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Detection of tetracycline and β-lactam resistance genes in *Shigella* species isolated from shigellosis cases in Iraq

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Abstract

Shigella is most commonly spread through the fecal-oral route, rather than through contaminated food, water, or swimming pools. A recent study collected 103 fecal samples of *Shigella* spp. from hospitals and laboratories across Iraq, spanning from July 2022 to June 2023. The samples were sourced from various cities, including Baghdad (65), Nasiriya (14), Babylon (11), Najaf (9), and Kirkuk (4). In this study, we examined *Shigella* species isolated from diarrhea samples for the presence of penicillin- and tetracycline-resistant genes. Tetracycline resistance genes (*tet*A and *tet*B) were detected in 96 (93%) and 84 (82%) isolates, respectively. Both genes (*tet*A and *tet*B) were detected in the same isolate 79 times (77%). While, β-lactam resistance genes ($bla_{\text{CTX-M}}$ and bla_{TEM}) were detected in 87 isolates (84.5%) and 83 isolates (81%), respectively. However, 68 isolates (66%) exhibit both genes ($bla_{\text{CTX-M}}$ and bla_{TEM}). Consequently, that detects the widespread use and overuse of penicillin and tetracycline. Thus, it is imperative to continue surveilling and regulating the spread of these genes to sustain the efficacy of existing antibiotics and develop new treatments for *Shigella* infections.

Keywords: Shigella, Shigellosis, Diarrhea, tet genes, bla genes

الكشف عن جينات مقاومة التتراسيكلين والبيتا لاكتام في أنواع الشيغيلا المعزولة من حالات داء الشيغيلات في العراق

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الخلاصه

عادة ما يكون انتشار بكتريا الشيغيلا من خلال الطريق البرازي الفموي، وليس من خلال الطعام أو الماء أو حمامات السباحة الملوثة. جمعت هذه الدراسة 103 عينة برازية ملوثة باحد انواع جنس الشيغيلا من المستشفيات والمختبرات في جميع أنحاء العراق، في الفترة من يوليو 2022 إلى يونيو 2023. تم الحصول على العينات من مدن مختلفة، بما في ذلك بغداد (65)، والناصرية (14)، وبابل (11)، والنجف (9)، وكركوك (4). كما تم فحص أنواع الشيغيلا المعزولة من هذه العينات بحثًا عن وجود جينات مقاومة للبنسلين والتتراسيكلين. تم تحديد جينات مقاومة للتتراسيكلين (*tet*A و *tet*B) في 96 عزلة (93%) و84 عزلة

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(82%) على التوالي. كما تم ملاحظة تواجد كلا الجينين (*tet*A و *tet*B) في نفس العزلة 79 مرة (77%). بينما تم تحديد جينات مقاومة بيتا لاكتام (bla_{CTX-M} و bla_{TEM}) في 87 عزلة (84.5%) و83 عزلة (81%) على التوالي. ومع ذلك، فإن 68 عزلة (66%) تحمل كلا الجينين (bla_{CTX-M} و bla_{TEM}) وبالتالي، فإن ذلك يكشف عن الاستخدام الواسع النطاق والإفراط في استخدام البنسلين والتتراسيكلين. وبالتالي، من الضروري الاستمرار في مراقبة وتنظيم انتشار هذه الجينات للحفاظ على فعالية المضادات الحيوية الموجودة وتطوير علاجات جديدة لعدوى الشيغيلا.

1. Introduction

Even in the 21st century, shigellosis remains one of the most significant threats to public health. According to a 2017 report from the World Health Organization (WHO), antimicrobial-resistant bacteria are responsible for approximately 700,000 deaths each year, with *Shigella* being one of these bacteria [1]. Most cases of shigellosis occur among children between the ages of 1-5 years [2-5]. A low infectious dose of *Shigella* (less than 100 bacterial cells) makes the infection spread faster than other diseases [6]. Furthermore, the ability of *Shigella* to tolerate stomach acid aids the transmission of the bacteria from person to person [6]. Although *Shigella* spp. have been detected in foods, water, swimming pools, and equipment, the fecal-oral route remains the most common way of transmission for the disease [7-8]. Four main species belonging to this genus are responsible for shigellosis. *Shigella sonnei* predominates in industrialized countries, *Shigella dysenteriae* has been implicated in travel infections, *Shigella flexneri* predominates in developing countries, and *Shigella boydii* is predominant in India [2, 9-12]. A previous investigation [13] revealed that the *inv*C gene is specific to the genus *Shigella* while each species may have a specific gene such as *rfc* for *S. flexneri*, *wbg*Z for *S. sonnei*, and *rfp*B for *S. dysenteriae*. Furthermore, the *omp*A gene was used as an internal control and considered a signaling marker for *S. boydii*. In addition, *Shigella* is an important pathogen associated with hospital community-acquired infection (HCAI) [14]. Tetracyclines were elucidated in 1945 by an American plant physiologist Benjamin Minge Duggar, which showed manifested effectual actions against various microorganisms. However, the first tetracycline-resistant bacteria were reported in the 1950s, shortly after the introduction of tetracycline as an antimicrobial drug. The first tetracycline-resistant bacteria isolated were species of *Shigella* and *Salmonella*. Since then, tetracycline resistance has become widespread among many different species of bacteria, including *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Escherichia coli* [15, 16]. A major concern in the treatment of bacterial infections is the ability of bacteria to resist antimicrobials. In other words, the presence of beta-lactamase genes like bla_{CTX-M} and bla_{TEM} can considerably reduce the effectiveness of antibiotics like penicillins and cephalosporins [17, 18]. These genes have been found in many different bacterial species, including *Shigella* spp. The prevalence of bla_{CTX-M} and bla_{TEM} genes in *Shigella* spp. is increasing globally. In some regions, these genes have been found in over 50% of *Shigella* isolates, making it difficult to treat infections effectively [18]. The spread of these genes is likely due to horizontal gene transfer, where bacteria can share genetic material, including resistance genes [17, 19-21]. The first tetracycline-resistant strain of *Shigella* was ascertained in 1953, and since then its numbers have augmented dramatically, reducing the effectiveness of tetracycline against *Shigella* infection [16]. The resistance of *Shigella* species to tetracycline depends on the presence of a plasmid gene known as the *tet* gene, which includes the *tet*A and *tet*B genes [1]. The aim of this study was to determine the prevalence of tetracycline and penicillin genes among *Shigella* species and to develop a strategy to reduce their prevalence.

2. Material and Methods

This investigation analyzed *Shigella* species isolated from diarrhea samples for the presence of penicillin- and tetracycline-resistant genes. A total of 103 samples of *Shigella* spp. were collected from different hospitals and laboratories in Iraq during the period July 2022 - June 2023 (65 from Baghdad, 14 from Nasiriya, 11 from Babylon, 9 from Najaf, and 4 from Kirkuk). The study was conducted under the approval of the Research Ethics Committee of Osol Aldeen University College No. OS28 dated 6/7/2022.

2.1. Molecular diagnosis

The genus and species of the isolates were validated by multiplex PCR technology. First, brain heart infusion (BHI) broth was used to propagate the isolates and incubated overnight at 37°C. Later, the Promega genomic DNA extraction and purification kit was used to isolate the DNA of the samples. Briefly, the mixture for multiplex PCR was prepared by mixing Ready to Load 5x FIREPol® Master Mix, 3µL DNA sample, 7 µL Milli Q water, and 1µL of forward and reverse primer for each gene (*inv*C, *rfc*, *rfp*B, *wbg*Z, and *omp*A). Moreover, the PCR program was set for 30 cycles (each cycle has denaturation, annealing, and elongation temperatures, (94°C/60sec), (60°C/30sec), and (72°C/90sec), respectively, with an initial temperature (94°C/5min), and ended with a final extension (72°C/7min).

2.2. Detection of resistance genes

PCR mixtures were formulated separately for each gene (*tet*A, *tet*B, $bla_{\text{CTX-M}}$, and bla_{TEM}) as follows: 5X master solution, 3µL DNA, 15µL Milli Q water, and 1µL (10pmol/µL) of each primer (F and R). Moreover, the PCR program was set for 30 cycles (each cycle has a denaturation temperature (94°C/60 sec), an annealing temperature as shown in Table 1, and an elongation temperature (72°C/90 sec) with an initial temperature (94°C/5min), and ended with a final extension (72°C/7 min).

Table 1: Primer sequences.

Gene	Target	Sequences (5'-3')	Annealing °C/sec.	Amplicon pb	Ref.
*inv*C	*Shigella* genus	F- TGCCCAGTTTCTTCATACGC	60/30	875	[13]
		R- GAAAGTAGCTCCCGAAATGC			
Rfc	*S. flexneri*	F- TTTATGGCTTCTTTGTCGGC		537	
		R- CTGCGTGATCCGACCATG			
*wbg*Z	*S. sonnei*	F- TCTGAATATGCCCTCTACGCT		430	
		R- GACAGAGCCCGAAGAACCG			
*rfp*B	*S. dysenteria*	F- TCTCAATAATAGGGAACACAGC		211	
		R- CATAAATCACCAGCAAGGTT			
*omp*A	*S. boydii*	F- GCAGGCATTGCTGGGTAA		1319	
		R- ACACTTGTAAGTTTTCAACTACG			
*tet*A	Tetracycline resistance gene	F- GCTGCAAGCAATGTTGTCCA	59/30	190	[22]
		R- CAGGCAGAGCAAGTAGAGGG			
*tet*B		F- CGCTAACCACTTTGGCGTAT	59/30	211	
		R- AGCTCCTGTGATCCCTGAAA			
$bla_{\text{CTX-M}}$	β-lactam resistance gene	F- CGCTTTGCGATGTGCAG	60/30	550	[23]
		R- ACCGCGATATCGTTGGT			
bla_{TEM}		F- GAGTATTCAACATTTCCGTGTC	60/30	800	
		R- TAATCAGTGAGGCACCTATCTC			

2.3. Antimicrobial sensitivity test

Bacterial suspensions were prepared and adjusted to 0.5-McFarland standard. A 100 µL sample of the adjusted bacterial suspension was carefully pipetted onto BHI agar and spread evenly with a sterile swab. Then, the agar was coated with antibiotic discs (Tetracycline 30µg, Penicillin 10 units, Cephalosporin 5µg, Ciprofloxacin 10µg, Azithromycin 15µg,) and incubated overnight at 37°C. The SPSS software (Ver. 21) was used to analyze the statistical significance of the data based on a P-value less than 0.05.

3. Results and Discussion

3.1. Detection of Shigella spp.

The multiplex PCR showed that 46 isolates (44.7%) were *S. sonnei*, 41 isolates (39.8%) were *S. flexneri*, 14 isolates (13.6%) were *S. dysenteriae* and 2 isolates (1.9%) were *S. boydii* as shown in Fig. 1. Moreover, *S. sonnei* was the most frequented species in Baghdad, Babylon, and Kirkuk, while *S. flexneri* predominated in Nasiriya and Najaf. In addition, shigellosis was prevalent in children aged 3-10 years (86 cases; 83.5%) (See **Fig. 2)**.

Shigellosis is a highly contagious disease, which is common in children over two years of age, especially in third-world countries [5]. Research has previously demonstrated the presence of *S. sonnei* and *S. flexneri* among children in Baghdad and other Iraqi provinces, as reported in studies [1, 18, 20, 24-26]. The present study revealed that 83.5% of shigellosis cases were among children aged 3-10 years. Moreover, regardless of gender, the incidence was prevalent among children of preschool and primary age (4-7 years). This indicates poor sanitary conditions in kindergartens and primary schools such as inadequate sanitation and children's ignorance of personal hygiene.

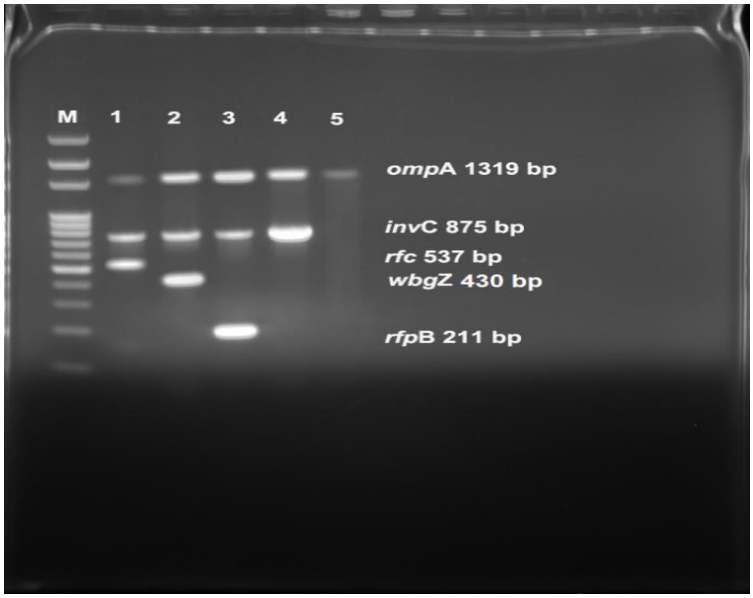

Figure 1: Multiplex PCR, *omp*A was used as an internal control. M, 100 bp plus marker; lane 1, *S. flexneri* (*rfc* and *inv*C); lane 2, *S. sonnei* (*wbg*Z and *inv*C); lane 3, *S. dysenteriae* (*rfp*B and *inv*C); lane 4, *S. boydii* (*omp*A and *inv*C); lane 5, *E. coli* negative control (*omp*A). Results were obtained after running samples on 2% agarose gel at 120 V for 1 h.

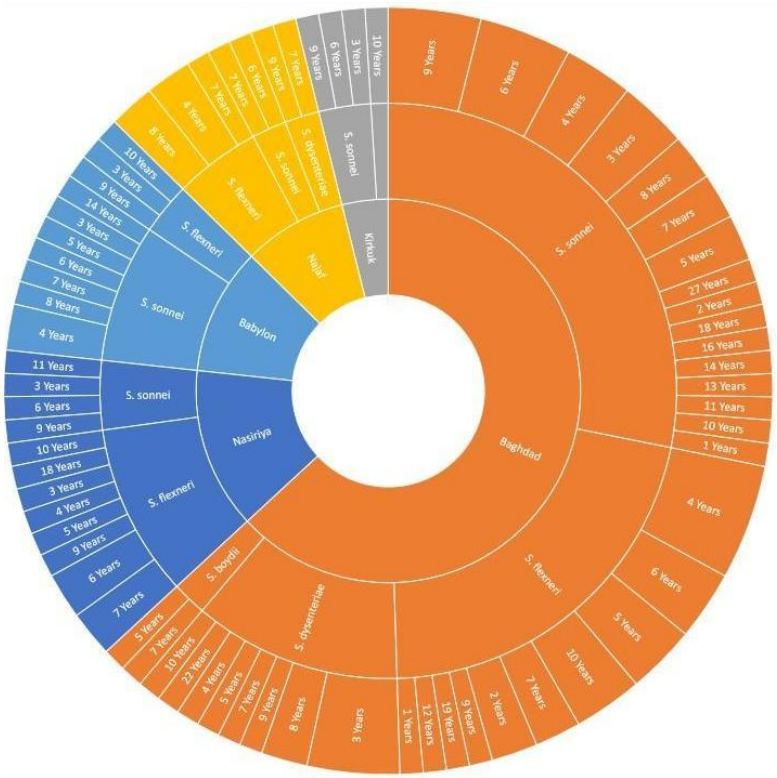

Figure 2: Distribution of *Shigella* species in different Iraqi governorates. As shown, most cases were in children under 10 years of age. Moreover, *S. sonnei* was the most prevalent species followed by *S. flexneri*.

3.2. Detection of resistance genes

Tetracycline resistance genes (*tet*A and *tet*B) were identified in 96 (93%) and 84 (82%) isolates, respectively. Both genes were recognized together in the same isolate 79 times (77%).

Due to the misapplication of antimicrobial agents, tetracycline-resistant *Shigella* spp. has been increasing exponentially since 1953 [27]. A previous investigation [1] detected *tet*A gene in 100% and *tet*B gene in 90% of *Shigella* isolates in Iraq. Our study detected *tet*A and *tet*B genes in 93% and 82% of *Shigella* isolates regardless of species, respectively. Moreover, all *S. sonnei* isolates harbored the *tet*A gene, while 90% of the *S. flexneri* isolates harbored the *tet*B gene. Similar results were identified in a previous study [22], which reported that the ability to resist tetracycline in *Shigella* isolates was mediated by the presence of *tet*A gene in *S. sonnei* and *tet*B gene in *S. flexneri*.

β-lactam resistance genes ($bla_{\text{CTX-M}}$ and bla_{TEM}) were detected in 87 isolates (84.5%) and 83 isolates (81%), respectively. Both genes were detected together in the same isolate 68 times (66%).

CTX-M and TEM enzymes can degrade third and fourth-generation cephalosporins [14]. The current study detected the $bla_{\text{CTX-M}}$ gene in 87 isolates but only 9 were penicillin-resistant and 3 were cephalosporin-resistant while the bla_{TEM} gene was detected in 83 isolates but only 7 were penicillin-resistant and 11 were cephalosporin-resistant. Similarly, a previous study [18] reported that CTX-M- and TEM-producing *Shigella* isolates have increased significantly in Iraq, reflecting increased resistance to penicillin and cephalosporin of *Shigella* spp. Moreover, we observed both genes together in the same isolates 67 times, and it surfaced that 80.7% and 98.5% of these isolates were penicillin-resistant and cephalosporins-resistant, respectively. Suggesting that resistance to penicillin and cephalosporins was mediated by the

presence of both genes (*bla*$_{CTX-M}$ and *bla*$_{TEM}$), implies that the deficiency of one of these genes might hinder effective resistance despite the presence of the other gene.

3.3. Antimicrobial activity analysis

The isolates showed resistance to antibiotics; penicillin 83 isolates (81%), tetracycline 78 isolates (76%), azithromycin 69 isolates (67%), cephalosporin 67 isolates (65%), and ciprofloxacin 33 isolates (32%).

Most developing countries suffer from HCAI, especially those caused by extended-spectrum ß-lactamase (ESBL)-producing bacteria [14]. Previous studies [1, 18, 20] showed that MDR *Shigella* spp. were detected in different provinces in Iraq. Our study revealed that 78 isolates (75.7%) have shown resistance to more than 3 antibiotics, which prompts us to seriously consider the risks of antibiotic misuse.

As a recommendation to reduce the risk of infection, further plans should include improved sanitation and personal hygiene, hand washing, and proper food preparation. In addition, routine immunization of those at risk should be encouraged. Moreover, the widespread use and overuse of penicillin and tetracycline, as well as the emergence of MDR bacteria, have led to the need for new antimicrobial drugs and strategies to combat bacterial infections. It is necessary to conduct further research to determine the virulence factors of *Shigella* spp. Continuous surveillance and containment of these genes are crucial to preserve the efficacy of existing antibiotics and facilitate the development of novel treatments for *Shigella* infections.

Conclusion

Children between the ages of 3-7 years are most susceptible to shigellosis, due to poor personal hygiene and hand washing. Moreover, *S. sonnei* is the most predominant species followed by *S. flexneri*. With this pattern of drug misuse, the antibiotics normally used to treat *Shigella* infections will not be beneficial against these microorganisms.

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