



Antimicrobial susceptibility profile of *Salmonella enterica* isolated from poultry farms using Vitek- 2

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Abstract

Background: The increase emergence and dissemination of antibiotic resistant *Salmonella* strains may contribute to a great risk on public health and impair classical treatment regime. The present study is aimed to investigate the prevalence of NTS in feces of broilers in four provinces in Iraq, and the resistance profile of isolates to clinically important antibiotics.

Method: a total of 185 fecal samples were collected during the period from October2017 to March2018, from 28 broiler farms distributed in four governorates (Al- Najaf Al-Ashraf, AL-Qadyssia, Al-Muthana, and Babylon). Suspected Isolates of *Salmonella* were assayed for, detection of *invA* gene PCR, antibiotic susceptibility by Vitek-2.

Results: The prevalence of NTS infection in 28 examined poultry flocks was 75,7%. High prevalence rate of NTS was observed in Babylon (91,7%), followed by Al-Qadyssia (72,7%) and Al-Muthana (70%), which significantly differed ($P \leq 0.01$) than reported in AL- Najaf (50%). While the isolation rate was 21.6%. All *Salmonella* isolates were positive for the *invA* gene. The majority of isolates were resistant to Minocycline (87.5%) and Ciprofloxacin (85%) with P value ≤ 0.01 . in addition to these, 55% of strains were resistant to Pipracillin, 52,5% to Ticarcillin ($p \leq 0.05$), and 37,5% to Gentamycin. Fortunately, all isolates were susceptible to Amikacin, Meropenem, Imipenem, Piperacillin/Tazobactam and Ticarcillin/Clavulanic acid, followed by 97.5% of strains were sensitive to Ceftazidime, Cefepime and Aztreonam. Twenty (50%) isolates were exhibited multiple resistance to three or more different antibacterial classes with different resistance profiles.

In conclusion, high prevalence of *Salmonella* and MDR *Salmonella* was found in broiler farms located in four Iraqi provinces.

Keywords: *Salmonella*, MDR, *inv A* gene, vitek-2

مظاهر الحساسية للمضادات الحيوية لميكروبات السالمونيلا المعزولة من مزارع الدجاج Vitek - 2

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الخلاصة:

الخلفية: إن زيادة ظهور سلالات السالمونيلا المقاومة للمضادات الحيوية وانتشارها قد يسهم في خطر كبير على الصحة العامة ويضعف نظام العلاج الكلاسيكي. تهدف هذه الدراسة إلى التحقيق في مدى انتشار السالمونيلا غير التيفوئيدية (NTS) في براز دجاج الاحم في أربع محافظات في العراق ، ودراسة مقاومة العزلات للمضادات الحيوية المهمة سريرياً باستخدام جهاز Vitek-2.

الطريقة: تم جمع ما مجموعه 185 عينة برازية خلال الفترة من أكتوبر 2017 إلى مارس 2018 ، من 28 مزرعة دواجن في أربع محافظات (النجف الأشرف ، القادسية ، المثنى ، وبابل). تم تعريض الجراثيم المشتبه بها من السالمونيلا لفحوصات الكشف عن *invA* جين بتقنية تفاعل البلمرة المتسلسل PCR، والحساسية المضادات الحيوية بواسطة جهاز Vitek-2.

النتائج: كان معدل انتشار عدوى NTS في 28 من قطعان الدواجن ما يقارب 75.7%. لوحظ ارتفاع معدل انتشار NTS في بابل (91,7%) ، تليها القادسية (72,7%) والمثنى (70%) ، والتي تختلف اختلافا كبيرا ($P < 0.01$) مما سجل في النجف (50%). بينما كان معدل العزل 21,6%. كانت جميع عزلات السالمونيلا ايجابية بالنسبة لجين *invA*. كانت غالبية العزلات مقاومة للمينوسايكلين (87% و 5%) والسيبروفلوكساسين (85%) مع قيمة P اصغر من 0,01 ، بالإضافة إلى ذلك ، كانت 55% من السلالات مقاومة للبييراسيلين ، و 52,5% للتيتكارسيلين ($P < 0.05$) ، و 337,5% للجنتاميسين. لحسن الحظ ، كانت جميع العزلات حساسة للأميكاسين ، الميروبيينيم ، الإيمبيينيم ، البيبيراسيلين / تازوبكتام والتيتكارسيلين / حمض كلافولانيك ، تليها 97,5% من السلالات كانت حساسة للسيفتازيديم ، السيفبيم والأزترينام. أظهرت 20 عزلة (50%) مقاومة متعددة لثلاثة أو أكثر من الاصناف المختلفة لمضادة للجراثيم مع مظاهر مقاومة المختلفة.

في الختام ، تم العثور على نسبة عالية من السالمونيلا و السالمونيلا المتعددة المقاومة للمضادات في مزارع اللاحم الواقعة في أربع محافظات عراقية.

كلمات مفتاحية: السالمونيلا، MDR، *invA* gene، Vitek-2

Introduction

Non-typhoidal *Salmonella* (NTS) are group of enteric pathogens transmitted primarily by contaminated food of animal origin (1). Moreover, plant products such as raw fruits and vegetables have been implicated in transmission cycle (2). NTS can circulate through the entire food chain that impact public health (3). Globally, they are associated with significant morbidity and mortality in humans and animals. Nowadays, there are over 2,610 *Salmonella* serovars (4). In developing countries, domesticated animals and poultry are considered the main reservoirs of NTS that can impact human health (5, 6).

Antibiotic resistance represents a great threat on the health of human and animal, as well as food security. Reports issued around the world indicate that there are dangerous levels of antimicrobial resistance. The emergence and spread of new resistance mechanisms globally, may reduce the effective treatment of infectious diseases (3).

Under the effect of antibiotic selective pressure, multidrug resistance (MDR) is frequently associated with horizontal transfer genetic elements such as plasmids, integrons, bacteriophages and transposons that may carry multiple resistance genes. The zoonotic MDR bacteria can be transmitted to human through poultry products, which can colonize the human gut and cause disease or transfer the resistance determinants to other enteric bacteria (7-9).

Classical pattern of MDR in *Salmonella* is resistance to the first-line

antibiotics chloramphenicol, ampicillin, and trimethoprim/sulfamethoxazole. The high prevalence of MDR in typhoidal *Salmonella* and NTS necessitates the use of second-line antibiotics (10). The fluoroquinolone (FQ), ciprofloxacin and the third-generation cephalosporin ceftriaxone are now the recommended drugs to treat invasive *Salmonella* infections or patients at risk of developing an invasive infection (11, 12).

World health organization (WHO) was developed a global priority list of antimicrobial resistant pathogens to help in prioritizing the world efforts that aim to fight antibiotic resistant bacteria. *Enterobacteriaceae* (carbapenem-resistant and third generation cephalosporin-resistant strains) and *Salmonella* species (fluoroquinolone-resistant strains) were listed as critical (first) and high (second) priority by the WHO (13).

Because of the growing problem of antibiotic resistance worldwide and the urgent need to apply "One Health" approach in order to limit or reduce the spread of resistance and its effect on public health. Therefore, the present study is aimed to investigate the prevalence of NTS in feces of broilers in four provinces in Iraq, and the resistance profile of isolates to clinically important antibiotics.

Materials and Methods

Sample collecting: A cross-sectional study was conducted to collect 185 fecal samples during the period from October 2017 to

March 2018, from 28 broiler farms distributed in four governorates including, 4 flocks from Al-Najaf Al-Ashraf (AL-Hayderyia (one flock), AL-Kufa (2 flocks), and Al-Manathara (one flock); 11 flocks from AL-Qadyssia province that including 8 districts: Ghammas (one flock), and Al-Shamyia (4 flocks), Al-Mahnawiyah (one flock), Al-Shinafiyah (one flock), and Al-Diwanyia (one flock); 10 flocks from Al-Muthana province which including: Al-Sumawa (3 flocks), and Al-Rumaitha (6 flocks), and AL-Khdier (one flock); and 12 flocks from Babylon province that including: AL-Kefel (one flock), AL-Hashimyia (one flock), Jubala (one flock) AL-Musayiab (one flock), AL-Tahmazia (one flock), and AL-Jarbuia (one flock). Five cloacal swabs were collected from each flock using sterile disposable cotton swabs, each swab immediately inoculated onto 10ml Selenite-F broth, and transported in cold box to the Laboratory of Department of Microbiology in the Faculty of Veterinary medicine, University of Kufa.

Isolation and biochemical identification of Salmonella: Selenite F broth contained the animal specimens was incubated at $41.5^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ overnight, then sub-cultured by streaking onto CHROM agar *Salmonella* (CAS) agar media (CHROM agar Company, Paris, France), Xylose-Lysine desoxycholate (XLD), All plates were incubated at 37°C for 18 to 24 hr. (14, 15). The growing colonies on CAS, XLD and were examined by naked eye for their color, shape and size. Then the suspected colonies were Gram stained and the biochemical testing were done that including Triple Sugar Iron, Urease test, Simmons-citrate test and Lysine Iron Agar (LIA) (16, 17).

Whole genomic DNA extraction: The DNA from the enriched culture was obtained by using a DNA extraction kit (Genaid, Korea) and the purified DNA was used as a template for the PCR assay.

Salmonella genus-specific PCR: The *invA* gene of *Salmonella* contains sequences unique to this genus and has been proved as a suitable PCR target, with potential diagnostic

applications (18). The primer sequence was: **invA-F** 'GTG AAA TTA TCG CCA CGT TCG GGC AA', and **invA-R** 'TCATCGCACCGTCAAAGGAACC', to produce an amplicon size 284 base pair (19).

The PCR was performed under the following conditions: primary denaturation: 94°C for 3 min, then 35 cycles of: denaturation: 94°C / 30 sec, annealing: 64°C / 30 sec min, and extension: 72°C / 40 sec., then final extension at 72°C for 5 min, then the amplified PCR products were electrophoresed in 1.5 % agarose gel and photographed by a gel documentation system.

Antimicrobial susceptibility by Vitek-2: Antibiogram testing was performed with the automated VITEK-2 compact system based on MIC technique determination using AST-N222 cards. The following antibiotics were tested: Amikacin, Aztreonam, Cefepime, Ceftazidime, Ciprofloxacin, Gentamicin, Imipenem, Meropenem, Minocyclin, Pefloxacin, Piperacillin, Piperacillin/Tazobactam, Rifampicin, Ticarcillin, Ticarcillin/Clavulanic acid, Tobramycin and Trimethoprim/Sulfamethoxazole. All isolates were delivered into Central Health Laboratory in Al-Najaf city. The instructions of manufacturer's were followed.

Statistical analysis: The chi-square test was used to determine any significant differences in resistance or prevalence where appropriate. Differences were considered significant at $p \leq 0.05$.

Results:

The prevalence of NTS infection in 28 examined poultry flocks was 75,7% . Considering each province flocks separately, high prevalence rate of NTS was observed in Babylon (91,7%), followed by Al-Qadyssia (72,7%) and Al-Muthana (70%), which significantly differed ($P \leq 0.01$) than reported in Al-Najaf (50%) as presented in table 1. Among 185 fecal (cloacal swabs) samples collected from broilers, exactly 40 isolates of NTS were recovered with an isolation rate of 21,6% .

Table-1: Distribution of *Salmonella* isolates in relation to geographic location of broiler flocks from four provinces of Iraq, during the period from October 2017 to March 2018.

Geographic location of Broiler flocks (age of birds)	No. of flocks	No. of samples	No. of isolates
AL-Najaf			
- Al-hayderyia (20 days)	1	5	1
- Al-Kufa (27 days)	1	5	-
- Al-Kufa (2 days)	1	5	1
- Al-Manathera (2 days)	1	5	-
Total	4	20	2^{b*}
Al-Qadyssia			
- Al-Shamyia (35 days)	3	15	(-)
- Al-Shamyia (13 days)	1	5	1
- Al-Shamyia (13 days)	1	5	1
- Al-Shamyia (13 days)	1	5	1
- Mhanawia (21 days)	2	10	2+3
- Ghamma (6 days)	1	5	2
- Shannafia (30 days)	1	5	2
- Diwanyia (25 days)	1	5	4
Total	11	55	16^{a**}
Al-Muthana			
- Al- Rummytha (15 days)	1	5	-
- Al- Rummytha (22 ays)	1	5	3
- Al- Rummytha (20 days)	1	5	-
- Al- Rummytha (15 days)	1	5	1
- Al- Rummytha (16 days)	1	5	1
- Al- Rummytha (15 days)	1	5	1
- Al-Sumawa (6 days)	1	5	1
- Al-Sumawa (22 days)	1	5	2
- Al-Sumawa (27 days)	1	5	-
- Al- Khdier (18 days)	1	5	1
Total	10	50	10^{a**}
Babylon			
- Al-Kefel (26 days)	3	15	3
- Al- Hashimyia (36 days)	1	5	2
- Jubala (24 days)	3	15	2
- Al -Musayiab (36 days)	2	10	2
- Al-Tahmazia (1 days)	1	5	-
- Al- Jarbueia (26 days)	2	10	3
Total	12	60	12^{a**}
Total (All)	28	185	40

**p < 0.01

* p < 0.05

Following PCR amplification of 40 suspected isolates of *Salmonella*, all strains yielded PCR products indicative of the presence of the *invA* gene. The band size obtained after PCR confirmation was 284 bp corresponding to the size of *invA* gene (Figure 1).



Figure 1: Ethidium bromide-stained agarose gel of monoplex PCR amplified products from extracted DNA of *salmonella* spp isolates and amplified with one gene primers. The electrophoresis was performed at 75 volt for 1 hr. Lane (M), DNA molecular size marker (100 bp ladder), Lanes (1, 2, 3, 4, 5, 6, 7, 8, 9, 10) show positive results with *InvA* gene (284bp).

As shown in Table 2, all isolates were totally sensitive to Amikacin, Meropenem, Imipenem, and Piperacillin/Tazobactam. Moreover, 97.5% of strains were sensitive to Ceftazidime, Cefepime and Aztreonam. Also, 80% of isolates were susceptible to Tobramycin and 78.5% to Trimethoprim-sulfamethoxazole. The statistical analysis

revealed that the susceptibility to these antibiotics significantly was differed than others (P value ≤ 0.01). However, majority of isolates were resistant to Minocycline (87.5%) and Ciprofloxacin (85%) with P value ≤ 0.01 . in addition to these, 55% of strains were resistant to Pipracillin, 52,5% to Ticarcillin ($p \leq 0.05$), and 37,5% to Gentamycin.

Table-2: Antibiogram of NTS strains collected from broiler farms using Vitec 2 compact.

Antimicrobial	Resistance	susceptibility
Trimethoprin/Sulfamethoxazole	5(12,5%)	35(78,5%) **
Amikacin	0	40(100%)**
Meropenem	0	40(100%) **
Imipenem	0	40(100%) **
Piperacillin/Tazobactam	0	40(100%) **
Ticarcillin/Clavulanic acid	0	40(100%)**
Aztreanam	1(2,5%)	39(97,5%) **
Ceftazidime	1(2,5%)	39(97,5%) **
Cefepime	1(2,5%)	39(97,5%) **
Minocycline	35(87,5%) **	5(12,5%)
Ciprofloxacin	34(85%)**	6(15%)
Ticarcillin	21(52,5%)*	19(47,5%)
Piperacillin	22(55%) *	18(45%)
Tobramycin	8(20%)	32(80%)**
Gentamicin	15(37,5)	25(62,5%) *

**($P < 0.01$) *($P < 0.05$)

Twenty (50%) isolates were exhibited multiple resistance to three or more different antibacterial classes with eight pattern as the profile of resistance presented in table 3. However, twelve (30%) strains were resistant to two different classes of antibiotics and only two NTS strains were susceptible to all tested antimicrobials.

Table: Antibiotic resistance profile of *Salmonella enterica* isolated from broilers.

GM/MNO/CIP/PIP/TIC/SXT/FEP/CAZ	1
GM/TM/MNO/CIP/PIP/TIC	7
GM/MNO/CIP/PIP/TIC/SXT	2
GM/MNO/CIP/PIP/TIC	4
MNO/CIP/PIP/TIC/SXT	1
MNO/CIP/PIP/TIC	3
MNO/CIP/PIP	1
CIP/PIP/TIC	2
GM/TM/MNO	1
MNO/CIP	10
MNO	3
CIP	3
Susceptible	2

CIP= Ciprofloxacin, MNO= Minocycline, PIP= Piperacillinm TIC= Ticarcillin, SXT= Trimethoprim/Sulfamethoxazole, FEP= Cefepime, CAZ= Ceftazidime, GM= Gentamicin, TM= Tobramycin,

Discussion

Salmonella enterica bacteria are one of the most important foodborne pathogens that circulate in the food chain, the increase emergence and dissemination of antibiotic resistant *Salmonella* strains may contribute to a great risk on public health and impair classical treatment regime (20).

Although, high levels (75.7%) of *Salmonella* colonizing intestine of broilers in this study, several other studies done in Gambia and Burkina Faso observed relatively high prevalence (67% and 55% respectively) (21, 22). Lower prevalence raters were observed in Vietnam by (23).

The bacteriological data on the prevalence of NTS in various studies are difficult to compare due to differences in geographic regions, collection seasons, the nature of samples, isolation methodologies, culture methods, culture media and environmental conditions.

With regard to the difference in prevalence between the different

geographic regions under study, the low prevalence rate of NTS in Al-Najaf was probably due to small number of farms compared to the rest of the governorates.

The current isolation rate was similar to that reported in other developing countries (24). In addition, nearly similar isolation rate was observed in broilers at slaughter age (25). However, higher isolation rate has been found by (26). Generally, the poultry industry is poorly managed in many parts of Iraq, where the biosecurity and disease prevention are still lagging behind and do not receive the valuable attention breeders. On the other hand, there is lacking of routine examination of broiler breeder farming for NTS shedding in Iraq and depending on vaccination with killed vaccine that provide short term protection against *Salmonella* Enteritidis.

The antibiotics resistance in *Salmonella* and emergence of many clones of multidrug-resistant isolates are becoming a great public concern worldwide (27).

In the present study, the highest frequency of resistance was observed to Minocycline that could be due cross-resistance with tetracycline, the antibiotic to which most isolates were resistant (data not shown). Other researchers have been found a correlation between the presence of tetracycline resistance determinants and resistance to Minocycline (28).

Also, the current data revealed high resistance to piperacillin and ticarcillin, similar results were reported among *Salmonella* isolated from poultry in India (29). Nevertheless, low resistance rate for both antibiotics was observed in Algeria, (30). The high resistance rate to these kinds of antimicrobial agents might be due to the uncontrolled use of beta-lactam as growth promoters or in the treatment of viral and bacterial infections, even the farmer can use them without veterinary supervision (31, 32). Unfortunately, such bad practices can select for expression of beta-lactamases or extended-spectrum beta-lactamases by enteric bacteria.

However, our results showed less resistance to Trimethoprim/sulfamethoxazole was (12,5%), these results were similar to other research findings like (33), on the other hand, these data disagreed with (26), who found high resistance rate to Trimethoprim/sulfamethoxazole among *Salmonella enterica* isolated from poultry.

Otherwise, all of the isolates in this study were susceptible to amikacin, Meropenem, Piperacillin / Tazobactm and Azteromene which may be due to the fact that these antimicrobials are not used for therapeutic purposes in veterinary medicine or as a growth promoter in conventional animal fattening, and the result was consistent with other reports (34).

The present study revealed moderate resistance to aminoglycosides (gentamicin and Tobramycin, this result

similar to that reported by (35), but higher than recorded by (36, 37).

The development of MDR zoonotic bacteria represent a multifaceted risk, as they pose a threat to animal productivity and food security, and on the other hand, pose a public health risk through their transmission through the food chain. In addition, they may be horizontally transfer resistance genes to other pathogens.

In the present study, 50% of isolates were MDR, which in coexistence with that observed by (36), however, this result was lower than recorded by (38), who found 67% of *Salmonella* Infantis isolated from broiler flocks were MDR. These findings support that the increased antibiotic resistance in zoonotic bacteria could be due to indiscriminate and unrestricted use of antimicrobial agent in veterinary medicine.

In summary, our findings showed high prevalence of *Salmonella* and MDR *Salmonella* in broiler farms that distributed in four provinces in Iraq. Accordingly, the poultry production in Iraq should get attention and development of monitoring and surveillance programs of antimicrobial resistance as well as the development of biosecurity and public health standards. On the other hand, the use antibiotic in veterinary sector should be restricted under obligatory guidelines.

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