

STUDIES ON BACTERIA ASSOCIATED WITH DIARRHEA IN BROILER CHICKENS WITH SPECIAL REFERENCE TO SALMONELLA

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ABSTRACT

A total of 750 samples were collected from liver, intestine and coloaca from 250 broiler chickens. 510 samples were collected from Diarrheic chickens and 240 samples from apparently healthy chickens from farms in Elgharbia governorate (Egypt). Samples were examined bacteriologically, seventy four sample (43.5%) were found to be positive for Escherichia coli that identified biochemically, thirty samples (17.6%) were found to be positive for Salmonella species that identified biochemically and seven samples were detected serologically and eleven samples detected by PCR. Twenty three samples (13.5%) were found to be positive for Proteus mirabilis that identified by biochemical and serological tests.

INTRODUCTION

Salmonella enterica serovar Typhimurium, is one of the major most significant infectious bacterial pathogen affecting poultry (*fatma et al., 2012*) and characterized by it's Zoonotic importance (*Moussa et al., 2012*).

Escherichia coli is one of the normal bacterial flora in the gastrionestinal tract of poultry. 10-15% of the intestinal coliforms in chickens are of pathogenic serotypes (*Alimehr et al., 1999*).

The outer membrane siderophore receptor gene *ironN*, which was first reported in *Salmonella enterica* affect the virulence of avian pathogen *Escherichia coli* (**Baumler et al., 1998 and Dozois et al., 2003**).

Proteus isolates and its microbial resistance from poultry were recorded (**Mostafa, 2013**).

Klebsiella organisms are known to play an important role as etiological agents of various diseases in birds and are found to be associated with different diseases as respiratory affections, septicemia, peritonitis, salpingitis, air sacculitis, omphalitis, arthritis, panophthalmitis and intestinal disturbances .such diseases cause great economic losses in poultry industry not only due to high mortality rates in young birds, slow growth and poor food conversion rates in growing birds, but also due to decrease in egg productions and hatchability of the infected eggs, (**Plessner et al., 1975, Reninie et al., 1990 and Mahalingam et al., 1998**).

Pseudomonas aeruginosa is an opportunistic pathogen that can invade fertile eggs causing death of embryos and virulent strains can cause diarrhea dehydration, dyspnea, septicemia, and death to newly hatched chickens (**Hebat – Allah Abd, 2004**).

The use of antimicrobials in poultry production, both to compact diseases as growth promoters, has been associated with the appearance antimicrobial resistance (**Gyles, 2008**).

Due to the significance of *Salmonella* spp. Infection in poultry industry and its zoonotic importance and affecting the virulence of normal flora opportunistic pathogens, Evaluation of enteric microbial

susceptibility of *Salmonella* spp isolated from broiler chickens contributes to its resistance and control. So the aim of the work is:

1. Isolation, biochemical and serological identification of recovered *Salmonella* species.
2. Molecular confirmation of *Salmonella* spp.
3. Antibiogram of isolated *Salmonella* spp.

MATERIAL AND METHODS

Samples collection:

A total of 750 samples from 250 broiler chickens were collected from farms in EL-Gharbia governorate (Egypt), 510 samples from diarrheic chickens. The samples were collected from liver, intestine of dead birds and coloacal swabs from living birds .Samples were collected in sterile buffered peptone water and taken to the laboratory on the day of collection under refrigeration with minimum of delay and incubated at 37°C for 24 hours.

Bacteriological examination:

Isolation of *Salmonella*: (Collee et al., 1996) and (Waltman, 1999).

After prenrichment 0.1 ml of the broth culture was transferred into 10 ml Rappaport vassiliadis broth and incubated at 42°C for 24-48 hours .Rappaport vassiliadis broth samples were streaked onto XLD agar plates and incubated overnight at 37°C .Typical colonies were picked and further investigation by morphological identification using Gram stain and biochemical identification.

Serological typing of *Salmonella* organisms was performed according to (Kauffman, 1974):

DENKA SEIKEN CO.,Japan antisera was obtained from food analysis center,Benha University ,faculty of veterinary medicine.

A- Identification of somatic (O) antigen " Slide agglutination test ".

B- Identification of flagellar (H) antigen " Tube agglutination test ".

In vitro antibiotic sensitivity of *Salmonella* isolates according to National committee for clinical laboratory standards "NCCLS" (2001) and (Srivani, 2011).

Determination of the susceptibility of the isolated *Salmonella* to antibiotic discs was adopted using diffusion break point technique, the discs that used for *Salmonella* were Amoxicillin, Ampicillin, Chloramphenicol, Ciprofloxacin, Erythromycin, Gentamycin, Kanamycin, Nalidixic acid, Neomycin, Norfloxacin, Oxytetracycline, Pencillin, Streptomycin and Salphamethoxazol.

Polymerase chain reaction technique:

DNA of the *Salmonella* was extracted and specific primers for *Salmonella* organism were used (Alvaez et al.,2004).

Sequence of primer:

| Primer | Sequence | Amplicon size (bp) | Target | Reference |
|------------------------------|--|--------------------|-------------------|-----------------------|
| <i>Salmonella</i> serotyping | | | | |
| OMPCF OMPCR | ATCGCTGACTTATGCAATCG CGGTTGCGTTATAGGTCTG | 204 | <i>Salmonella</i> | Alvarez et al. (2004) |
| ENTF ENTR | TGTGTTTTATCTGATGCAAGAGG TGAACACGTTCTGTTCTCTGG | 304 | Entertidis | Alvarez et al. (2004) |
| TYPHF TYPHR | TTGTTCACITTTTACCCTGAA CCCTGACAGCCGTTAGATATT | 401 | Typhimurium | Alvarez et al. (2004) |

DNA amplification:

PCR were performed in a final volume of 25 µl. the optimized PCR mixture consisted of 1.5 mM MgCl₂, 200 µM deoxynucleoside triphosphates, 1 U of Taq polymerase, 60 pmol of 1C DNA per sample and DNA template 5 µl. Distilled water was added to bring the final volume 25 µl.

PCR protocol:

Consisted of the following steps : (i) an initial denaturation step of 2 min at 95°C, (ii) 30 cycles, with 1 cycle consisting of 1 min at 95°C, 1 min at 57°C and 2 min at 72°C and (iii) final elongation step of 5 min at 72°C.

Detection of PCR product:

The PCR products were electrophoresed in 2.5% agarose gel, stained with 2 µg Ethidium bromide and photographed under UV light.

RESULTS

Table (1): Incidence of bacteria isolated from diarrheic chickens

| Enteric bacteria | N. of examined DC | N. of Samples | Numbers and incidences of enteric bacteria | |
|--------------------------|-------------------|---------------|--|----------|
| | | | No | % per DC |
| <i>Escherichia coli</i> | 170 | 510 | 74 | 43.5 |
| <i>Salmonella</i> spp. | 170 | 510 | 30 | 17.6 |
| <i>Proteus mirabilis</i> | 170 | 510 | 23 | 13.5 |

- DC = Diarrheic Chickens.

- AHC = Apparently Healthy Chickens as a control

- Incidence according to number of birds.

Table (2): Results of serological identification of isolated *Salmonella* species.

| Identified Strains | Number | Group | Antigenic Structure | |
|-------------------------------|--------|----------------|---------------------|-------------|
| | | | O | H |
| <i>Salmonella</i> Anatum | 1 | E ₁ | 3, 10, 15, 34 | e, h : 1, 6 |
| <i>Salmonella</i> Typhimurium | 2 | B | 1, 4, 5, 12 | i : 1, 2 |
| <i>Salmonella</i> Dublin | 1 | D ₁ | 1, 9, 12 | g, p : |
| <i>Salmonella</i> Enteritidis | 2 | D ₁ | 1, 9, 12 | g, m : 1, 7 |
| <i>Salmonella</i> Kentucky | 1 | C ₃ | 8, 20 | i : z6 |

Table (3): Results of polymerase chain reaction of the isolated *Salmonella* spp.

| Organism | No. of positive samples | % of positive sample * |
|-------------------------------|-------------------------|------------------------|
| <i>Salmonella</i> | 11/30 | 36.6 |
| <i>Salmonella</i> Enteritidis | 1/30 | 3.33 |
| <i>Salmonella</i> Typhimurium | 2/30 | 6.6 |

* % Calculated from number of isolated *Salmonella* spp. strains

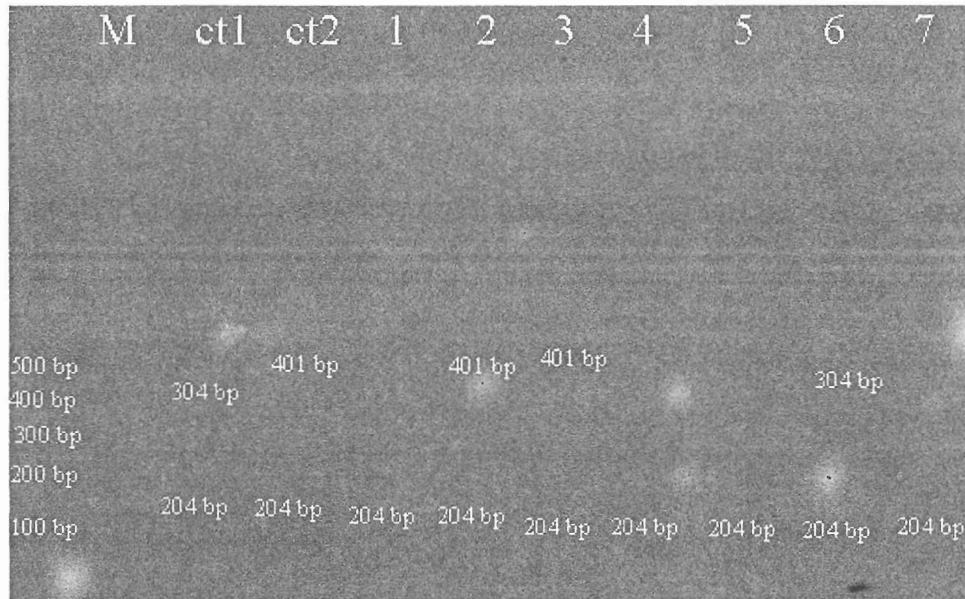


Fig.(1): Multiplex PCR amplification profile, (M=100 bp, ct1 = control positive of *S. Enteritidis* (*Salmonella* spp. 204bp and *S. Enteritidis* 304bp), ct2 = control positive of *Salmonella* spp. 204bp, *S. Typhimurium* 401bp), lane 2 and 3 positive *Salmonella* Typhimurium lane 6 positive *S. Enteritidis*, while lanes 1,4,5 and 7 are positive *Salmonella* species. 204bp)

Table (4): Antibiotic susceptibility of *Salmonella* Anatum, *Salmonella* Dublin, *Salmonella* Kentucky, *Salmonella* Enteritidis and *Salmonella* Typhimurium isolated from diseased chickens to chemotherapeutic agents :

| Antimicrobial agent | Diffusion zone break point (mm) | <i>S.</i> Typhimurium | <i>S.</i> Enteritidis | <i>S.</i> Kentucky | <i>S.</i> Anatum | <i>S.</i> Dublin |
|------------------------|---------------------------------|-----------------------|-----------------------|--------------------|------------------|------------------|
| Amoxicillin(AMX) | ≤ 14 | 8 (R) | 15 (I) | 9 (R) | 12 (R) | 5 (R) |
| Ampicillin (AM) | ≤ 13 | 5 (R) | 11 (R) | 8 (R) | 10 (R) | 5 (R) |
| Chloramphenicol (C) | ≤ 12 | 1 (R) | 6(R) | 4 (R) | 3 (R) | 3 (R) |
| Ciprofloxacin (CP) | ≤ 15 | 9 (R) | 18 (S) | 16 (I) | 19 (S) | 11 (R) |
| Erythromycin (E) | ≤ 13 | 2 (R) | 3 (R) | 5 (R) | 3 (R) | 1 (R) |
| Gentamycin (GM) | ≤ 12 | 16 (S) | 20 (S) | 19 (S) | 13 (I) | 16 (S) |
| Kanamycin (K) | ≤ 13 | 14 (I) | 17 (S) | 7 (R) | 10 (R) | 18 (S) |
| Nalidixic acid (NA) | ≤ 13 | 5 (R) | 14 (I) | 8 (R) | 15 (I) | 9 (R) |
| Neomycin (N) | ≤ 12 | 9 (R) | 9 (R) | 14 (S) | 13 (I) | 7 (R) |
| Norfloxacin (NOR) | ≤ 12 | 13 (I) | 13 (I) | 11 (R) | 16 (S) | 9 (R) |
| Oxytetracycline (T) | ≤ 14 | 6 (R) | 14 (I) | 6 (R) | 15 (I) | 8 (R) |
| Penicillin (P) | ≤ 20 | 7 (R) | 13 (R) | 10 (R) | 9 (R) | 3 (R) |
| Streptomycin (S) | ≤ 11 | 2 (R) | 3 (R) | 5 (R) | 3 (R) | 1 (R) |
| Sulphamethoxazol (SXT) | ≤ 10 | 5 (R) | 6 (R) | 3 (R) | 11 (I) | 2 (R) |

DISCUSSION

Results in table (1) revealed that *Escherichia coli* isolated with an incidence of 43.5% our results partial similar with *Barbour et al. (1985)* and *Cardoso et al. (2006)* reporting incidence 40.4% and 47.1% respectively while higher incidence recovered by *El- Boray and Abo-Taleb (2002)* and *Hany (2013)* with an incidence 74.54% and 58.33% respectively. On the other hand lower incidence recovered by *El Morsi (1998)* with an incidence 20%.

Also in table (1) results revealed that *Salmonella* spp. isolated with an incidence of 17.6% that agree with *Abeer (2004)* and *Kassay et al. (2010)* with an incidence 18.8% and 16.1% while higher incidence was recovered by *Abd El.Galil et al. (1993)* with an incidence 25%. on the other hand lower isolation rate obtained from *walid et al. (2010)* and *Habtamu et al. (2011)* with and incidence 5.6% and 2.7% respectively.

Proteus isolated with an incidence 13.5% and this result was partial similar with *Abeer (2004)* who isolated it with an incidence 14.9%. while higher rate was recovered by *Mohamed (1994)* and *Cardoso et al.(2006)* with an incidence 25.77% and 66.7% respectively.

In table (2) our study found that serological identification of *Salmonella* spp. Showed that 7 isolates serologically typed and 2 isolates belonged to *S. Typhimurium*, 2 isolates belonged to *S. Enteritidis*, 1 isolates belonged to *S. Kentucky*, 1 isolates belonged to *S. Anatum* and 1 isolates belonged to *S. Dublin*. While *Mohamed (2003)* the serological identification of *Salmonella* revealed *S. Typhimurium* and *S. Enteritidis* were the only isolated serovars but in *Rianatou et al. (2006)* serological typing of 21 of *Salmonella* isolates showed that the most prevalent were *S. Kentucky* 30%, *S. Muenster* 13.3%, *S. Brancaster* 8.8% and *S. Enteritidis* and *S. Hader* 6.6%.

Results in table (3) revealed that *Salmonella* examination giving PCR product of 204 bp size and multiplex PCR amplification results using primers specific for *Salmonella* Enteritidis giving PCR product of 304 bp size and *Salmonella* Typhimurium 401 bp size. And this agree with *Alvarez et al. (2004)* and *Hassan (2011)*, and disagree with and *Siddique et al. (2009)* and *Ahmed et al. (2012)* who revealed that *Salmonella* examination giving PCR product of 284 bp size.

In table (4) *S. Typhimurium*, *S. Enteritidis*, *S. Kentucky*, *S. Anatum* and *S. Dublin* isolates were resistant to ampicillin, chloramphenicol, erythromycin, penicillin, streptomycin, sulphamethoxazole except *S. Anatum* is moderately resistant and amoxicillin except *S. Enteritidis* is moderately resistant and sensitive to gentamycin except *S. Anatum* while *S. Enteritidis* and *S. Anatum* are moderately resistant to nalidixic acid, *S. Typhimurium* and *S. Enteritidis* are moderately resistant to norfloxacin and *S. Enteritidis* and *S. Anatum* are moderately resistant to oxytetracycline this results disagree with the results given by *Ludovico et al. (2005)* and *Fernanda et al. (2006)* and partial similar to *Akter et al. (2007)* and *Fatma et al. (2012)*. *S. Enteritidis* sensitive to gentamycin and this agree with *Ahmed et al. (2012)*.

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دراسات على البكتيريا المصاحبة للإسهال في دواجن التسمين مع الاهتمام ببكتريا السالمونيلا

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تم تجميع 750 عينة من الكبد والأمعاء ومسحات من فتحة المجمع منهم 510 عينة من طيور تعاني من الإسهال و240 عينة من طيور سليمة ظاهريا وتم تجميعهم من 250 فرخه تسمين من مزارع بمحافظة الغربية. وتم عمل تشخيص معملى باستخدام الفحص الميكروسكوبى والزرع باستخدام الأوساط البكتيرية المتعددة ، والاختبارات البيوكيميائية والسيرولوجية واستخدام تقنية البى سى آر .

والنتائج أظهرت أن نسبة الإصابة بالإشريشيا كولاي 43.5% ونسبة الإصابة بالسالمونيلا 17.6% ونسبة الإصابة بالبروتيس ميرابيلس 13.5%.

وتم تصنيف عترات السالمونيلا وتبين أنها سالمونيلا تايفيموريم وسالمونيلا انترتيدس وسالمونيلا دابلن وسالمونيلا أناتم وسالمونيلا كينتاكى ولوحظ أن عترات السالمونيلا حساسة لبعض المضادات الحيوية مثل الجنتاميسين ماعدا السالمونيلا كينتاكى أقل حساسية له .

كما أنها مقاومة لبعض المضادات الحيوية مثل أمبسيلين وكلورمفنيكول وإيريثرومايسين وبنسيلين وستريبتومايسين. تم أيضاً إجراء اختبار البى سى آر لبعض عترات السالمونيلا المعزولة للتشخيص السريع والدقيق.