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ISOLATION AND IDENTIFICATION OF *SALMONELLA SPP.* FROM POULTRY FARMS BY USING DIFFERENT TECHNIQUES AND EVALUATION OF THEIR ANTIMICROBIAL SUSCEPTIBILITIES

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ABSTRACT

This study aimed to isolate and identify *Salmonella spp.* from various sources of poultry farms by using four different techniques (conventional biochemical tests, API 20E system, serology and polymerase chain reaction) the total number of isolates was 44(9%). The Salmonellae including 4 species, *S. gallinarum* 9(1.85%), *S. typhimurium* 7(1.44%), *S. newport* 21(4.3%) and *S. ohio* 1(0.21%). The highest isolation rate was in first week of chicks life 18(25.7%), however, the highest isolation rate of salmonella was from liver 13(28.8%). There are similarity in identification rate of *Salmonella spp.* between API 20 E system and PCR assay using *flic* gene. In this study using PCR amplification of *rfbsg* and *rfbsp* genes in differentiation of *Salmonella serovar gallinarum* into *S. gallinarum* and *S. pullorum* biovars very useful. Results of antimicrobial susceptibility revealed high resistance of isolates against seven antibiotics arranged in descending from high to low resistance (Azithromycin, Florfenicol, Trimethoprime-sulphamethaxezole, Tetracycline, Ciprofloxacin, Ampicillin and Gentamycin).

INTRODUCTION

Meat of poultry is an important food product and the broiler chicken-related industry is an economically important component of the agro-industry (1). *Salmonella* species have been considered as one of the most important foodborne pathogens all around the world (2). Meat and poultry products are recognized as the major sources for transmitting *Salmonella* species to human with 40 % of the clinical cases attributed to the consumption of egg and poultry products (3, 4).

Salmonellae are gram negative, non-lactose fermenting and non-sporing bacteria. With exception of *Salmonella pullorum* and *Salmonella gallinarum*, all Salmonellae are actively motile (5). Identification of *Salmonella spp.* can be performed via both

serotyping and molecular methods. Serotyping offers a reliable method for differentiating *Salmonella* strains, but this procedure is time-consuming. However, molecular methods are fast, as well as highly sensitive and very specific(6). Most *Salmonella* strains have structural gene *flic* that encode flagellins. Non-motile strains generally exhibit these structural genes, but are unable to build up a functional flagellum (7). The primers for allele-specific PCR amplification of *rfbsg* and *rfbsp* genes were based on *rfbS*, these primers used to differentiation of *Salmonella gallinarum* from *Salmonella pullorum* (8).

This study aimed to: 1) isolation and identification of *salmonella spp.* by using four methods conventional biochemical tests, API 20 E system, serotyping and molecular analysis; 2) antibiotic susceptibility of *salmonella spp.* against seven antimicrobials commonly used in poultry production plants.

MATERIAL AND METHODS

-Sample collection

For isolation of *Salmonella spp.* different samples were taken from three poultry flocks in AL- Mudinah district in Basrah governorate, a total of 485 samples were collected as in the following table:

Table (1): type and number of samples and age of broiler chicken.

Specimen	Type of sample	No. of sample	Chicken age
Dead chicken	Liver tissue	45	1-4days
	Yolk sack	45	1-4days
	Caecal content	45	1-4days
Live chicken	Cloacal swab pre-treatment	100	1-4days
	Cloacal swab after treatment	100	24days
egg	Egg shell	30	Before incubation
	Egg content	30	
Ration	From fodder	30	1-30 days
Litter	Saw dust with feces	30	1-30 days
Water	From watering place	30	1-30 days
Total		485	

-Isolation and identification of *Salmonella spp.*

Cultural methods for detection of Salmonellae involve a nonselective pre-enrichment, followed by selective enrichment and plating onto selective and differential agars. Suspect colonies are confirmed biochemically, serologically and by Polymerase chain reaction (9). One gram of solid material was added to 9 ml of nutrient broth, whereas, swabs were inoculated into 10ml nutrient broth and incubated at 37◦C for 18 h (10,11). The swabs of egg shell and contents were prepared according to (12). After pre-enrichment, 1 ml of enriched cultures of all sample types were transferred to 9 ml of Selenite F broth and incubated at 37°C for 18 h.

A loop-full of culture from Selenite F broth was streaked into plates of XLD, The plates were incubated at 37°C for 18 h and checked for growth of typical colonies of *Salmonella Spp*. (13).

Conventional biochemical methods including; urase test, triple sugar iron (TSI) slant reaction, lysine decarboxylase test, ornithin decarboxylase test, indole test, Citrate utilization test using Simmon's citrate agar, motility test and carbohydrates fermentation tests(xylose, lactose, sucrose, arabinose, trehalose, rhamnose), all these tests were done according to (14).

Suspected colonies on XLD agar were further identified by using API 20E test kit (bioMérieux, Inc., France), the plastic strips holding twenty mini-test tubes were inoculated with the saline suspensions of the cultures according to manufacturer's directions. After incubation in a humidity chamber for 18 hours at 37°C, the color reactions were read (some with the aid of added reagents as supplied by the kit). The data were analyzed by the manufacturer's keys and positive results with ≥89% probabilities were confirmed as *Salmonella*.

Serological identification of isolates was done in the Department of Microbiology, Central Public Health Laboratory in Baghdad, according to method described by (15).

Bacterial DNA was extracted according to manufacture of bacterial extraction kit (Genaid,korea). Conditions of PCR for *rfbsg* and *rfbsp* genes amplification of *Salmonella gallinarum* and *Salmonella pullorum* was done according to (8) and for *flic* according to (7) .

Table (2); oligonucleotide primers for PCR amplification.

Primer		Sequence (5´→ 3´)	product (bp)	Reference
Flic	F	CTGGTGATGACGGTAATGGT	197	(7)
	R	CAGAAAGTTTCGCACTCTCG		
rfbsp	F	GTA TGG TTA TTA GAC GTT GTT		
	R	TAT TCA CGA ATT GAT ATA TCC	187	(8)
rfbsg	F	GTA TGG TTA TTA GAC GTT GTT		
	R	TAT TCA CGA ATT GAT ATA CTC	187	(8)

-Determination of the antibiotic susceptibility of isolates

All the isolates that were identified as *Salmonella spp*. by serotyping were tested for antibiotic susceptibility using Kirby-Bauer disc diffusion assay (16). The antibiotic tested were from (Bioanalyse/ Turkey), including; Azithromycin (15µg), Trimethoprime + sulphamethoxazole (25 µg), Gentamycin (10 µg), Ciprofloxacin (5 µg), Florfenicol (30 µg), Ampicillin (10 µg) and Tetracycline (30 µg).

-Statistical analysis

All statistical calculation were carried out with the statistical package MINITAB V. 16.

RESULTS AND DISCUSSION

Results of this study Tables (3&4) indicate that, the total number of *Salmonella spp*. identified by conventional techniques was 51/485 (10.5%), by API 20E system was 44/485 (9%), by serotyping was 38/485(7.8%) and by molecular identification was 44/485 (9%) figure (1-B), these results are in agreement with (10) in Basrah city who found that the overall presence of *Salmonella spp*. was 9.2%. Moreover, (17) found that, the prevalence of *Salmonella spp*. in south of Iran was (8%) and in west of Iran was (9.4%).

Table (3): Isolation rate of *Salmonella Spp*. according to age of chickens by conventional biochemical tests, API 20 E, Serotyping and PCR (*flic*).

Age/ day	No. of examined samples	No. of positive isolates identified by conventional biochemical tests		No of isolates identified by API 20E system		No of positive isolates identified by serology		No of positive isolates identified by PCR- *flic* gene	
		NO.	%	NO	%	NO	%	NO	%
1	54	4	7.4	4	7.4	3	5.5	3	5.5
2	60	7	11.6	7	11.6	5	8.3	7	11.6
3	65	7	10.7	6	9.7	6	9.2	7	10.7
4	70	19	27.1	19	27.1	16	22.5	18	25.7
6	24	6	25	3	12.5	3	12.5	4	16.6
7	29	5	17.2	2	6.8	2	6.8	2	6.8
14	10	0	0.0	0	0.0	0	0.0	0	0.0
24	80	3	3.7	3	3.7	3	3.7	3	3.7
28	33	0	0.0	0	0.0	0	0.0	0	0.0
Total	485	51	10.5	44	9.0	38	7.8	44	9.0

Note: the rate of each number obtained by dividing the number on total number of raw.

$P < 0.01$

The highest isolation rate in first week 25.7% (fourth day) table (3), this result is in accordance with that of (18, 19 & 20) who concluded that, the highest isolation rates were in one-week-old of chicks.

Table (4) display that, the highest isolation rate of Salmonella spp. was from the liver (28.8%) this result is in agreement with results of (23 & 24). Salmonellae are intracellular facultative organisms (21), also (22) mention that, Salmonella organism localize in the visceral organs such as liver, spleen, ovaries, kidneys, heart , lungs etc.

Table (4): isolation rate of *Salmonella Spp.* from different sources according to conventional biochemical tests, API 20 E, Serotyping and PCR (*flic*).

Source of samples	No. of examined sample	No. of positive isolates identified by conventional biochemical tests		No of positive samples identified by API 20 E		No of positive isolates identified by serology		No of positive isolates identified by PCR- *flic* gene	
		No	%	No	%	No	%	No	%
Cloacal swab	200	10	5	10	5	8	4	9	4.5
Liver	45	15	33.3	15	33.3	12	26.7	13	28.8
Yolk sack	45	5	11.1	5	11.1	4	8	5	11.1
Caecal content	45	6	13.3	6	13.3	5	11	6	13.3
Litter	30	6	20	5	16.6	6	20	6	20
Ration	30	6	20	3	10	3	10	4	13.3
Water	30	3	10	0	0.0	0	0.0	1	3.33
Egg shell	30	0	0.0	0	0.0	0	0.0	0	0.0
Egg content	30	0	0.0	0	0.0	0	0.0	0	0.0
Total	485	51	10.5	44	9.0	38	7.8	44	9.0

Note: the rate of each number obtained by dividing the number on total number of raw.

$P < 0.01$

According to the results of serotyping, table (5), the overall percentage of *Salmonella gallinarum- pullorum* is (1.85%), this result in accordance with (25) who reported that, the percentage of *Salmonella gallinarum* was (1.45 %) and disagree with (26). Isolation rate of *Salmonella typhimurium* was (1.44%) table (5), this results is in agreement with (10&27). *Salmonella newport* isolation rate was the highest percentage(4.32%) in comparison with other isolated serotypes, a total of 22 *Salmonella newport* from 38 *Salmonella* isolates which identified by serology table (5), this result compatible with results of (28). *Salmonella Ohio* was isolated from one cloacal swab (0.21 %) table (5).

Table (5): Number and percent of different *Salmonella spp.* which identified by using serology according to source of sample.

Source of sample	No of examined sample	*Salmonella gallinarum-pullorum*		*Salmonella typhimurium*		*Salmonella newport*		*Salmonella ohio*	
		No.	%	No.	%	No.	%	*No.*	%
Cloacal swab	200	0	0.0	3	1.5	4	2	1	0.0
Liver	45	6	13	0	0.0	6	13.3	0	0.0
Yolk sack	45	1	2.0	0	0.0	3	6.0	0	0.0
Caecal content	45	1	2.0	0	0.0	4	8.0	0	0.0
Litter	30	0	0.0	3	10	3	10.0	0	0.0
Ration	30	1	3.3	1	3.3	1	3.0	0	0.0
Water	30	0	0.0	0	0.0	0	0.0	0	0.0
Egg shell	30	0	0.0	0	0.0	0	0.0	0	0.0
Egg content	30	0	0.0	0	0.0	0	0.0	0	0.0
Total	485	9	1.85	7	1.44	21	4.3	1	0.21

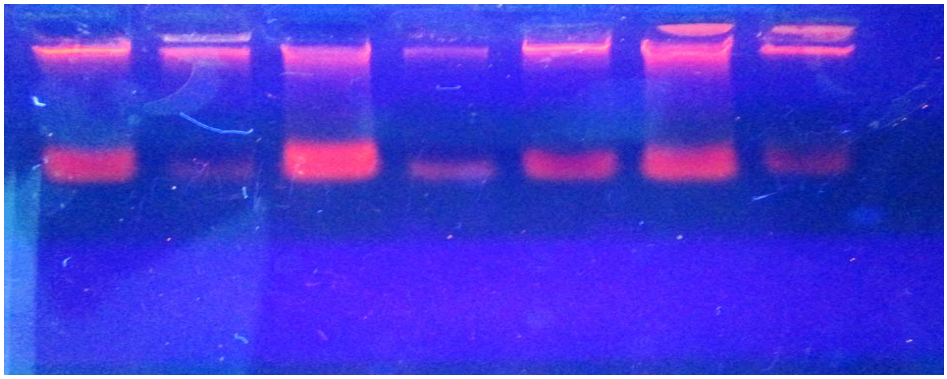
Note: the rate of each number obtained by dividing the number on total number of raw.

Although *Salmonella pullorum* and *Salmonella gallinarum* cause different diseases in poultry, they are very similar. *Salmonella gallinarum* is responsible for fowl typhoid, and *Salmonella pullorum* causes pullorum disease, both are non-motile and present the same somatic antigenic structure (7). The differentiation between *Salmonella pullorum* and *Salmonella gallinarum* is very important from epidemiological and preventive perspectives. They are very similar, and cannot be distinguished by conventional serological methods (7). Moreover, (22) reported that, the results of serotyping able to reaches only to identification of *Salmonella serovar gallinarum* without of differentiation of it into their biovars pullorum and gallinarum. Serotypes gallinarum, pullorum and enteritidis are very similar from the point of view of their antigenic structure, so polymerase chain reaction (PCR) can particularly be a useful tool to provide rapid and definitive detection of these avian *Salmonella* serotypes (8). Table (6), reveal that, the all isolates 9(1.86%) which identified as *Salmonella serovar gallinarum* by serotyping re-identified as *Salmonella gallinarum* by using PCR (*rfbsg*) figure (1-C). These results are in congruence with (8) who concluded that, the results obtained by the allele-specific

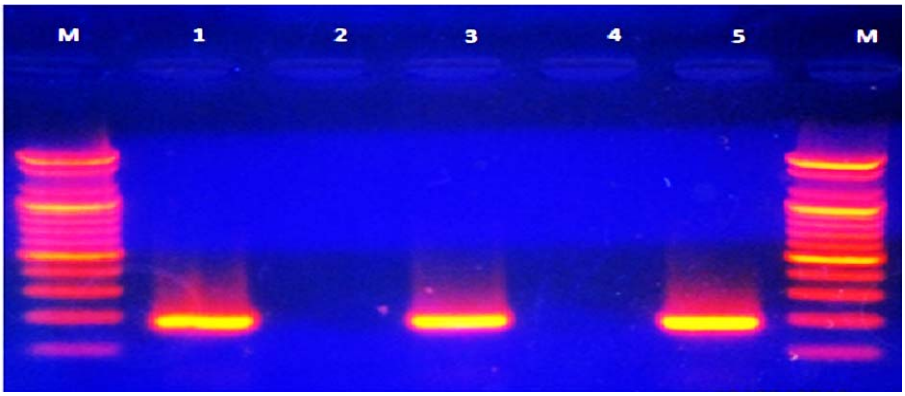
PCR using *S. gallinarum* specific primer (*rfbsg*) for the serotype-specific detection of *S. gallinarum* are conclusive

Table (6): Molecular identification of *Salmonella serovar gallinarum* isolates by using *rfbsg* and *rfbsp* according to total number and source of samples.

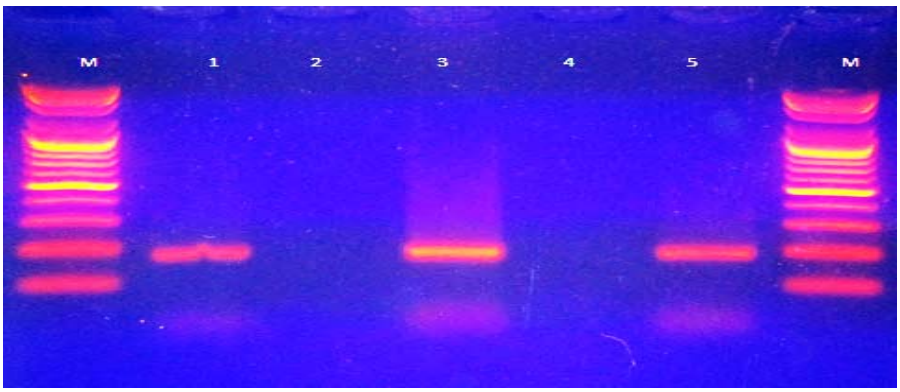
Sample	Total collection sample.	*rfbsg*		*rfbsp*	
		No.	%	No.	%
Cloacal swab	200	0	0.0	0	0.0
liver	45	6	13.3	0	0.0
Yolk sack	45	1	2.2	0	0.0
Caecal content	45	1	2.2	0	0.0
Litter	30	0	0.0	0	0.0
Ration	30	1	3.3	0	0.0
Water	30	0	0.0	0	0.0
Egg shell	30	0	0.0	0	0.0
Egg content	30	0	0.0	0	0.0
Total	485	9	1.85	0	0.0



A- Total genomic DNA extract of *Salmonella spp*. isolates on 0.8% agarose gel stained with ethidium bromide.



B- PCR amplification mixture was run on 1.5% agarose gel stained with ethidium bromide. Lanes: M, (100 bp) DNA ladder molecular weight marker;1and 3 and 5 positive for *flic* gene (197 bp) as *Salmonella Spp.* 2& 4 ; negative control.



C- PCR amplification mixture was run on 1.5% agarose gel stained with ethidium bromide. Lanes: M, (100 bp) DNA ladder molecular weight marker; lanes1and 3and 5; positive for *rfbsg* gene (187 bp) as *S. gallinarum*, 2& 4; negative control.

Figure (1): Electropherogram of total genome DNA, PCR amplification of *flic* gene and PCR amplification of *rfbsg* gene.

-Comparison the effectiveness of four techniques in identification of *Salmonella spp.*

Comparison the results of different methods clarify by table (7), there are similarity in the results of identification rate between API 20E and PCR assay 44/51(86.3%), these results are in accordance with (29) who found that, the API 20E had the highest agreement with PCR tests at the (99.9%) likelihood level.

Table (7): Overall results obtained by different methods of isolation and identification of *Salmonella spp.*

Type of test	Positive tested samples	%
Positive Cultural result on XLD	216/485	44.5
Conventional Biochemical tests	51/51	100
API 20 E	44/51	86.3
Serological	38/51	74.5
PCR	44/51	86.3

-Antimicrobial susceptibility test

Acquired resistance in *Salmonellae* can originate from chromosomal mutation or from acquisition of transferable genetic materials (30), many scientists reported that the main cause of acquired resistance is using of antibiotics in poultry for different purposes such as growth promotion, prophylaxes or therapeutics (31 & 32).

Results of this study shows that 32/38 (84.2%) of isolates were resistant to azithromycin, 71% to florfenicol, 68.4% to trimethoprime-sulphamethaxezole, (63.2 %) to tetracycline, (60.5%) to ciprofloxacin, (55.3%) to ampicillin , and (31.6%) to gentamycin, table (8). These results are in accordance with (33&34).

Table (8): Antibiogram of 7 antimicrobials were tested against (38 isolates) of *Salmonella Spp*. which identified by serotyping.

No.	Antimicrobials	Number of resistant isolates		Number of intermediate isolates		Number of susceptible isolates	
		No.	%	No.	%	No.	%
1	Azithromycin	32	84.2	6	15.8	0	0.0
2	Trimethoprime-sulphamethaxezole	26	68.4	0	0.0	12	31.6
3	Ampicillin	21	55.3	2	5.3	15	39.4
4	Tetracycline	24	63.2	6	15.8	8	21
5	Florfenicol	27	71	0	0.0	11	28.9
6	Gentamycin	12	31.6	14	36.8	12	31.6
7	Ciprofloxacin	23	60.5	0	0.0	15	39.5

P< 0.001

عزل وتشخيص انواع السالمونيلا من مزارع الدواجن باستخدام تقنيات مختلفة وتقييم حساسياتها لمضادات الميكروبات

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الخلاصة

هدفت هذه الدراسة لعزل و تحديد أنواع السالمونيلا من مصادر مختلفة من مزارع الدواجن باستخدام أربع تقنيات مختلفة (الاختبارات البيو كيميائية التقليدية، نظام API 20E ، علم الأمصال و تفاعل البلمرة المتسلسل) وكان العدد الكلي للعزلات ٤٤ (٩ ٪) . أنواع السالمونيلا تضمنت ٤ أنواع ، *S. gallinarum* ٩ (١.٨٥ %) ، *S. typhimurium* ٧(١.٤٤ %) ، *S. newport* ٢١(٤.٣%) و *S. ohio* ١(٠.٢١ %) . وكان أعلى معدل للعزل في الأسبوع الأول من عمر الكتاكيت ١٨ (٢٥.٧ ٪)، من جانب اخر ، كان أعلى معدل لعزل السالمونيلا من الكبد ١٣ (٢٨.٨ ٪) . هناك تشابه في معدل تحديد السالمونيلا بين نظام API 20 E وفحص PCR باستخدام الجين *flic* . في هذه الدراسة كان تضخيم الجينات *rfbsg* و *rfbsp* باستخدام PCR لغرض تفريق النمط المصلي *Salmonella serovar gallinarum* إلى الانماط الحيوية *S. gallinarum* و *S. pullorum* مفيدا" جدا . نتائج الحساسية لمضادات الميكروبات كشفت مقاومة عالية من العزلات ضد سبعة مضادات حيوية مرتبة بصورة تنازلية من الأعلى إلى الاقل مقاومة (أزيثروميسين ، فلورفنيكول ، ترايميثوبريم - سلفاميثاكسيزول، تتراسيكلين ، سيبروفلوكساسين ، أمبيسلين و جنتاميسين) .

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