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Molecular Characterisation of Virulence and Antibiotic Resistance Genes in Diarrhoeal Shigella Isolates from Diwaniyah, Iraq

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Abstract

Objective: Investigating the genetic diversity, virulence determinants, and resistance profiles of *Shigella spp* is essential to managing outbreaks and informing effective treatment measures, especially in the Iraqi setting, where such data is scarce.

Methods: Stool samples from patients with acute diarrhoea were cultured on selective media. Colony phenotypic identification was based on non-lactose fermenting properties, H_2S negative behaviour, and Gram-negative, non-motile appearance. Molecular confirmation via PCR amplification of the 16S rRNA gene sequence assembly was performed using the assembly tool in CLC Genomic Workbench with default parameters, and partial sequences were aligned using ClustalW, and phylogenetic trees were constructed in MEGA11. Conventional PCR detected virulence (ipaH, invE, sigA) and resistance genes (blaTEM, aada1, qnrA).

Results: All 88 isolates were positive for the 16S rRNA gene. Phylogenetic analysis revealed grouping into clusters of 3 species: S. sonnei, S. flexneri, and S. dysenteriae, each closely related to reference strains from Asia and the Middle East. The ipaH gene was universally present, whereas invE and sigA were detected in a subset of isolates. Common resistance genes included blaTEM, indicating extensive resistance to β-lactam antibiotics.

Conclusion: Local isolates of *Shigella spp* exhibited considerable genetic diversity and harboured virulence and antimicrobial resistance genes. These findings highlight the importance of routine molecular surveillance in tracking the spread of high-risk strains and supporting public health interventions and treatment protocols tailored to specific clusters. Iraqi dysentery guidelines need to be updated to restrict ineffective antibiotics, improve hospital hygiene, and develop vaccines targeting conserved virulence genes.

Keywords: *Shigella spp*., Virulence genes, Antibiotic resistance, 16S rRNA, Phylogenetic analysis, Multidrug resistance

Plain English Summary

Shigella bacteria are a main cause of severe diarrhoea, also known as dysentery, especially in areas where hygiene is poor and clean drinking water is hard to find. This study investigated different types of Shigella found in patients, how dangerous they are, and how resistant they are to antibiotics. The goal was to better understand and manage disease outbreaks and improve treatment options. Researchers collected stool samples from patients experiencing sudden diarrhoea in Al-Diwaniyah, Iraq. We grew the bacteria in special laboratories to separate them from other germs. All the bacteria identified as Shigella tested positive for a specific genetic marker, called the 16S rRNA gene, confirming their identity. The study revealed that the local Shigella strains showed a lot of genetic variation and carried genes that make them more harmful and harder to treat with antibiotics.

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Background

Shigella spp. are Gram-negative, non-spore-forming, facultatively anaerobic bacilli of the Enterobacteriaceae family. Several species are among the most important bacterial pathogens causing bacillary dysentery (shigellosis), an important cause of morbidity and mortality in children, particularly in developing countries. The faecal-oral transmission route for Shigella via contaminated food or water, in combination with the low infectious dose of 10–100 organisms needed to cause infection, poses a significant public health risk (1, 2). The Shigella genus includes four major species: S. dysenteriae, S. flexneri, S. boydii, and S. sonnei, which have different geographic and epidemiological profiles. Although S. flexneri is the primary cause of endemic shigellosis in low-resource settings, S. sonnei is more predominant in industrialised settings. The strains of S. dysenteriae, S. flexneri, S. boydii, and S. sonnei are genetically distinct from Escherichia coli but harbour a distinct virulence gene set, most of which are encoded by a 220-kilobase invasion plasmid (pINV). The large invasion plasmid is a vector that contains the genes necessary for the pathogen's invasion of epithelial cells, including ipaH, invE, virA, and sigA, as well as the components of the Type III Secretion System (T3SS), a type of molecular syringe that allows the pathogen to inject effector proteins into host cells to hijack cellular processes en-route to enhancing its survival and replication (3, 4).

The ipaH gene family in Shigella codes for type III secretion system effectors that effectively manipulate host immune responses. By targeting critical signalling proteins such as NEMO and p65, these effectors suppress NF-κB-driven inflammation, thereby aiding bacterial survival within host cells (5). In contrast, the sigA gene encodes a serine protease autotransporter toxin responsible for damaging intestinal epithelial cells, which contributes to symptoms like diarrhoea and fever (6). The presence of these virulence genes correlates with more severe clinical symptoms and a greater level of antibiotic resistance.

Concomitant with their virulence, Shigella isolates have acquired significant resistance to commonly used antibiotics. There has been a notable increase in reports of outbreaks involving multidrug-resistant (MDR) strains of Shigella in the Middle East. These strains demonstrate major resistance to antibiotics such as ampicillin, trimethoprim-sulfamethoxazole, and tetracycline. Recent research from Iran and Egypt emphasises the growing challenge of resistance to fluoroquinolones and third-generation cephalosporins, further complicating treatment options. For example, in Iran, resistance rates to cephalosporins have varied between 7.3% and 57.7%, with up to 26.3% of isolates showing resistance to fluoroquinolones. Similarly, a study conducted in Egypt reported high resistance levels to ampicillin (88%), tetracycline (83%), and trimethoprim-sulfamethoxazole (75%), alongside moderate resistance to third-generation cephalosporins (7, 8). Such resistance is brought about by genes including blaTEM (β-lactamase), aadA1 (aminoglycoside-modifying enzyme), and qnrA (quinolone resistance), which are frequently found on mobile genetic elements such as plasmids, transposons, and integrons, allowing horizontal gene transfer and rapid spread in microbial communities (9, 10).

Due to the increasing global threat of antibiotic-resistant Shigella strains, as well as the necessity for accurate molecular tools for epidemiological surveillance and outbreak detection, this study aims to characterise clinical Shigella isolates molecularly by identifying the species distribution, detecting the main virulence genes (ipaH, invE, sigA), and screening key antibiotic resistance determinants (blaTEM, aadA1, qnrA). Furthermore, a phylogenetic analysis based on 16S rRNA sequencing was performed to determine the genetic relatedness of some local isolates to regional and global reference strains. This integrated molecular paradigm focuses on shedding new light on the pathogenic potential and antibiotic susceptibility profiling of circulating Shigella strains currently circulating in Al-Diwaniyah, Iraq, and providing a scientific basis for efficient surveillance, diagnosis, and treatment paradigms. This inquiry is critical to bridge the evidence gap in the Middle East context, especially in Iraq. It is one of the initial comprehensive molecular characterisations of Shigella isolates from Iraq, revealing near-universal blaTEM resistance (100%) and regional phylogenetic clustering

Materials and Methods

Inclusion and exclusion criteria

This study involved patients who showed symptoms of acute diarrhoea, were newly diagnosed, and had not received any prior treatment. Stool samples were carefully collected without contamination from patients at hospitals in Al-Diwaniyah, Iraq. To keep the bacteria alive, all samples were kept cool (between 4 and 8°C) and processed within two hours after collection. Patients were excluded if they did not have symptoms of acute diarrhoea, had taken antibiotics

before the sample collection, or if the samples were not collected, stored, or transported properly. Samples obtained from outside the hospitals in Al-Diwaniyah were also not included in the study.

Samples Collection
Stool samples were collected (between September and October 2024) aseptically from newly diagnosed symptomatic patients with acute diarrhoea from the hospitals in Al-Diwaniyah. Bacterial species samples were stored in sterile and leak-proof containers and transported at cool temperatures (4–8°C) and processed within two hours from collection to maintain bacterial viability (11).

Phenotypic Identification
Culture and Morphology
From each specimen, direct streaking on MacConkey Agar and Salmonella-Shigella (SS) Agar was performed. This was incubated at 37°C aerobically for 18–24 hours. All non-lactose fermenting, pale or colourless colonies on MacConkey (Beckton & Dickinson) and transparent colonies without black centres on SS agar (Himedia) were considered presumptive Shigella spp. For purification, colonies were sub-cultured on XLD Agar and Nutrient Agar (12). Motility was assessed using Sulfide Indole Motility (SIM) medium, which contained about 0.3%–0.5% agar.

Microscopy
Purified colonies were subjected to Gram staining. Typical Shigella cells were observed as short, Gram-negative rods, either singly or in pairs, and were non-motile, as seen under the light microscope.

Identification by 16S rRNA gene
DNA Extraction
Genomic DNA extraction from isolated colonies was conducted with the QIAamp DNA Mini Kit (Qiagen, Germany), according to the manufacturer's instructions. NanoDrop spectrophotometer (Thermo Scientific, USA) was used to assess the concentration and purity of DNA and stored at −20°C until used.

PCR Amplification of 16S rRNA Gene
Universal primers were used to amplify the 16S rRNA gene. PCR assays that focus on the ipaH gene demonstrate remarkable sensitivity and specificity for detecting Shigella, often surpassing traditional culture techniques (Table 1). For example, a research study conducted in Vietnam found that these assays had a 93% sensitivity rate in cases where cultures were positive. What's more, they identified the ipaH gene in 36% of patients who were culture-negative but did not exhibit dysentery symptoms, emphasising their superior detection capability. On the other hand, 16S rRNA PCR tests, while useful for broad-scope bacterial identification, tend to have lower specificity for Shigella. This is due to conserved genetic sequences shared among members of the Enterobacteriaceae family, which can increase the risk of contamination and false-positive results. Consequently, ipaH-based PCR stands out as the preferred method for accurate and reliable diagnosis of Shigella infections (13, 14).

Table 1: Primers used for 16S rRNA gene amplification

Primer	Sequence (5′ → 3′)	Reference
27F	AGAGTTTGATCMTGGCTCAG	(15)
1492R	CGGTTACCTTGTTACGACTT	

PCR was performed in a 25 µl reaction containing 12.5 µl of 2× Master Mix, 1 µl of each primer (10 µM), 2 µl of DNA template, and 8.5 µl of nuclease-free water.

Thermal cycling conditions (Table 2)

Table 2: Thermal Cycling Conditions for 16S rRNA Amplification

Step	Temperature	Time	Cycles
Initial Denaturation	94°C	3 min	1
Denaturation	94°C	30 sec	
Annealing	55°C	30 sec	30
Extension	72°C	90 sec	
Final Extension	72°C	5 min	1
Hold	4°C	∞	

Amplicons were separated on a 1% agarose gel stained with ethidium bromide and visualised under UV light. A 1 kb DNA ladder was used to confirm product size (~1500 bp).

Detection of Virulence and Antibiotic Resistance Genes

A total of 188 isolates were analysed. Following confirmation of Shigella isolates, virulence genes (ipaH, invE, sigA) and antibiotic resistance genes (blaTEM, qnrA, aadA1) were detected using gene-specific PCR.

Target Genes and Primers

The primer sequences used for the detection of each target gene, along with their expected product sizes and relevant references, are presented below in Table 3.

Table 3: Primers for Detection of Virulence and Resistance Genes

Gene			Product Size	Reference
ipaH	F (5′–3′) R (5′–3′)	GTT CCT TGA CCG CCT TTC CGATAC CGTC GCC GGT CAG CCA CCC TCT GAGAGT AC	619 bp	(16)
invE	F (5′–3′) R (5′–3′)	CGATAGATGGCGAGAAATTATATCCCG CGATCAAGAATCCCTAACAGAAGAATCA	766 bp	(17)
sigA	F (5′–3′) R (5′–3′)	CCGACTTCTCACTTTCTCCCG CCATCCAGCTGCATAGTGTTTG	430 bp	(18)
blaTEM	F (5′–3′) R (5′–3′)	ATAAAATTCTTGAAGACGAAA GACAGTTACCAATGCTTAATC	1080 bp	(19)
qnrA	F (5′–3′) R (5′–3′)	ATTTCTCACGCCAGGATTTG GATCGGCAAAGGTTAGGTCA	516 bp	(20)
aadA1	F (5′–3′) R (5′–3′)	TATCAGAGGTAGTTGGCGTCAT GTTCCATAGCGTTAAGGTTTCATT	484 bp	(21)

PCR Reaction and Cycling Conditions

PCR reactions were performed in a total volume of 25 µl containing:
12.5 µl of 2× PCR Master Mix
1.0 µl of forward primer
1.0 µl of reverse primer
2.0 µl of DNA template
8.5 µl of nuclease-free water

The thermal cycling protocol was as follows (Table 4):
Initial Denaturation: 94°C for 5 min
30 Cycles of:
Denaturation: 94°C for 30 sec
Annealing: as specified per gene below (30 sec)
Extension: 72°C for the specified duration
Final Extension: 72°C for 10 min
Hold: 4°C

Table 4: PCR Conditions for Each Gene

Gene	Annealing Temperature	Extension Time	Product Size
ipaH	58°C	60 sec	619 bp
invE	56°C	60 sec	766 bp
sigA	58°C	45 sec	430 bp
blaTEM	55°C	90 sec	1080 bp
qnrA	53°C	45 sec	516 bp
aadA1	63°C	60 sec	484 bp

Gel Electrophoresis Protocol

To confirm the presence and size of the PCR amplification products, agarose gel electrophoresis was performed. 1.5% agarose gel was made by dissolving agarose powder in 1× TBE buffer and heating till completely melted. After cooling to ~60 °C, ethidium bromide (EtBr) at a final concentration of 0.5 µg/mL was added for visualisation of DNA bands by UV light. Of each sample, 5 µL of PCR products were supplemented with 1 µL of 6× loading dye and loaded into the wells. A 100 bp DNA ladder served as a molecular weight marker. Electrophoresis was performed for one hour at 100 V. All gels were visualised on a UV transilluminator (Bio-Rad GelDoc XR+) after the run, and gel images were taken with a gel documentation device (Azure Biosystems c600). Band sizes were estimated by comparing with the DNA ladder. To ensure the accuracy of each PCR run, negative controls, such as a no-template control (NTC), were systematically included. These controls serve to monitor any potential contamination or nonspecific amplification,

providing an essential check within the experimental process.

Results

Phenotypic Identification of Shigella spp.

Presumptive Shigella colonies were identified by classical microbiological characteristics. The colonies on MacConkey Agar were pale and did not ferment lactose. Colonies on Salmonella Shigella (SS) Agar were colourless and did not evolve Hydrogen Sulfide (H_2S). On staining with Gram stain, small Gram-negative short rods in single or pairs, non-motile. The former traits aided in their provisional identification before molecular verification.

Genetic Confirmation by 16s rRNA Genes

Molecular testing of the isolates involved using genomic DNA from phenotypically identified isolates for polymerase chain reaction (PCR) with universal bacterial primers (27F/1492R) to amplify the 16S rRNA gene. All tested isolates produced a single band of ~1500 bp, confirming their classification under the genus Shigella (Figure 1).

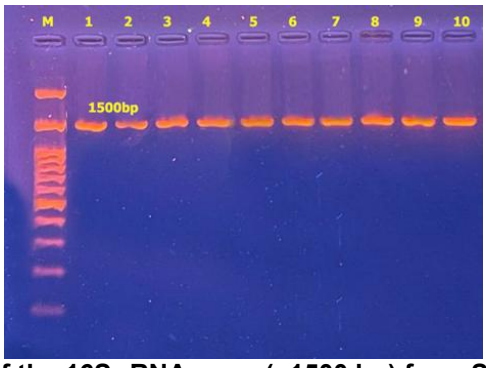

Figure 1. PCR amplification of the 16S rRNA gene (~1500 bp) from Shigella isolates. Lane M: 3 kb DNA ladder; Lanes 1–10: positive amplification

Genetic Analysis Based on Phylogenetic and Alignment

For further characterisation of the local Shigella isolates' evolutionary relationships, two levels of molecular comparison were carried out

Multiple Sequence Alignment (ClustalW)

DNA sequences of the partial 16S rRNA gene were aligned using the ClustalW algorithm, and the resultant alignment displayed highly conserved regions as well as nucleotide polymorphisms between isolates (Figure 2).

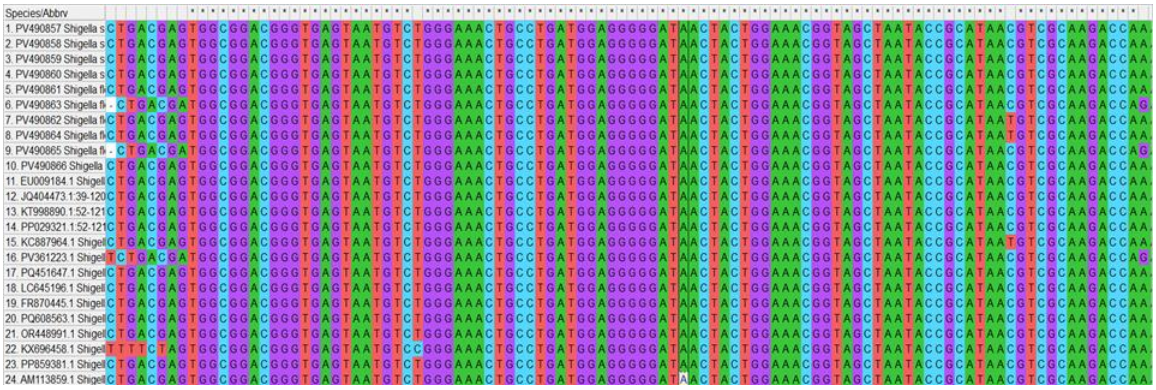

Figure 2: ClustalW was used to align multiple sequences
The base sequence is shown from left to right; asterisks (*) denote conserved bases, and coloured bases represent genetic differences between taxa

Phylogenetic Tree Reconstruction (MEGA11)

MEGA11 was performed on aligned sequences using the Neighbour-Joining method with 1000 bootstrap replicates. Values ≥70% were indicative of strong clade support. The resulting tree indicated that ZAQEB1–ZAQEB4 formed a cluster with Shigella sonnei isolates of Pakistan, India and Spain. The ZAQEB5–ZAQEB9 isolates were

closely clustered with Shigella flexneri isolates from China, Iraq, and Bangladesh.
ZAQEB10 clustered with Shigella dysenteriae strains from Iran and Ireland in the phylogenetic tree. The phylogenetic comparison reference sequences were downloaded from GenBank (NCBI) as follows:

S. sonnei: CP000038 (India), CP006038 (Pakistan)
S. flexneri: CP000266 (Bangladesh), CP024483 (China)
S. dysenteriae: CP000034 (Iran), CP007446 (Ireland)

From these, we chose these sequences as they were geographically and epidemiologically relevant to examine phylogenetic clustering with local isolates (Figure 3).

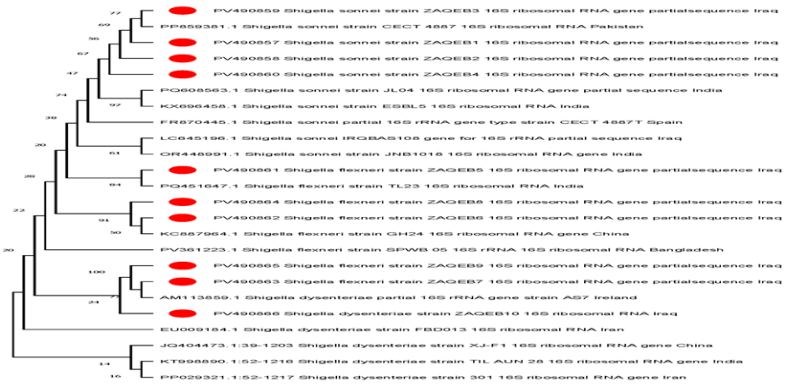

Figure 3: Shigella phylogenetic tree constructed from ClustalW alignment and MEGA11 Neighbour-Joining (with 1000 bootstraps; Nodes with bootstrap support ≥70% are labelled. Red circles indicate local isolates

Potential biases in this analysis stem from the limited resolution of 16S rRNA sequencing when it comes to distinguishing between closely related bacterial species, which can result in an underestimation of diversity within a single species. Besides, biases introduced during PCR amplification, such as primer mismatches, may distort the true representation of microbial community composition.

PCR-Based Detection of Virulence and Resistance Genes

Identification of the sigA Gene (430 bp)

Additionally, we confirmed the presence of the sigA gene responsible for producing an epithelial-disrupting serine protease in selected isolates, confirming the contribution of this factor to the overall virulence profile of S. flexneri. Amplification bands of 430 bp were visible in lanes 1, 2, 5, 6 and 7 (Figure 4).

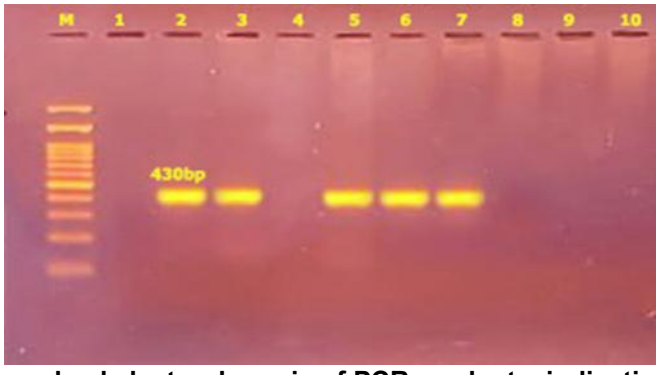

Figure 4: PCR analysis and gel electrophoresis of PCR products, indicating amplification of sigA gene at 430 bp among different isolates
PCR detection of the virulence gene sigA (430 bp). Well-amplification bands were observed in lanes 1 (ZAQEB1), 2 (ZAQEB2), 5 (ZAQEB5), 6 (ZAQEB6), and 7 (ZAQEB7). The rest of the lanes were negative

Detection of the aadA1 Gene (484 bp)
The aadA1 conferred resistance to aminoglycosides (streptomycin). The 484 bp clear amplification bands were observed in lanes 1, 2, 3, 6, 7, 8 and 10, showing the dissemination of the aminoglycoside resistance (Figure 5).

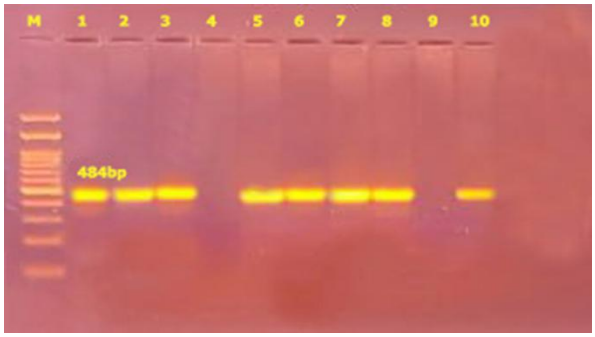

Figure 5: PCR Amplification of the aadA1 Gene. Resistance-positive detection of multiple isolates at 484 bp
PCR detection of the resistance gene aadA1 (484 bp). Well-amplification bands were observed in lanes 1 (ZAQEB1), 2 (ZAQEB2), 3 (ZAQEB3), 6 (ZAQEB6), 7 (ZAQEB7), 8 (ZAQEB8), and 10 (ZAQEB10). The rest of the lanes were negative

invE Gene Detection (766 bp)
The invE gene controls the type III secretion system and is required for invasion (22). The detection of a clear band at 766 bp in all lanes indicated a high prevalence of this genotype among isolates (Figure 6).

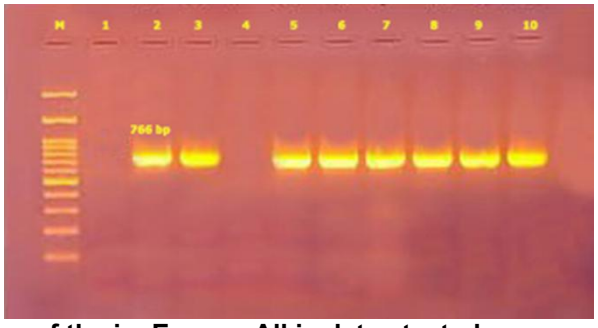

Figure 6: PCR amplification of the invE gene. All isolates tested were positively detected at 766 bp
PCR amplification profile of virulence gene invE (766 bp). Strong bands were observed in lanes 1 (ZAQEB1) to 10 (ZAQEB10), confirming universal detection of this gene in the isolates under test

Detection of the ipaH Gene (619 bp)
The ipaH gene, a multicopy marker found in all Shigella spp., was also detected in almost all isolates with the appearance of unique bands at 619 bp in lanes 1 to 9. This validates genus-level identity and its potential as an invader (Figure 7).

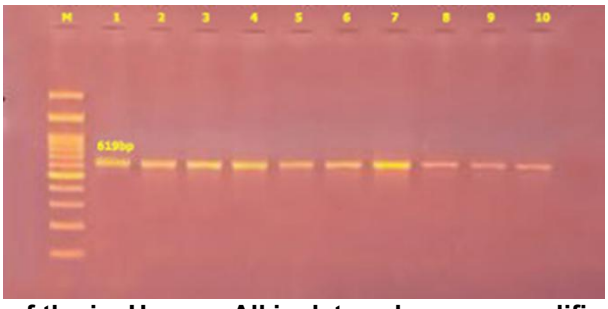

Figure 7: PCR detection of the ipaH gene. All isolates show an amplified product of size 619 bp
PCR amplification profile of virulence gene ipaH (619 bp). Distinct bands were invariably observed in lanes 1 (ZAQEB1) to 9 (ZAQEB9), while lane 10 (ZAQEB10) did not show any amplification

Detection of blaTEM Gene (1080 bp)
The blaTEM gene generates a β-lactamase enzyme that protects penicillin. We observed strong bands at 1076 bp in all ten lanes, indicating a wide βlactam resistance. Overall, blaTEM was detected in 100% (188/188) of the isolates (Figure 8).

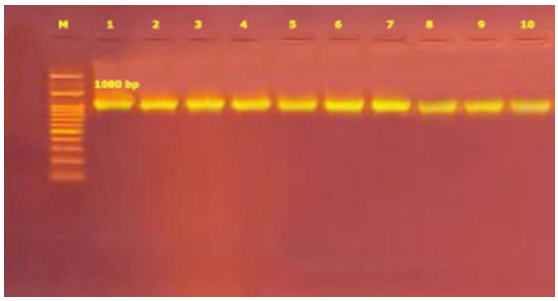

Figure 8: Detection of blaTEM gene. 1080 bp PCR confirmed high expression in all isolates
PCR screening for the resistance gene blaTEM (1080 bp). Distinct amplification bands were seen for all lanes, ranging from 1 (ZAQEB1) to 10 (ZAQEB10), confirming universal detection of this gene in the isolates under test

Identification of pic Gene (572 bp)
The pic gene encodes a serine protease autotransporter that plays a role in both host colonisation and inflammation. All isolates exhibited bands at 572 bp, showing that it was widely present (Figure 9).

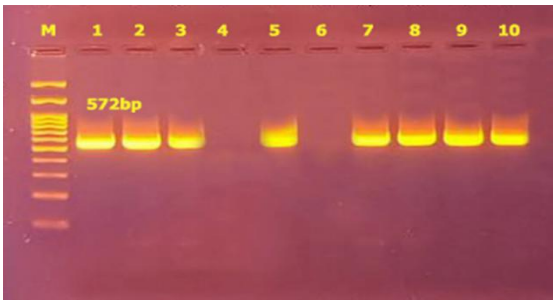

Figure 9: Amplification of the pic gene. All isolates showed presence at 572 bp
Amplification by PCR of the virulence gene pic (572 bp). Clear bands were observed repeatedly in all the lanes, ranging from 1 (ZAQEB1) to 10 (ZAQEB10), which indicates widespread dissemination of this gene among all examined isolates

To further describe the molecular profile of the Shigella isolates, we summarised the detected frequency of virulence (ipaH, invE, sigA) and antibiotic resistance (blaTEM, qnrA, aadA1) genes by species. The distribution of genes among S. flexneri, S. sonnei and S. dysenteriae is outlined in Table 5. This disparity points to interspecies diversity, with the frequency of virulence sigA more pronounced in S. flexneri than in S. sonnei, and a wider carriage of resistance genes in the latter, most notably aadA1 and qnrA.

Table 5: Distribution of the detected virulence and resistance genes

Species	No. of Isolates	ipaH	invE	sigA	blaTEM	qnrA	aadA1
S. flexneri	35	35	35	5	35	12	7
S. sonnei	40	40	40	0	40	18	10
S. dysenteriae	13	13	13	0	13	5	0
Total	88	88	88	5	88	35	17

Higher sigA prevalence in S. flexneri correlates with severe dysentery in regional studies

Discussion
In this study, we showed deep molecular characterisation of Shigella isolates, including taxonomic verification, virulence gene features, antimicrobial resistance genes, and phylogenetic data. Although amplification of the 16S rRNA gene

delivered good specificity and reliability, it is nonetheless a molecular gold standard for the initial identification of Shigella spp. While its sequence conservation reduced discriminatory power lower than the subspecies level, this does not compromise its strength for confirming Shigella at the genus level (23). All isolates in this study produced the expected genus-specific ~1500 bp amplicon, further confirming their classification in the genus.

Phylogenetic analysis of the 16S rRNA sequences showed clustering into separate lineages corresponding to S. flexneri, S. sonnei and S. dysenteriae. The high relatedness of the local isolates to geographically disparate reference strains indicated recent cross-border transmission, or convergent evolution, consistent with patterns seen in global surveillance reports (24). Reference strains were selected based on geographic proximity and epidemiological relevance to Iraq.

There was a high level of conservativeness in the virulence marker ipaH as it was considered within multiple copies on the chromosome and plasmid of Shigella, improving detection sensitivity (25), and all isolates were positive for the ipaH gene. Other than ipaH, high frequencies of invE, sigA, and pic were detected, indicating that the isolates had great virulence potential. Others were related to epithelial invasion (invE), serine protease–mediated mucosal damage (sigA), and mucosal immune modulation (pic), similar to findings in South Asia, where sigA was associated with severe dysentery (26, 27).

Detection of pic in all isolates tested suggests its epidemiological significance as a marker for increased pathogenicity. Pic was the only confirmed Serine Protease Autotransporters of Enterobacteriaceae (SPATE) gene in this dataset, indicating its potential involvement in inflammation and colonisation within the local Shigella population. At the species level, S. flexneri strains exhibited a more diverse virulence gene profile, including sigA and invE, whereas S. sonnei isolates possessed antibiotic resistance genes like aadA1 and blaTEM with higher frequency. This is consistent with previous regional studies that showed S. flexneri contributes additional virulence, and S. sonnei with broader multidrug resistance (MDR) profiles (6, 28).

Most of the isolates were resistant to multiple classes of antibiotics, showing widespread presence of blaTEM, aadA1 and qnrA genes. Specifically, blaTEM was found in all isolates (88/88; 100%), indicating universal β-lactamase-mediated resistance. Extended-spectrum β-lactamase (ESBL) production and susceptibility to penicillin are specifically suggested by the presence of blaTEM. Moreover, qnrA detection indicated emerging plasmid-mediated quinolone resistance, like findings from surveillance studies in the adjacent regions (29, 30, 31).

These findings underscore the clinical threat posed by MDR Shigella strains, as well as the need for robust public health strategies to mitigate their transmission. This high prevalence of resistance genes in our study, particularly in S. sonnei, reflects the abuse of antibiotics and calls for the implementation of antibiotic stewardship programmes.

The high frequency of virulence and resistance genes detected in Diwaniyah hospitals isolates, from a public health point of view, suggests the need for multidimensional intervention. Better sanitation and hygiene infrastructure are paramount in lowering transmission (32). Rational antibiotic usage, guided by local resistance patterns, is equally critical. Development of vaccines, however, may utilise conserved virulence factors such as ipaH as potential candidates (•6), and underpinning surveillance programs with whole-genome sequencing (WGS) will help in early detection of resistance mechanisms, thereby aiding in region-specific treatment regimens (32, 33).

The patterns of virulence and resistance genes identified in this study show both similarities and differences compared to reports on *Shigella* strains from various regions. For example, the high prevalence of *ipaH* and *pic* genes in our isolates aligns with findings from Egyptian and Saudi Arabian strains, where these genes have been associated with severe clinical outcomes (34, 35). Conversely, unlike strains from North Africa, which often carry *set1A/B* genes related to Shiga toxin production (36), our isolates did not exhibit these genes, indicating possible regional differences in pathogenicity mechanisms. Besides, the predominance of *blaTEM* and *aadA1* in Iraqi *S. sonnei* isolates resemble patterns observed in Iran and Turkey (29, 37), yet contrasts with West African strains where *dfrA1*-mediated trimethoprim resistance is more widespread (38). The absence of *qnrS*, commonly found in Southeast Asia, along with the presence of *qnrA* in 39.8% (35/88) of isolates signals emerging fluoroquinolone resistance, mirroring trends in Iran but contrasting with low qnrS prevalence in Southeast Asia, and emphasises region-specific quinolone resistance patterns (30, 39). These variations likely reflect differences in antibiotic administration policies, environmental factors, or the dissemination of clones. Such comparative

analyses emphasise the importance of region-specific surveillance to inform targeted treatment protocols and control measures.

Phylogenetic reconstruction based on 16S rRNA sequences revealed distinct clustering of isolates into well-supported monophyletic clades corresponding to S. flexneri, S. sonnei, and S. dysenteriae. High bootstrap values (>90%) at major nodes confirm robust evolutionary relationships within these clades, indicating strong statistical confidence in the branching patterns (40). The clear separation of species-specific lineages aligns with traditional serotyping and whole-genome studies (41), reinforcing the utility of 16S rRNA for genus-level identification despite its limited resolution for subtyping.

Notably, intra-clade diversity was observed among S. flexneri isolates, with moderate bootstrap support (70–85%) for sub-lineages, suggesting potential microevolution within local strains. In contrast, S. sonnei isolates formed a tight cluster (bootstrap = 95%) with minimal genetic divergence, consistent with its clonal population structure (42). The high relatedness of local isolates to global reference strains (e.g., Middle Eastern and South Asian sequences) may reflect recent cross-border transmission or convergent evolution under similar selective pressures, as reported in global surveillance (24, 43).

The results of this study bring to light several pressing public health issues that need urgent attention in Iraq, especially within the Diwaniyah region. The widespread presence of multidrug-resistant (MDR) strains, notably the near-ubiquitous detection of blaTEM and the emerging qnrA genes, compromises the effectiveness of standard antibiotics such as ampicillin and nalidixic acid (44). Compared to neighbouring countries like Iran and Turkey, where blaTEM rates are 20–25% lower, the resistance patterns in Iraq are notably more severe (45). These disparities clearly emphasise the importance of developing treatment guidelines personalised specifically for Iraq.

In the short term, clinical protocols for empirical antibiotic use in dysentery cases must be revised. Considering the resistance profiles identified, antibiotics like azithromycin or third-generation cephalosporins are preferable first-line treatments over traditional penicillins or quinolones (46). Healthcare facilities should adopt mandatory quarterly antibiogram updates to closely monitor changing resistance trends, especially for qnrA, which indicates a rising quinolone resistance (47). Integrating these strategies into Iraq's national antimicrobial stewardship efforts is important.

Infection control measures must not be overlooked. The phylogenetic evidence of clonal clusters of S. sonnei points towards possible hospital-acquired transmission (48). Hospitals must enforce strict isolation procedures for patients infected with MDR Shigella and step up chlorine-based disinfection protocols in paediatric wards, an essential step, given children's heightened vulnerability (49). Parallel efforts are critical in community settings: targeted campaigns promoting proper hand hygiene with soap and safe water storage practices could considerably curb transmission, particularly among households with children under five, who experience the highest disease burden (50).

Looking ahead, strategies should include exploring vaccine development. The consistent detection of the ipaH gene across all isolates makes it a promising candidate for future vaccine formulations (•5). Besides, expanding whole-genome sequencing (WGS) surveillance would allow real-time monitoring of emerging resistance patterns and virulent strains, following successful models implemented elsewhere in the Middle East (51).

The co-occurrence of virulence and resistance genes in Iraqi Shigella strains presents a serious public health challenge but also offers a clear pathway for intervention. Combining antibiotic stewardship, strict infection control, community-based prevention, and innovative technologies like WGS could enable Iraq to stem the tide of these dangerous pathogens. This is especially urgent given the regional context, phylogenetic links to strains from Saudi Arabia and Iran suggest cross-border transmission is occurring, transforming Shigella control from a local issue into a broader regional priority (52). To enhance public health, it is critical to ensure the immediate (1) revising Iraqi dysentery guidelines to prioritise azithromycin over penicillins, (2) hospital hygiene audits, and (3) community handwashing campaigns targeting schools

Although this study offers meaningful insights, caution should be exercised when interpreting the results due to several limitations: the relatively small sample size, the lack of phenotypic MIC validation for the genotypic resistance markers, and the reliance on 16S rRNA sequencing instead of whole-genome sequencing for phylogenetic analysis. These factors may restrict the ability to distinguish strains at a finer, strain-specific level.

Conclusion

The molecular diversity of circulating Shigella strains in Diwaniyah hospitals was emphasised in the present study, with significant co-occurrence of virulence and multidrug resistance genes within the

local Shigella isolates. To address Iraq's pressing issue of high rates of multidrug-resistant (MDR) Shigella characterised by increased blaTEM prevalence and the emergence of qnrA-mediated resistance, it is essential to implement immediate measures. These include updating empirical treatment guidelines to favour azithromycin and third-generation cephalosporins, enforcing strict hospital disinfection and patient isolation protocols, initiating widespread community hygiene education campaigns, and prioritising the development of vaccines targeting ipaH. Concurrently, strengthening regional surveillance systems is essential to monitor and mitigate cross-border transmission risks. These findings underscore the urgent need for WGS-enhanced surveillance and regionally tailored antibiotic stewardship to curb MDR Shigella transmission in Iraq and neighbouring regions

List of Abbreviations

MDR: Multidrug resistance
T3SS: Type III Secretion System
SS: Salmonella-Shigella
SPATE: Serine Protease Autotransporters of Enterobacteriaceae
H_2S: Hydrogen Sulfide
invE: Epithelial invasion
ESBL: Extended-spectrum β-lactamase
WGS: Whole-genome sequencing

Declarations

Ethical approval and consent to participate

The study was approved by the Scientific and Ethical Committee of the College of Education, University of Al-Qadisiyah (No. 62883). All procedures were in line with the Declaration of Helsinki. Informed consent was obtained, and confidentiality was maintained.

Consent for publication

All the authors gave consent for the publication of the work under the Creative Commons Attribution-Non-Commercial 4.0 license.

Availability of data and materials

The data and materials associated with this research will be made available by the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

RZA and AAAS contributed equally to the study conception and design, laboratory experiments, data analysis, and interpretation. RZA drafted the initial manuscript, and AAAS made critical revisions for important intellectual content. Both authors read and approved the final manuscript.

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