COMPARATIVE DIAGNOSTIC STUDY OF AVIAN SALMONELLOSIS IN SALAHALDEEN PROVINCE

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ABSTRACT

The aims of this study were to detection of dominant Salmonella species that caused poultry infection Salahaddin province , and evaluation local prepared and manufactured serological kits that used in diagnosis of poultry salmonellosis, for this purpose 100 diarrheatic hen checked by culture methods, PCR, ELISA, Whole blood agglutination test and Slide agglutination test. The results showed that Salmonella isolation from hen in rate 34% , intestine is most suitable site for Salmonella isolation and *Salmonella. typhimurium* is most dominant spp. The sensitivity of ELISA test was 76.4%, while for other used tests were 100%. The specificity of ELISA test, Whole blood agglutination test, slide agglutination test for *S. typhimurium* and slide agglutination test for *S enteritidis* were: 80.3%, 86.3%,77.9% and 66.6% respectively.

Key words: *Salmonella. gallinarum* , *Salmonella pullorum*, Whole blood agglutination.

دراسة تشخيصية مقارنة لمرض السالمونيلا عند الدواجن في محافظة صلاح الدين

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المستخلص

هدفت هذه الدراسة الى تحديد النوع السائد لجنس السالمونيلا في الدواجن المصابة بالإسهال في محافظة صلاح الدين وتقييم العدد الجاهزة والمحضرة المستخدمة في التشخيص. ولهذا الغرض تم دراسة حالة 100 دجاجة اختبرت باستخدام الزرع الجرثومي و تفاعل البوليمرات المتسلسل، تفاعل التلازن الدموي، اختبارات التلازن في الشريحة. اظهرت نتائج الدراسة ان نسبة عزل السالمونيلا كانت 34%، وان الامعاء هي افضل مكان يمكن استخدامه للعزل الجرثومي وان نمط *Salmonella. typhimurium* هو الاكثر سيادة مقارنة بالانواع الاخرى. اظهرت الدراسة ان حساسية اختبار الاليزا كانت 76.4% في حين كانت للاختبارات المصلية الاخرى المستخدمة 100%، في حين كان نوعية اختبار الاليز ، واختبار التلازن الدمي، واختبار التلازن في الشريحة (*S. typhimurium*) واختبار التلازن في الشريحة (*S. enteritidis*) 80.3%، 86.3%، 77.9%، 66.6% على التوالي.

الكلمات المفتاحية: السالمونيلا الطيرية، السالمونيلا الفراضية ، اختبار التلازن الدموي

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INTRODUCTION

Salmonella is gram negative bacteria, non capsulated, non sporulated , motile by Peritrichous flagella except *S. gallinarum* and *S. pullorum* (19).The genus Salmonella belonged to Enterobacteriaceae, which have more than 2500 serotypes based on 16SrRNA sequence analysis (11). Salmonellosis is an infectious disease of humans and animals, it occur due to infection by Salmonella and main species of them: *Salmonella enterica*, and *Salmonella. Bongori* (18). Avian can infected by Salmonella vertically and horizontally via contamination food, water and hatching eggs, as well as infection may be occur via birds, rodent, insects, and even infected farm workers (26). Many factors influence with occurrence of avian salmonellosis, include host age, genetic, stress factors from environmental, treatment with antimicrobial and anti-inflammatory drugs and other infections (8). Main clinical signs of Salmonella in poultry were depression, somnolence, weakness, loss of appetite, drooping wings, breathing or gasping, diarrhea and dehydration. In some cases lameness, swelling of joint and blindness may be occur (5).

Salmonellosis diagnosed by:

-Direct staining of samples by gram stain, this methods is not specific because of Salmonella morphology share with other Gram negative bacteria (12).

-Culture methods: it is highly specialized methods but because of interrupted bacterial shedding, it gave false negative result (24).

-Whole blood agglutination test: this test can performed directly in field (18).

-ELISA: This test used for detection of IgG against Salmonella, it is more sensitive than other serological tests (17).

MATERIALS AND METHODS

This study performed in salahaldeen governorate in period from January to march-2018, in multi flocks.

-Sample: 100 diarrheatic hens from flocks contain 50000 hens, from each hen intestine, liver, spleen, and gallbladder were taken for bacterial isolation and blood for serology tests

-Culture methods : all samples cultivation in peptone water (HIMEDIA-INDIA) (pre enrichment medium) incubated in 37^{o}C for 24h, after that sub culturing on Selenite F broths (HIMEDIA-INDIA) as liquid selective enrichment medium and incubation on 43^{o}C for 24h. then sub culturing on selective sold media (Xylose lysine deoxycholate agar, Brilliant green agar and MacConkey agar) and incubation on 37^{o}C for 24h (18). A group of biochemical tests applied according to (20). These tests were used for recognition of isolate in genus and species .Antibiotic sensitivity test: applied according to (2).

-Serotyping of Salmonella isolate: performed by using Salmonella antisera (pro-lab Diagnostics- USA) which consist from two groups: Salmonella Polyvalent Somatic O Antisera and Salmonella Polyvalent flagellar H Antisera. These tests were used for confirmation species of Salmonella.

-PCR test: DNA template Prepared by reactivation of bacteria by culturing in brain-hart infusion broth, then DNA extraction by boiling lysis method and according to (23). Compound of reaction: as in Table 1. Thermo cycler programs as ias in Table 2.

Table 1. Compounds used in preparation of Reaction Mixture

Compounds used in preparation of Reaction Mixture	Volume (microliters)	Reference
Taq PCR Master Mix KIT: Which contain Taq DNA Polymerase (2.5 Unit), PCR Buffer with 3mM MgCL2, 200µMdNTP	25	(Qiagen, Germany).
Forward primer invA1 5'-GTG AAA TTA TCG CCA CGT TCG GGC AA-3'	2 from 100pM Solution	Shanmugasamy *et al*.,2011(22)
Primer Reverse invA13'-TCA TCG CAC CGT CAA AGG AAC C-5'	2 from 100pM Solution	
DNA Template	2	(Qiagen, Germany)
DNA free water	19	(Qiagen, Germany)
Total	50	

Table 2. Thermo cycler programs

Stage	Temperature (c)	Time	No.of cycles
First Denaturation	94	60second	1
Denaturation step	94	60second	
Primer-annealing step	64	30second	35
DNA extension step	72	30second	
Final DNA extension	72	7 mint	1
End Temperature	4		

Serology tests

ELISA: preformed by kit (BIO CHEK-CK218 SE/ST-UK) which detect antibodies to invasive strains of whole Salmonella cell group B and D in chickens and turkey, that include *S. typhimurium, S.heidelberg, S. enteritidis, S.gallinarum and S.pullorum*.

-Whole blood agglutination test: applied by mixing of 0.2 ml of blood with 0.2ml of Salmonella antigen (Nobillis®S antigen, Intervet, Holland). Appear of agglutination within 2mints refers to positive results. This test used for detection of *S. gallinarum* and *S. pullorum.*

-Slide agglutination test (for detection of *S. typhimurium*) preformed in the current study by preparation of somatic and flagellar antigen for *S.typhimurium*. Somatic antigen prepared by using heating 100C and according to (13). While flagellar antigen prepared by using formalin (BDH - England) and according to (4).

- Slide agglutination test (for detection of *S. enteritidis*) preformed in the current study by preparation of whole bacteria antigen by sonication of *S. enteritidis* bacteria for 50 minutes at intervals in a water-cooled sonicator(40 MHZ/second) and according to (15).

statistical analysis:

$$\textbf{Positive agreement} = \frac{N.of\ sampls\ gave\ positive\ results\ in\ first\ test}{N.of\ sample\ gave\ positive\ results\ in\ second\ test} X100$$

$$\textbf{Negative agreement} = \frac{N.of\ sampls\ gavenegative\ results\ in\ first\ test}{N.of\ sample\ gave\ negative\ results\ in\ second\ test} X100 \textbf{ (10).}$$

$$\textbf{Sensitivity} = \frac{True\ positive}{True\ positive+false\ negative} X100$$

$$\textbf{Specificity} = \frac{True\ negative}{True\ negative+false\ positive} X100$$

$$\textbf{positive predictive values} = \frac{True\ positive}{True\ positive+false\ positive} X100$$

$$\textbf{negative predictive values} = \frac{True\ negative}{True\ negative+false\ negative} X100 \textbf{ (7).}$$

RESULTS AND DISCUSSION

According to colony morphology, biochemical tests and result of PCR, Salmonella was isolated from hen in rate 34% (34:100) figure (1) (the case considered as positive case if Salmonella isolated from intestine or liver or spleen or gallbladder).

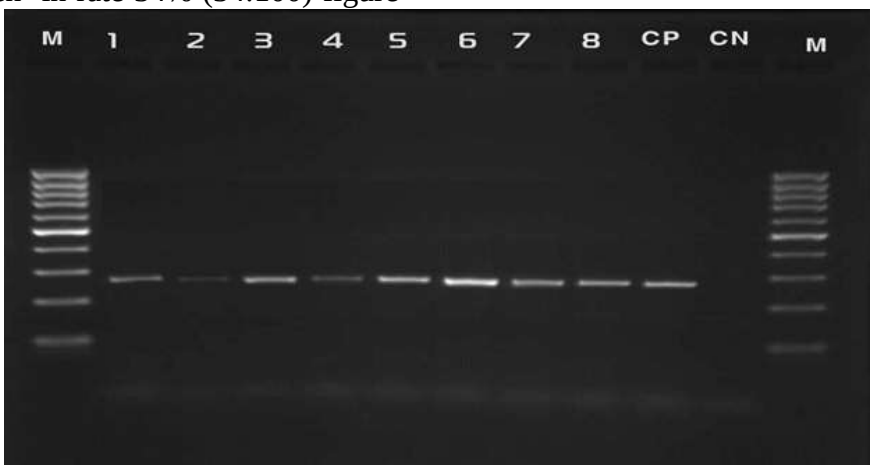

Figure 1: Electrophoresis on 2 % a garose gel and ethidium bromide staining, showing the results of PCR procedures. M: DNA marker, CP control positive, CN: control negative, wells 1-8 positive samples of Salmonella which showed band in size 284 bp.

In the current study Salmonella isolation was in high ratio in compare with other studies(1;15; 19; 21). That's may be due to types of samples, in our study the samples taken from clinically infected hens while other studies applied as survey. In the current study Salmonella isolation rate from intestine, gallbladder, spleen and liver were 91.1%, 55.8%, 32.% and 11.7% respectively. Table 3.

Table 3. Isolation of salmonella according to organ

Organs	Number of Salmonella isolate	rate of Salmonella isolate
Intestine	**31**	**91.1%**
Liver	**19**	**55.8%**
Gallbladder	**11**	**32.%**
Spleen	**4**	**11.7%**

High isolation rate was from intestine and liver in current study is in agreement with Menghistu et al(2011) (15). That s may be due to their pathogenesis pathway. In the first stage of pathogenesis, salmonella invaded mucus membranes and Linning peyer's patches then transmitted by macrophage to vital organs particularly liver (16). According to biochemical tests, and agglutination with antisera four species of Salmonella were isolated in the current study which are: *S. typhimurium*, *S. enteritidis*, *S. gallinarum* and *S. Pullorum*

-*S. typhimurium* appeared as motile, ferment Xylose, Arabinose, Trehalose with acid and gases, and agglutination with antisomatic antibody 1,4,5,12 and antiflagellar antibody i-1,2.

- *S. enteritidis* appeared motile, fermented Xylose, Arabinose, Trehalose and Maltose and produced acid with gases, non ferment Inosito and agglutination with anti somatic antibody 1,9, 12 and anti flagellar g,m

- *S. gallinarum* appeared non motile and fermented Xylose, Arabinose, Trehalose and maltose with out gases, and agglutination with anti somatic antibody 1,9,12

- *S. pullorum* appeared non motile and fermented Xylose, Arabinose, Trehalose with acid and gases, and agglutination with anti somatic antibody 9,12. Table 4. describe isolation rate for each salmonella spp.

Table 4. isolation rate of salmonella spp.

Salmonella spp.	Number of isolate	Rate of isolation
S. typhimurium	**14**	**41.1%**
S. enteritidis	**8**	**23.5%**
S. gallinarum	**7**	**20.5%**
S. Pullorum	**5**	**14.7%**
Total	**34**	**100%**

High incidence of *S. typhimurium* and *S. enteritidis* in current study in compare with other Salmonella spp. This result agrees with the result of other researchers (1). High incidence of *S. gallinarum* in compare with *S. pullorum* agrees with the result of others (19). Dominance of one species isolates upon other species refers to it is disruption, resistant to antibiotics, sensitivity of animals to that's species, types of animals, types of samples and season. In the current study, Salmonella is resistant to antibiotic in different ratios according to type of antibiotic and spp. of Salmonella. As in Table 5.

Table 5. Results of antibiotic resistant

Salmonella Spp / resistant rate	No. of isolate	Antibiotic types: Ciprofloxacin	Nitrofurantion	Tetracycline	Trimethoprim	Streptomycin	Nalidixic acid	Chloramphenicol	Cephalexin
S. typhimurium	**14**	**3 (21.4%)**	**5 (31.2%)**	**8 (57.1%)**	**6 (42.8%)**	**5 (31.2)**	**6 (42.8%)**	**4 (28.5)**	**3 (21.4%)**
S. enteritidis	**8**	**2 (25%)**	**4 (50%)**	**4 (50%)**	**4 (50%)**	**3 (37.5%)**	**6 (75%)**	**2 (25%)**	**2 (25%)**
S. gallinarum	**7**	**0 (0%)**	**3 (42.8%)**	**2 (28.5%)**	**2 (28.5%)**	**3 (42.8%)**	**7 (100%)**	**1 (14.3%)**	**0 (0%)**
S.. Pullorum	**5**	**0 (0%)**	**3 (60%)**	**2 (40%)**	**2 (40%)**	**2 (40%)**	**4 (80%)**	**0 (0%)**	**0 (0%)**

In current study, generally high rate of antibiotic resistant, that's may be due to mass using of antibiotic and genetic transfer of resistant gene between bacteria (6) Result of ELISA test: in compare with culture result, the Sensitivity, specificity, positive predictive values and Negative predictive values of ELISA were: 76.4%, , 80.3% , 66.6% and 86.8%. respectively. As describe in Table 6

Table 6. Compare between ELISA test and bacterial culture

Result of culture test		Result of ELISA test			
		Positive		Negative	
Culture results	No.	No.	Rate	No.	Rate
Positive culture	34	26	76.4%	8	23.5%
Negative culture	66	13	19.6%	53	80.3%
Total results	100	39	39%	61	61%

In the current study positive isolation case gave negative results in ELISA test (effect in sensitivity). That's due to early stage of infection or low efficiency of immune system (14). As well as ELISA kit used in current study detected IgG which appeared after 10 days from infection (20). When compare between whole blood agglutination test (for detection of *S. gallinarum* and *S. Pullorum*) and bacterial culture showed that the sensitivity, specificity, positive predictive values and negative predictive values of whole blood agglutination test were 100%, 86.3%, 75% and 76% respectively. As describe in Table7.

Table 7. Compare between Whole blood agglutination test and bacterial culture

Result of culture test		Result Whole blood agglutination test			
		Positive		Negative	
Culture results	No.	No.	Rate	No.	Rate
Negative culture to Salmonella spp.	66	9	13.6%	57	86.3%
Positive culture to *S. gallinarum* and *S. Pullorum*	12	12	100%	0	0%
Positive culture to *S. typhimurium* and *S. enteritidis*	22	4	18.1%	18	81.8%
Total positive culture to Salmonella spp	34	16	47.0%	18	52.9%

In compare between slide agglutination test (for detection *S. typhimurium*) and bacterial culture showed that the sensitivity, specificity, positive predictive values and negative predictive values of slide agglutination test were 100%, 77.9%, 73.6%,100% respectively. As describe in Table 8.

Table 8. comparison between slide agglutination test and positive bacterial culture for *S. typhimurium*

Result of culture test		Result slide agglutination test			
		Positive		Negative	
Culture results	No.	No.	Rate	No.	Rate
Negative culture to Salmonella spp.	66	13	19.6%	53	80.3%
Positive culture to *S. typhimurium*	14	14	100%	0	0%
Positive culture to *S. gallinarum , S. Pullorum S. enteritidis*	20	5	25%	15	755
Total positive culture to Salmonella spp	34	19	55.8%	68	44.2%

In compare between Slide agglutination test (for detection of *S enteritidis*) and bacterial culture showed that the sensitivity, specificity, positive predictive values and negative predictive values of Slide agglutination test were 100%, 82.5%, 66.6% and 100% respectively. As describe in Table 9.

Table 9. compare between Slide agglutination test and positive bacterial culture for *S. enteritidis*

Result of culture test		Result Slide agglutination test			
		Positive		Negative	
Culture results	No.	No.	Rate	No.	Rate
Negative culture to Salmonella spp.	66	8	12.1%	58	87.8%
Positive culture to *S. enteritidis*	8	8	100%	0	0%
Positive culture to *S.gallinarum, S.Pullorum S.typhimurium*	26	4	15.3%	22	84.6%
Total positive culture to Salmonella spp	34	12	35.2%	22	72%1

Appearance of positive result in serology tests that showed negative results in culture is due to interval bacterial shedding, or low number of bacteria, or treated with antibiotic(2). There are many factors lead to false positive results in serology test (low specificity) which due to cross reaction with other similar bacteria, vaccination, carrier birds and endemic area(2). In current study showed difference in sensitivity and specificity of serological tests. That's may be due to types of antigen used in serological test (whole cell or parts of bacteria). The low specificity of slide agglutination tests is due to cross reaction between salmonella with other bacteria particularly Enterobacteriaceae (2).

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