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DETECTION AND IDENTIFICATION OF *SALMONELLA* ISOLATED FROM CHICKENS BY POLYMERASE CHAIN REACTION (PCR)

(With 2 Tables and 9 Figures)

By

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الكشف والتعرف على معزولات السالمونيلا من الدجاج بإستخدام إختبار البلمرة

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يعتبر ميكروب السالمونيلا من الميكروبات الخطيرة لأنة أحد مسببات التسمم الغذائى للانسان والحيوان والطيور. أجريت هذه الدراسة لمعرفة نسبة تواجد السالمونيلا فى الدجاج فى محافظة أسيوط، وكذلك الأختلافات الجينية بين المعزولات المختلفة باستخدام اختبار البلمرة. وقد شملت الدراسة التعرف على التغيرات الباثولوجية التى حدثت على الانسجة المختلفة فى الدجاج المعدى صناعيا. تم فحص ٢٠٠ عينة من الأعضاء الداخلية للدجاج، كما تم عمل أختبار حساسية لمعرفة أفضل علاج للميكروب. أوضحت نتائج العزل البكتيريولوجى وجود عدد ٢٨ عترة من ميكروب السالمونيلا بنسبة ١٤٪. وللتفريق بين أنواع السالمونيلا المعزولة أستخدمت تقنية تفاعل البلمرة المتضاعف من النوع المتعدد m-PCR بالأستعانة ببادئات خاصة للاكتشاف النوعى للأنتيجين الجسدى ٠والسوطى لميكروب السالمونيلا. أظهرت النتائج وجود السالمونيلا تيفيميوريوم بنسبة ٥٧,١٤% والسالمونيلا أنتريديس بنسبة ٤٢,٨٦%. وعن طريق أختبار الحساسية تم معرفة أن الميكروب حساس للينكوسباكتين، كلورافنيكول، أريثرومايثين، سيبروفلوكساسين، ديوكسسيكلين، أسبكتينوميسين، كولستين، امبسلين، اموكسيسلين، حمض الكلافيولنيك، والجنتاميسين. كما أظهرت الدراسة الباثولوجية وجود سمك فى جدار الأوعية الدموية وتورم فى الرئتين. هذا بالإضافة إلى وجود إرتشاح من الخلايا وحيدة النواة فى العضلات القلبية، أيضا تورم وإرتشاح خلوى بالكبد.

SUMMARY

The occurrence and identification of *Salmonella* spp. using a multiplex PCR (m-PCR) in chickens, as well as histopathological changes in

experimentally infected chickens and antibiotic sensitivity test were studied. 200 samples (intestine, liver) were collected from freshly dead bird for bacteriological examination. 28 *Salmonella* isolates were found in chicken samples with overall percentage 14%, by conventional culturing and biochemical reactions. Seven representing isolates were confirmed by a multiplex-PCR (m-PCR) using the three primers pair (ST11-F, ST15-R), (S1-F, S4-R) and Fli15-F, Fli15-R for identification of the most frequent *Salmonella enterica* serovars. Our results revealed that isolation rate of serovar Enteritidis was 57.14% (16/28) and 42.86% (12/28) were identified as serovar Typhimurium. Histopathologically of the experimentally infected chicken showed thickening of the alveolar wall, blood vessel wall and edema in the lung. Moreover, mononuclear cell infiltration in cardiac muscles and hepatic edema with heterophil cells infiltration in infected liver was also seen. The current study demonstrated that all of 28 *Salmonella* strains were susceptible to lincospectin, chloramphenicol, erythromycin, ciprofloxacin, doxycyclin, spectinomycin, colistin but were resistant to ampicillin, amoxicillin/clavulanic acid and gentamycin.

Key words: *Salmonella, Chickens, Multiplex-PCR, Antibiotic Resistance.*

INTRODUCTION

Salmonella enterica is one of the most important leading pathogens of food-borne illness throughout the world that pose a significant health hazard to human. Infected poultry are the most frequently incriminated reservoirs of *Salmonellae* that can be transmitted through the food to human (Clavijo *et al.*, 2006; Humphrey, 2006). Food-borne diseases caused by *Salmonella* serotypes occur at high frequency in industrialized nations and developing countries and represent an important public health problem worldwide (White *et al.*, 2001; Lampel *et al.*, 2000). *Salmonella enterica* subsp. *enterica* serovar Typhimurium are the major dominating serotypes of *Salmonella* in poultry and poultry product (Young, *et al.*, 2003; Gürakan *et al.*, 2008). *Salmonella* spp. is one of the most important pathogens responsible for gastrointestinal infections in human, poultry and its derivatives being one of the best known sources of contamination. An increase of strains showing individual and multiple resistance against different antibiotics have been found from isolates from pigs, poultry, and cattle in recent years (Esaki *et al.*, 2004). Standard culture methods for detecting *Salmonella* spp. in poultry include non-selective pre-enrichment

followed by selective pre-enrichment followed by selective and differential agars (Whyte *et al.*, 2002). These methods take approximately 4-7 days (Harvey and Price, 1979; Perales and Audicana, 1989). Since *Salmonella* is closely related to both public and animal health, more rapid and sensitive methods for the identification of this bacterium are required (Schrank *et al.*, 2001). Several alternative, faster methods for the detection of *Salmonella* have been developed, the use of the polymerase chain reaction (PCR) being one of the most promising approaches (Candrian, 1995; Scheu *et al.*, 1998). Rapid identification methods are based on genomic amplification techniques using distinct target DNA sequences determined by PCR. Recently, specific identification of *S.enterica* serovars Typhi and Paratyphi A by multiplex PCR, which detects *rfbE, rfbS, viaB*, and *fliC* genes, has been reported, and this method correctly identify *S. enterica* serovars Typhi and Paratyphi A and differentiate these from other *Salmonella* serovars that have similar antigenic structures (Hirose *et al.*, 2002; Young *et al.*, 2003).

The aim of this study:

1- Determination of prevalence rate of *Salmonella* infection among broiler chickens in Assiut.
2- Using a multiplex PCR method for *Salmonella enterica* serovars identification.
3- Histopathological changes in intestine and liver post infection with *Salmonella*.
4- Antibiotic sensitivity test to select the best treatment of *Salmonella* infections in chickens.

MATERIALS and METHODS

Sample collections:

Two hundreds samples from internal organs (intestine and liver) were collected from diseased broiler chickens of different farms that were necropsied at Department of Poultry Diseases, Faculty of Vet. Med., Assiut University from different localities in Assiut province. The selected birds showed diarrhea, vent pasting and stunting in forty five days chickens. The post mortem examination of all the cases was performed for the all dead birds. At necropsy, gross lesions were recorded carefully, and representative tissue samples from clinically diseased birds and experimental infected birds containing lesions were fixed in 10% neutral buffered formalin for histopathological studies.

Culture procedure:

Samples were aseptically cultured into selenite F broth (Oxoid) and incubate at 41°C for 18-24 hours. Subsequently, a loop full of each broth was streaked on surface of MacConky agar, *Salmonella Shigella* agar (S.S. agar) and Xylose Lysine Desoxycholate agar (X.L.D. agar) for further incubation at 37°C for 24 h (FDA, 1992).

Bacteriological method for *Salmonella* identification:

Salmonella-typical colonies on the plates were confirmed with standard biochemical tests and procedures for *Salmonella* spp. (Rotger and Casadesús, 1999). Presumptive *Salmonella* colonies were kept at -70°C brain heart infusion broth (Difco) with the addition of 20% (v/v) glycerol. An aliquot of this storage solution was taken and incubated in 5 ml phosphate-buffered peptone water for 24 h at 37°C prior to multiplex PCR for further confirmation.

***Salmonella* Molecular identification**

Genomic DNA extraction

The liquid cultures were centrifuged at 8,700 x *g* for 15min, and the cell pellets were re-suspended in 1ml of sterile water. The re-suspended cells were re-centrifuged at 12,500 x*g* for 15min. The pelleted cells were then used for DNA extraction as followed by manufacturer instructions for QIAamp DNA miniprep kit and the concentration was determined using ultraviolet spectrophotometer at A_{260}.

Subtyping of *Salmonella* serotypes using multiplex-PCR (m-PCR):

To develop a multiplex-PCR for *Salmonella* serotypes identification, three pairs of primers were designed from published primer sequences as shown in (Table 1). The multiplex PCR reaction contained 5 µL of template DNA, 0.4 µM of each primer, 100 µM of each deoxynucleotide triphosphate, 1.25 mM of MgCl2 (25 mM), 2.5 U of Taq Polymerase, 5 µL of 10X PCR Buffer (Promega Corp., MI, USA), and water to bring the final reaction volume to 50 µL.

PCR was performed in a Techne Cyclogene PCR System thermocycler. The temperature program started with a PCR amplification was as follows: one initial denaturation cycle at 95 ◦C for 5 min, followed by 30 cycles of 95 ◦C for 40 s, 58 ◦C for 20 s and 72 ◦C for 20 s, and one final extension cycle of 72 ◦C for 7 min. Finally, the samples were cooled to 4°C. Fragments were separated in 1% agarose gel by unidirectional electrophoresis and visualized by staining with ethidium bromide. Fragment sizes were determined by comparison with a 1KB plus DNA ladder (Invitrogen).

Histopathology:

The tissues were collected from experimentally infected birds then subjected to fixation in neutral buffered formalin, trimming, washing and dehydration in ascending gr !es of ethanol, clearing in methylbenzoate and embedding in paraffin. 5µm thick sections were cut, stained with hematoxylin and eosin (Habib-ur-Rahman, *et al,* 2003). Sections were examined in the Department of Pathology, Faculty of Veterinary Medicine, Assiut University.

Antibiotic sensitivity test:

Salmonella-typical colonies on the plates were culture into brain heart infusion broth over night at 37°C and culture fluently over the entire surface of nutrient agar (Difco) with sterile cotton swab. Commercial antibiotic disks containing single concentrations of each antibiotic were then placed on to the inoculated plate surface. The zone of growth inhibition around each disk after over night incubation at 37°C, were measured in millimeters. The zone diameter was interpreted using a zone size interpretation chart (Lorian, 1996).The antibiotics and their concentration were as follow. lincospectin, chloramphenicol 30µg, erythromycin 10µg, ciprofloxacin 5µg, doxycyclin 30µg, spectinomycin 10µg, colistin 25µg, enrofloxacin 5µg, ampicillin 10µg, amoxicillin/ calvulanic acid 10µg, and gentamycin 10µg (Quinn *et al.,* 1994).

RESULTS

Isolation of *Salmonella* from the examined samples:

The isolation trials of *Salmonella* from collected samples yielded overall isolation rate 14% (28/200) of the examined birds. Using biochemical reactions the highest isolation rate of the 28 *Salmonella* isolates, 16 (57.14%) were identified as serovar Enteritidis, while 12 (42.86%) were identified as serovar Typhimurium. Of the above mentioned 28 isolates, 7 representing ones were used to confirm identification using multiplex PCR. Of these 7 isolates 4 were identified as serovar Enteritidis, while 3 were identified as serovar Typhimurium (Fig. 1).

Specificity of the multiplex polymerase chain reaction:

For multiplex PCR, 3 primer pairs were used, (ST11-F, ST15-R), (S1-F, S4-R) and (Fli15-F, Fli15-R). The results revealed that no amplification could be observed among non-*Salmonella* strains as a

negative control, but a specific amplification (429bp) could be detected in all *Salmonella* strains for the genus *Salmonella,* whereas (Fli15-F, Fli15-R) primer pair could amplify a 650bp fragment among the tested strains of *S.* Typhimurium only. However, (S1-F, S4-R) primer pair could amplify a 250bp fragment, examined strains of *S.* Enteritidis (Table2).

Pathology:

Gross examination of the infected birds showed marked lesions in many organs. The lung appeared severely congested (Fig.2). The intestinal and cecal walls were congested (Fig.3). Liver was enlarged with dark red discoloration and friable consistency (Fig.4). Hemorrhages were also demonstrated at the junction between proventriculus and gizzard (Fig.5).

The aim of histopathological examination is detect the pathological action of the *Salmonalla* microorganisms on different tissues and organs. Our results revealed severes acute systemic lesions in almost all body parts. H&E stained sections of intestine exhibited mucosal epithelial cell necrosis, the submucosal blood vessels appeared dilated, engorged with blood with edema in their walls. Extravascular red blood cells (RBCS) could be seen mixed with infiltration of a granular mononuclear cells and heterophils in the submucosa. Hepatic lesions were characterized by multifocal necrosis of hepatocytes and vascular congestion (Figs 6 & 7). The lung revealed thickening of the alveolar wall due to massive infiltration with heterophils and lymphocytes. Degenerated alveolar wall were fused forming emphysemas (Fig.8). Cardiac muscles demonstrated heterophilic and mononuclear cell infiltration (Fig.9).

Antibiotic sensitivity test:

All 28 *Salmonella* isolates were susceptible to the antimicrobial effect of lincospectin, chloraphenicol, erythromycin, ciprofloxacin, doxycyclin, spectinomycin, colistin, but they were resistant to ampicillin, amoxicillin/clavulanic acid and gentamycin.

Table 1: Primers used in this study for multiplex-PCR identification of the most frequent *Salmonella*.

Primer	Length (nucleotides)	Primer sequence	Amplicon Size (bp)	Reference
ST11-F (1)	24	GCC AAC CAT TGC TAA ATT GGC GCA	429	Aabo
ST15-R (1)	25	GGT AGA AAT TCC CAG CGG GTA CTG G		*et al.*, 1999
S1-F (2)	20	GCC GTA CAC GAG CTT ATA GA	250	Soumet
S4-R (2)	20	ACC TAC AGG GGC ACA ATA AC		*et al.*, 1999
Fli15-F (3)	22	CGG TGT TGC CCA GGT TGG TAA T	620	Soumet
Fli15-R (3)	16	ACT GGT AAA GAT GGC T		*et al.*, 1999

(1) *Salmonella* sp., (2) *S. entertidis*, (3) *S. typhimuriym*

Table 2: Specificity of the multiplex PCR

Strains	Number of strains	PCR-positive results by m-PCR with amplified products of		
		429 pb	620 pb	250 pb
S. Enteritidis	3	3	0	3
S. Typhimurium	4	4	4	0

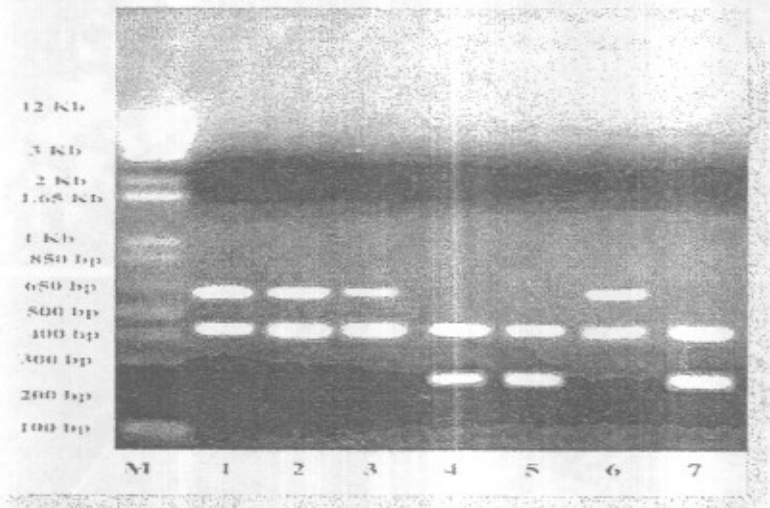
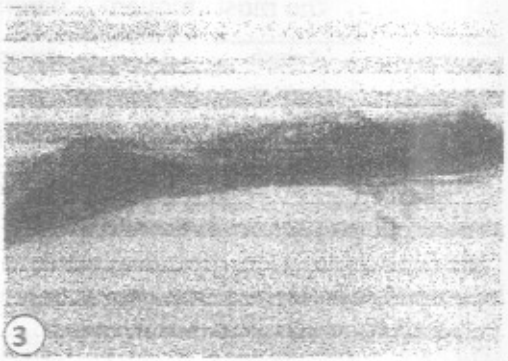


Fig. 1: Mutltiplex PCR amplification from various suspected *Salmonella* strains, *S.* Typhimurium lanes (1,2,3,6) and *S.* Enteritidis lanes (4 and 5), M, 1KB plus DNA ladder (Invitrogen).

2



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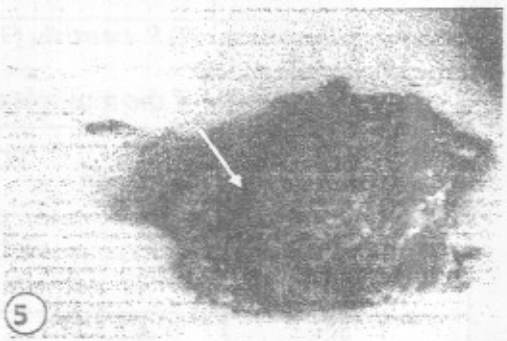

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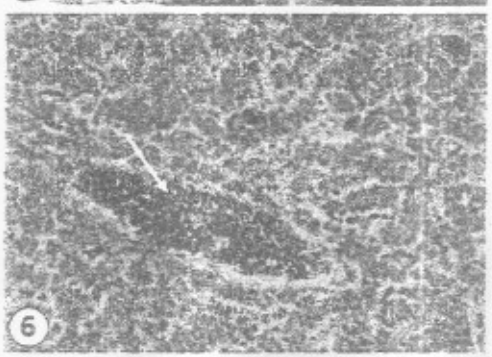

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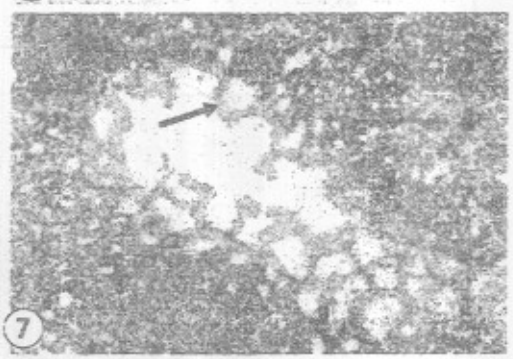

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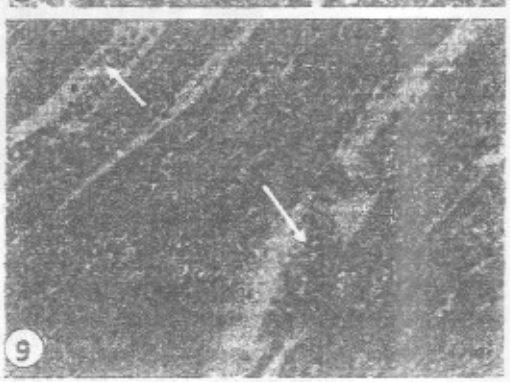

9

LEGENDS

Fig.2: Lungs of infected chickens showing different stages of pneumonia

Fig.3: Intestine of infected chickens showing focal necrotic intestinal lesions.

Fig.4: Liver and heart of infected chicken showing perihepatitis and pericarditis.

Fig.5: Hemorrhages in the junction between proventriculus and gizzard (arrow).

Fig.6: Liver of infected chicken showing congestion and oedema with heterophil cell infiltration.

Fig.7: Lung of infected chicken showing effusion and thickening of the degenerated alveolar wall due to massive heterophilic and mononuclear cell infiltration.

Fig.8: Lung of infected chicken showing septal and prevascular edema.

Fig.9: Cardiac muscles showing heterophilic and mononuclear cell infiltration.

DISCUSSION

Salmonella enterica are recognized as major food-borne pathogens in the world (Gatto *et al.*, 2006). The predominant serotypes change over time and differ from one geographical area to another. All over the world, as well as in our country, the most often isolated serotype is *S.* Enteritidis (Gatto *et al.*, 2006). In this study, the *Salmonella* detection rate was the highest (14%) in chicken carcasses. These results are in general agreement with previous results that *Salmonella* in poultry products were generally low and ranged from 8% in Albania (Beli *et al.*, 2001), 12% in turkey (Ozbey and Ertas, 2006), 23-34% in Belgium (Uyttendaele *et al.*, 1998, 1999), 25% in United Kingdom (Plummer *et al.*, 1995), 26% in Ireland (Duffy *et al.*, 1999), 36% Malaysia (Rusul *et al.*, 1996) to 43% in USA (Bokanyi *et al.*, 1990). However, Antunes *et al.* (2003) mentioned that poultry samples are frequently contaminated with *Salmonella* (60%) which belong to 10 different serotypes. The controversy in the results could be attributed to several factors such as differences in origin, time period and age of the diseased birds, sampling procedure, and differences in methodology

applied to detect the pathogen (Bryan and Doyle, 1995; Uyttendaele *et al.*, 1999).

The isolation trials adopted in the current study yielded that the overall isolation rate was 14% (28/200) of the examined birds. Using biochemical reactions, the serovar Enteritidis was identified from 16/28 with a percentage 57.14%, while 42.86% (12/28) were identified as serovar Typhimurium. These results simulates recent reports from England and Spain that *S.* Enteritidis and *S.* Typhimurium are the most frequent serotype with incidences of 60% and 86%, respectively (Gatto *et al.*, 2006).

A Multiplex PCR-based assay (m-PCR) with three sets of primers was developed for the detection of the most common serotypes of *S.* enterica as *S.* Enteritidis and *S.* Typhimurium. This is a very rapid and simple molecular method for serotyping common *Salmonella*, the specific sequence could be detected in all *Salmonella* enterica serotypes. The time for serotyping is dramatically reduced to only 5 hrs. The method is basic and does not need specialized staff and a large collection of antisera. The assay may be applied in any clinical facility which has PCR and electrophoresis equipment.

In accordance with (Hirose, *et al,* 2002) a multplix PCR gave a positive result for all *Salmonella* strains yielding a specific fragment of 429 bp at the genus level and an additional distinct 250 bp amplified product of *sefA*, which allowed identification of *S.* Enteritidis, whereas an additional 620-bp amplified band was observed only in samples of *S.* Typhimurium serotype. Random amplified polymorphic DNA analysis with primer 3 is of potential use as a serotype-specific marker for *S.* Typhimurium, (Gürakan *et al.*, 2008).

Our result reveals that all the 28 *Salmonella* isolates were susceptible to the antimicrobial effect of lincospectin, chloramphenicol, erythromycin, ciprofloxacin, doxycyclin, spectinomycin, colistin, but they were resistant to ampicillin, amoxicillin/clavulanic acid and gentamycin. This is partially consistent with results of (Zahraei Salehi *et al.*, 2005) that 30 *Salmonella* strains were susceptible to the antimicrobial effect of cefotaxime, tylosin, colistin, ciprofloxacin, enrofloxacin, gentamycin, chloramphenicol, cephalotin and cefotaxime and resistent to trimethoprim, nalidixic acid, flumequine, tetracycline, neomycin, streptomycin, kanamycin and amikacin. However our results disagrees with the findings of Kristiansen *et al.* (2003) that *Salmonella* Typhimurium DT104, is resistant to streptomycin, chloramphenicol, amoxicillin, sulfonamides, and tetracyclines.

Both gross and histopathological lesions of *Salmonella* infections in chicken are a great diagnostic tool besides bacteriological studies (Dhillon *et al.*, 2001). The gross lesions demonstrated in the current study are in a general agreement with those of Talha *et al.* (2001), Habib-ur-Rahman *et al.* (2003) that include hepatic enlargement, hepatic and intestinal congestion besides dark red discoloration and fleshy consistency of the lung. Histopathologically, the present observations support previous studies (Talha *et al.*, 2001; Habib-ur-Rahman *et al.*, 2003; Haider *et al.*, 2004) and are expressed by congestion and hemorrhages with infiltration of mononuclear cells in the intestinal mucosa and submucosa, myocarditis, extravasated blood aggregates and edema. These pathological lesions could be attributed to the direct action of *Salmonella* which is a facultative intracellular microorganism (Buxton and Jean, 1963; Ruby *et al.*, 2003).

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