTGGCACCCTGTAGGC		*qnrS*	Robicsek et al. (2006)

Table (2): PCR Conditions

Gene Integrons	Hot start	Denat.	Anneal.	Prim. ext.	Cy.	Final ext.	Target
Class 1 integ	94ºC/10 min	94ºC/1 min	55ºC/1 min	72ºC/3 min	30	72ºC/10 min	Variable
Class 2 integ	94ºC/10 min	94ºC/1 min	55ºC/1 min	72ºC/3 min	30	72ºC /10 min	Variable
β-lactamases							
CMY	94ºC/10 min	94ºC/1 min	55ºC/1 min	72ºC/1 min	35	72ºC /7 min	1007bp
TEM	94ºC/10 min	94ºC/30 sec	50ºC/30 sec	72ºC/1 min	30	72ºC /10 min	1080bp
CTX-M	95ºC/10 min	95ºC/30 sec	55ºC /30 sec	72ºC/30 sec	30	72ºC /5 min	550bp
OXA	94ºC/10 min	94ºC/1 m	56 ºC/ 1 min	72ºC/30 sec	35	72ºC/10 min	591bp
SHV	94ºC/10 min	94ºC/30 sec	50ºC/30 sec	72ºC/1 min	30	72ºC /10 min	795bp
Plasmid mediated quinolones							
QnrA	94ºC/10 min	94ºC /1 min	54ºC /2 min	72ºC/1.5 min	35	72ºC /7 min	626bp
QnrB	94ºC/10 min	94ºC /1 min	54ºC /2 min	72ºC/1.5 min	35	72ºC /7 min	495bp
QnrS	94ºC/10 min	94ºC /1 min	54ºC /2 min	72ºC/1.5 min	35	72ºC /7 min	565bp
Salmonella genus	95ºC/2 min	95ºC/1 min	57ºC/1 min	72ºC/2 min	35	72ºC /5 min	204bp
Salmonella* serotype *Enteritidis	95ºC/2 min	95ºC /1 min	57ºC /1 min	72ºC /2 min	35	72ºC /5 min	304bp
Salmonella* serotype *Typhimurium	95ºC/2 min	95ºC /1 min	57ºC /1 min	72ºC /2 min	35	72ºC /5 min	401bp

Table (3): Illustrated and serotyping of isolated *Salmonella spp.* from examined samples

Serotypes	Fresh poultry meat	Frozen poultry meat	Fresh poultry organs	Fresh beef meat	Frozen beef meat	Fresh beef organs	Total		
							No	% of *Salmonella* isolates	% of total isolate
S. typhimurium	2	0	1	3	1	2	9	60	5
S. paratyphi A	0	1	1	0	0	1	3	20	1.67
S. enteritidis	1	0	0	1	0	0	2	13.3	1.11
S. kentucky	1	0	0	0	0	0	1	6.7	0.56
Total	**4**	**1**	**2**	**4**	**1**	**3**	**15**	**100**	**8.3**

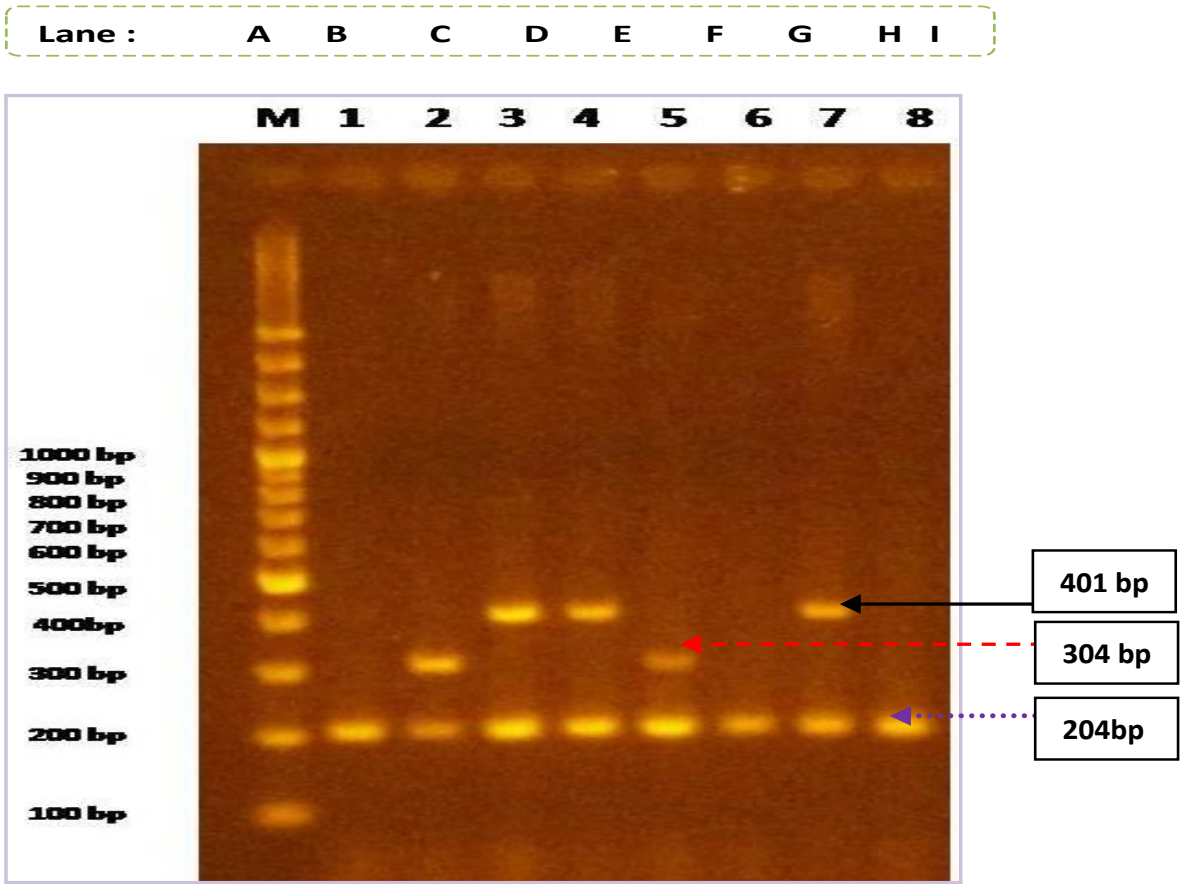
Table (4): Results of antimicrobial sensitivity tests for *Salmonella spp.*

Number of resistant strains 15 (100%)	**Antimicrobial class**	**Antimicrobial agent(s) tested**
1 (6.6%)	Fluoroquinolones	**ENR**
4 (26.6%)	Aminoglycosides	**S**
11 (73.3%)	Cephalosporins	**CRO**
9 (60%)	Cephalosporins	**CPD**
10 (66.7%)	Cephalosporins	**CAZ**
12 (80%)	Penicillins	**AMP**
0	Phenicols	**C**
12 (80%)	Cephalosporins	**CTX**
6 (40%)	Tetracycline	**TE**
0	Fluoroquinolones	**CIP**
5 (33.3%)	Quinolones	**NA**
8 (53.3%)	β-Lactamase inhibitors	**AMC**

Table (5): Resistance phenotype and incidence of resistance genes in *Salmonella spp.*

Sample no.	**Isolate no.**	**Source**	**Serotype**	**Resistance phenotype**	**Resistance genes**
15	1	Fresh poultry meat	*S. kentucky*	S, AMP, TE,NA, AMC	
32	2	Fresh beef meat	*S. enteritidis*	S,CRO, CPD, CAZ, AMP, CTX, TE, NA	bla_{TEM}, bla_{CMY}, bla_{CTX-M}
44	3	Fresh poultry organs (liver)	*S. typhimurium*	CRO, CPD, AMP, CTX, NA, AMC	bla_{TEM}, bla_{CTX-M}
69	4	Fresh beef meat	*S. typhimurium*	CRO, CAZ, AMP, CTX	bla_{CTX-M}
73	5	Fresh poultry meat	*S. enteritidis*	CRO, CAZ, AMP, CTX, TE	bla_{CTX-M}
85	6	Frozen poultry meat	*S. paratyphi A*	CRO, CPD, CAZ, AMP, CTX, AMC	bla_{TEM}, bla_{CTX-M}
100	7	Fresh beef meat	*S. typhimurium*	CAZ, AMP, CTX	bla_{CTX-M}
115	8	Fresh beef organs (liver)	*S. paratyphi A*	CRO, CPD, CAZ, CTX, AMC	bla_{CTX-M}
124	9	Frozen beef meat	*S. typhimurium*	ENR, S, CRO, CPD, CAZ, AMP, CTX, TE, NA, AMC	bla_{TEM}, bla_{CMY}, bla_{CTX-M}
129	10	Fresh beef organs (spleen)	*S. typhimurium*	S, CRO, CPD, AMP, CTX, TE	bla_{TEM}
141	11	Fresh poultry organs (liver)	*S. paratyphi A*	CRO, CPD, CAZ, AMP, CTX, AMC	bla_{CTX-M}
157	12	Fresh poultry meat	*S. typhimurium*	CRO, CPD, CAZ, CTX, NA	bla_{CTX-M}
162	13	Fresh beef organs (liver)	*S. typhimurium*	AMP,AMC	

169	14	Fresh poultry meat	*S. typhimurium*	AMP	
173	15	Fresh beef meat	*S. typhimurium*	CRO,CPD,CAZ,CTX,TE,AMC	bla_{TEM},bla_{CTX-M}



Figure(1): Gel electrophoresis photograph showing Identification of ***Salmonella*** serotypes by using multiplex PCR: lane A:(**M**)100 bp ladder, lanes B, G and I: Samples 1, 6 and 8 were positive for ***Salmonella spp.*** other than ***S. enteritidis*** and ***S. typhimurium***, lanes C and F: Samples 2, 5 were positive for ***S. enteritidis*** (target size 304 bp), lanes D, E and H: Samples 3, 4, 7 were positive for ***S. typhimurium*** (target size 401 bp).

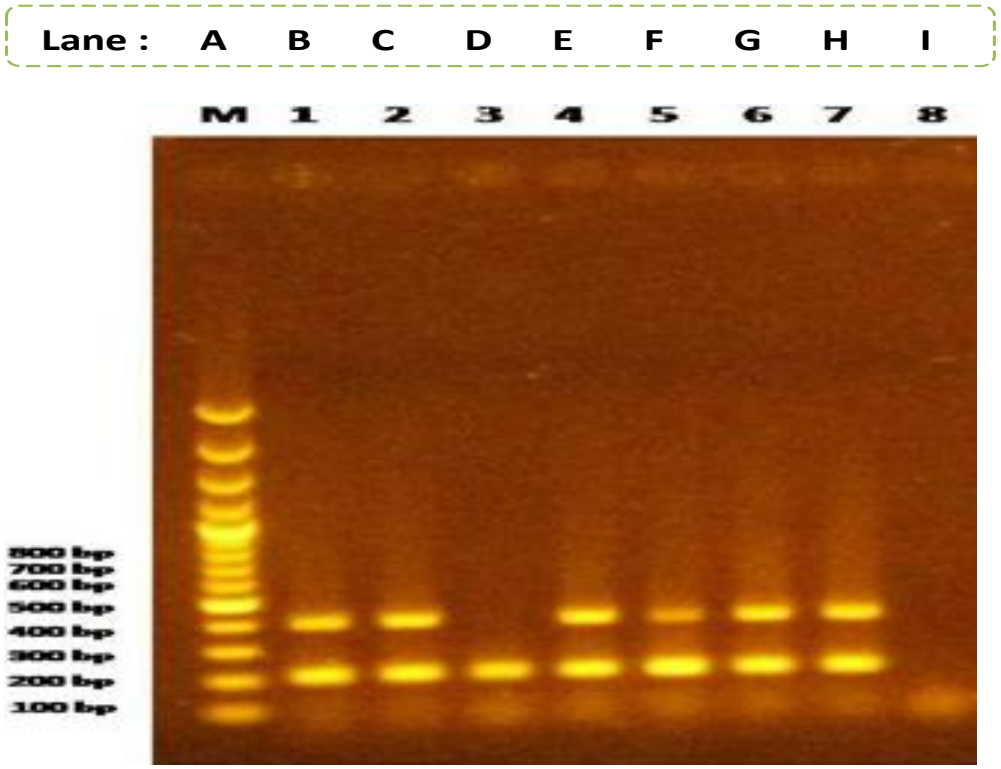


Figure (2): Gel electrophoresis photograph showing Identification of ***Salmonella*** serotypes by using multiplex PCR: lane A: (**M**)100bp ladder, lanes B, D, E, F, G and I: Samples 1, 2, 4, 5,6 and 7were positive for ***S. typhimurium*** (target size 401 bp).lane D: sample 3 was positive ***Salmonella spp***. other than ***S. enteritidis*** and ***S. typhimurium***.

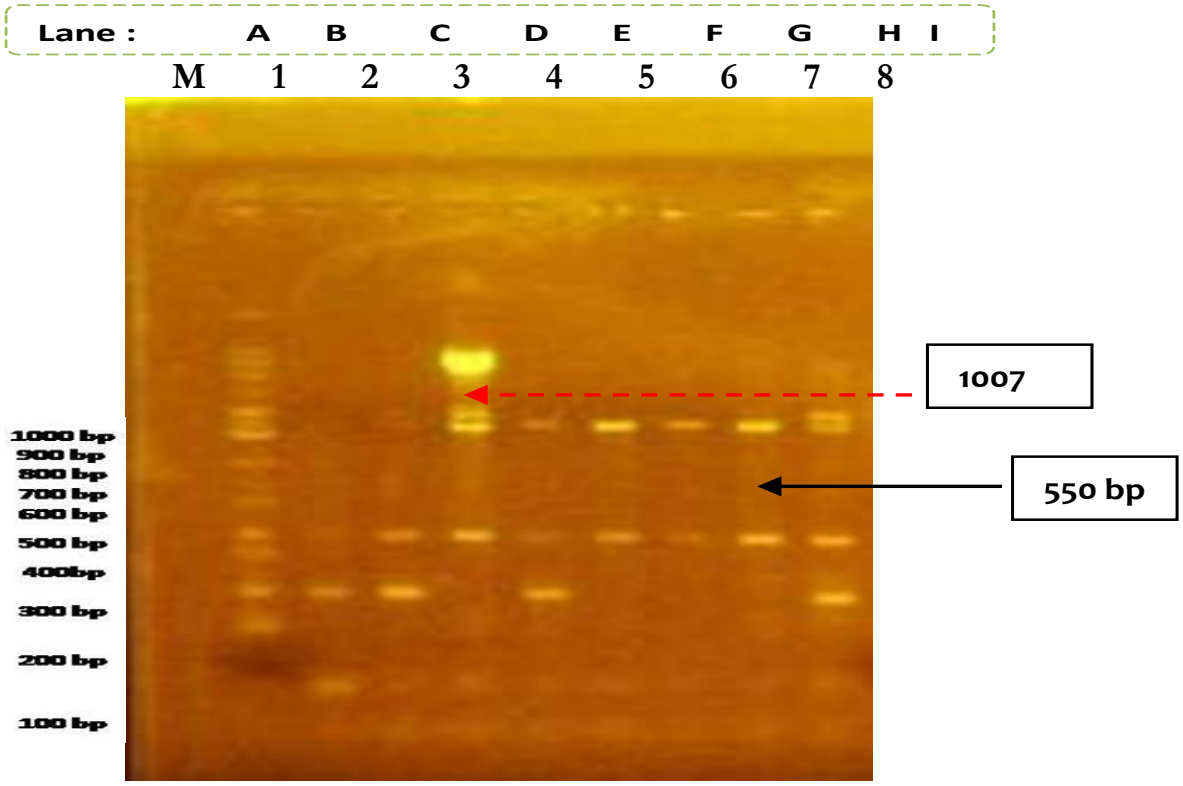


Figure (3): Gel electrophoresis photograph showing Identification of β-Lactamase resistance genes : lane A: (**M**)100bp ladder, lane D: Sample 3 was positive for bla_{CMY} (target size 1007 bp).lanes D, E, F, G, H and I sample 3, 4, 5, 6, 7, 8 were positive for bla_{CTX-M} (target size 550 bp).

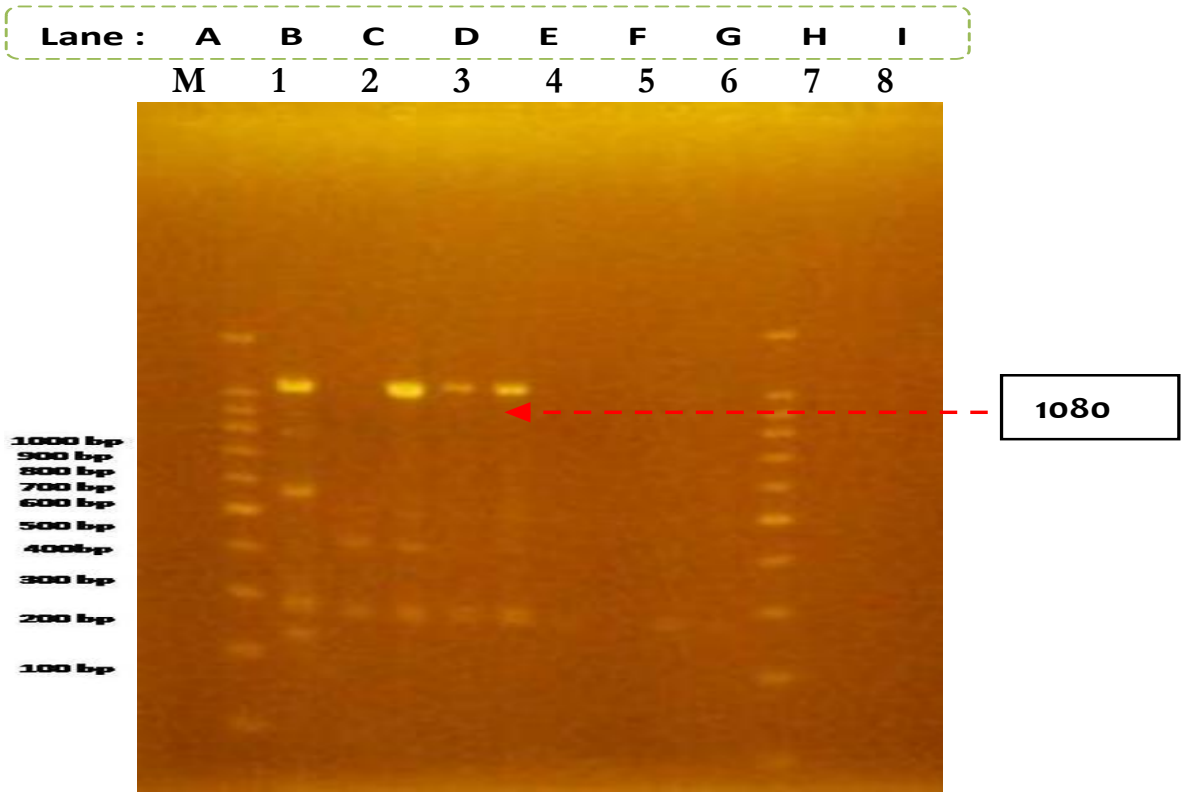


Figure (4): Gel electrophoresis photograph showing Identification of β-Lactamase resistance genes : lane A: (**M**)100bp ladder, lanes B, D, E and F: Samples 1, 3, 4 and 5 positive for bla_{TEM} (target size 1080 bp), while all samples are negative for bla_{SHV}.

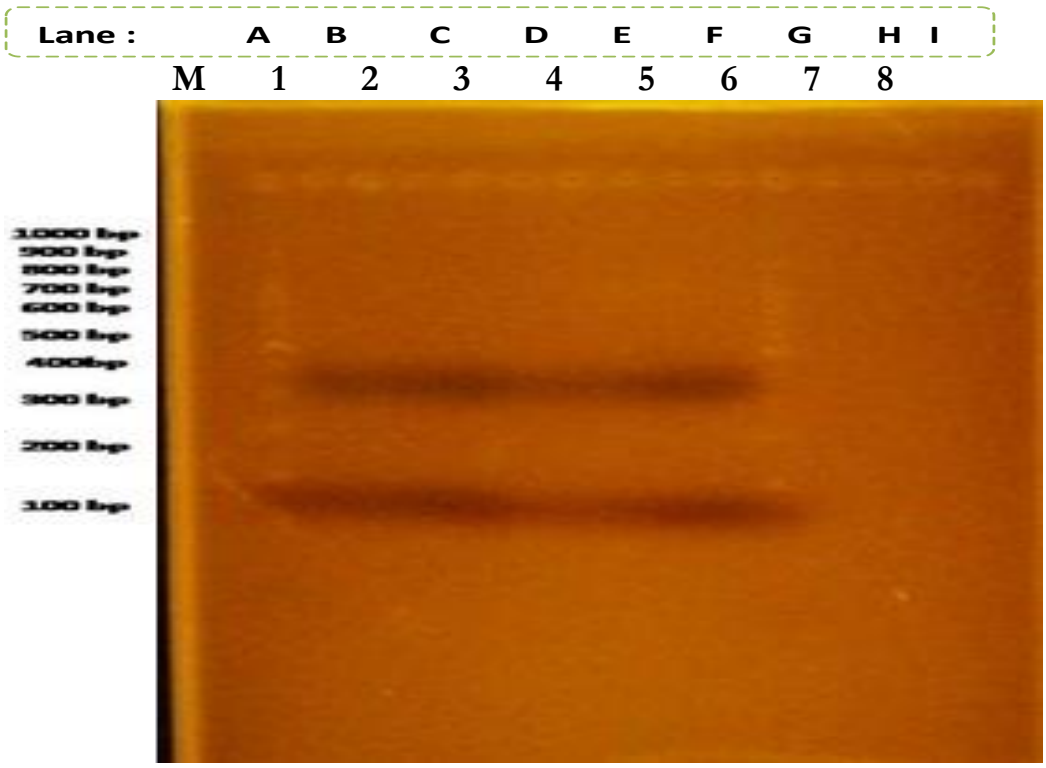


Figure (5): Gel electrophoresis photograph showing Identification of β-Lactamase resistance genes : lane A: (**M**)100bp ladder, all samples are negative for $bla_{OXA.}$

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