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MOLECULAR ANALYSIS OF VIRULENCE GENES IN *SHIGELLA* SPECIES ISOLATED FROM FAECAL SAMPLES OF GASTROINTESTINAL TRACT PATIENTS

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Abstract

Shigellosis is still a serious public health issue in countries with middle and low incomes, particularly in South Asia, where it is spread by crowded cities and poor sanitation. Shigella species are major causes of bacillary dysentery and are associated with considerable morbidity and mortality, especially in children. Shigella species are linked to significant morbidity and mortality, particularly in children, and are primary causes of bacillary dysentery. Molecular epidemiology studies are crucial for directing treatment and control methods since the spread of virulence genes and the advent of MDR (multidrug-resistant) strains make clinical management even more difficult. Between March and August 2025, this study examined the molecular traits, virulence factors, and patterns of antibiotic resistance of Shigella species isolated from patients suffering from gastrointestinal tract infections in District Peshawar, Khyber Pakhtunkhwa, Pakistan. This study investigated the molecular characteristics, virulence determinants, and antimicrobial resistance patterns of Shigella species isolated from patients with gastrointestinal tract infections in District Peshawar, Khyber Pakhtunkhwa, Pakistan, between March and August 2025. Total 400 stool samples were collected from major tertiary hospitals, of which 120 isolates were confirmed as Shigella spp. through biochemical testing and serological analysis. The predominant species was S. sonnei (74%), followed by S. flexneri (24%) and S. boydii (2%). Molecular screening of virulence genes revealed that ipaH (95.4%) and ial (75.1%) were the most prevalent, while set1B was detected at lower frequency (7.8%). Notably, eight S. sonnei isolates carried the Shiga toxin (STX) gene, traditionally restricted to S. dysenteriae type 1, and were associated with severe clinical outcomes. Antimicrobial susceptibility testing showed alarming resistance to cotrimoxazole (90.4%) and tetracycline (79.5%), alongside moderate resistance to nalidixic acid (45.8%) and ampicillin (42.2%). Lower resistance rates were recorded for azithromycin (17.0%), ciprofloxacin (12.0%), and cefotaxime (6.0%), with gentamicin (3.0%) and cefixime (3.6%) remaining highly effective. Furthermore, PMQR genes were detected in 45.8% among Shigella species, with qnrA, qnrS, and qnrD being the most common, and multi-gene combinations frequently observed. The strong association between PMQR positivity and nalidixic acid resistance highlights a growing threat of

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fluoroquinolone resistance. Overall, these findings underscore the predominance of S. sonnei, the heterogeneity of virulence genes, and the emergence of multidrug resistance, emphasizing the urgent need for rational antibiotic stewardship and continuous molecular surveillance to guide effective treatment strategies against shigellosis in Pakistan.

INTRODUCTION

Consumption of contaminated food causes the diseases which in severe case may lead to hospitalisation, or life loss. Foodborne diseases constitute the significant worldwide health issue, and it is caused by the intake of contaminated food or water that houses microorganisms, toxins, chemicals, or parasites. They are also one of the most prevalent popular health issues with millions of individuals being affected annually in developed as well as developing nations. Shigellosis is an extremely contagious diarrheal infection brought about by bacteria of the genus *Shigella*. It remains a major international health issue especially in the low and middle-income nations that are characterised by poor sanitation, clean water availability and health facilities. It is the cause of approximately two million ailments and 23,000 mortalities annually (Dallal *et al.*, 2020) it is mostly transmitted through fecal-oral route with contaminated food, water or direct contact between a person and another. Due to its low infectious dose, any exposure of small quantity on the pathogen may result in symptomatic infection, hence outbreaks occur very often in congested and resource-limited environments (Mardaneh *et al.*, 2013). Poor hygiene habits, a lack of accessibility to clean water, and insufficient food safety laws all are contributing to the high prevalence of foodborne illnesses in low- and middle-income nations. The elderly, those with compromised immunity, pregnant women, and children younger than five are particularly susceptible to serious consequences. Common causative agents include bacteria such as Salmonella, *Shigella*, Escherichia coli, and Listeria, as well as viruses like norovirus and hepatitis A, in addition to a range of parasites and chemical contaminants (Libby et al., 2023). The high transmissibility of the disease is explained by its rapid spread, as well as by a low infectious dose; only 200 cells are required to be infected (Shane et al., 2017). More than 90% of *Shigella* infections worldwide are caused by the strains *S. flexneri* and *S. sonnei*, which also have the greatest frequency of any of the four *Shigella* species worldwide. Both *Shigella boydii* and *Shigella dysenteriae* are two more species. *S. flexneri* is the most frequent cause of diarrhea, particularly in underdeveloped nations, particularly in Asia, while *S. sonnei* is more common in developed nations (Kahsay & Muthupandian, 2016). *Shigella* causes some 80 million cases and 600,000 deaths each year worldwide, a large percentage of which are in children. Despite the fact that the mortality rate of shigellosis remains low in different environments and among different populations. Its level is still quite high, especially among children younger than five years. Geographic location, environmental factors and serotype of the involved *Shigella* all contribute to the burden of disease (Dabbagh et al., 2014).

The management of shigellosis primarily focuses on rehydration, symptomatic relief, and targeted antimicrobial therapy. Since the disease often presents with diarrhea and fluid loss, oral or intravenous rehydration remains the cornerstone of treatment, especially in children and elderly patients who are at a greater risk of dehydration. Nutritional support is equally important to prevent malnutrition and aid recovery in vulnerable populations (Guarino *et al.*, 2020). Shooraj *et al.*, (2024) believe that the correct antibiotic prescribed by the doctor can be used to treat the infection, thus preventing many symptoms and the possible fatal outcome. In the large majority, a decent antimicrobial therapy has become more difficult to choose since the number of clinical cases of *Shigella* resistant to a range of antibiotics has increased. In the backdrop of increasing antimicrobial resistance, research on the effective vaccines and new

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therapeutic agents has gained a priority as a public health concern. There are preventive measures such as safe water supply, sanitation, and high-level hygiene measures which should be applied to minimize disease burden and limit the transmission of resistant strains (Gonabadi *et al.*, 2024).

Pathogenesis of *Shigella* is both toxinogenic and invasive and interspecies variation is significant. Invasion of cells through the T3SS is the basic process through which non-dysenteria species such as *S. flexneri* and S. sonnei cause the manifestation of diseases. The system produces effector proteins including IpaA-D and IcsA. The proteins assist bacteria entry into epithelial cells, escape of the bacteria out of the vacuoles, intracellular motility through actin, and localized inflammation and mucosal damage that eventually results in dysentery (Aslam et al., 2024). Conversely, Shiga dysenteriae type 1 produces Shiga toxin (Stx) is a strong exotoxin that results from the binding of B subunits by enzyme A. The B subunit targets the glycolipid receptors in host namely Gb3. Conversely, A subunit causes inactivation of the 60S ribosomal subunit leading to the termination of protein synthesis, cell death and intense mucosal damage (Scott et al., 2025). Once systemic, Shiga toxin damages endothelial cells in the kidneys and central nervous system, leading to microvascular thrombosis, hemolysis, thrombocytopenia, and renal failure, clinically manifesting as hemolytic-uremic syndrome (HUS) (Travert et al., 2021). Additionally, Shiga toxin induces innate immune responses, including the unfolded protein response, apoptosis, complement activation, and cytokine release, which further amplify tissue injury and inflammation (Lee & Tesh, 2019).

Shigella pathogenicity hinges on a dual mechanism: intracellular invasion and, in some species, toxin-mediated injury. Non-dysenteriae species such as Effector proteins (IpaB, IpaC, and IcsA) are injected into epithelial cells by the bacteria S. flexneri and S. sonnei, which use a T3SS to accomplish this triggering actin remodeling, cell-to-cell spread, and localized colonic inflammation (Miles et al., 2024). In contrast, S. dysenteriae type 1 produces the potent Shiga toxin (Stx), an AB5 exotoxin whose B subunit binds to Gb3 on host cells (especially renal and neural tissues), and whose A subunit halts protein synthesis by inactivating the 60S ribosomal subunit resulting in epithelial damage and systemic injury such as hemolytic–uremic syndrome (HUS) (Menge, 2020). Disease severity in shigellosis is shaped by a convergence of bacterial, host, and environmental factors. Bacterial determinants include toxin type, virulence gene profile, and antibiotic resistance mechanisms, which modulate pathogenic potential and treatment outcomes (Nasser et al., 2022). From the host perspective, extremes of age, malnutrition, and immunosuppression significantly heighten risk and morbidity. Environmental factors, including inadequate sanitation, water contamination, crowding, and lack of health care services contribute to transmission and the negative impact of shigellosis on the general population, especially within low-income environments (Bengtsson et al., 2022).

During the period between the years 2000 and 2020, antibiotic-resistant Shigella species were particularly prevalent. Empirical treatment guidelines are no longer based on the prescription of antibiotics such as β-lactam antibiotic, doxycycline, Minocycline, sulfadiazine, Sulfamethoxazole, and Co-trimoxazole. This is because the global resistance of Shigella isolates is increasing at an alarming rate. Quinolones taken orally are currently the treatment of choice for practically all cases of shigellosis, whether they are suspected or proved. Furthermore, as a consequence of the inappropriate utilization of ciprofloxacin in the treatment of shigellosis, fluoroquinolone-resistant Shigella isolates are becoming increasingly prevalent all over the world. Since these strains are thought to have originated from a common ancestor in South Asia, it is hypothesized that they have spread beyond their initial geographic region after the year 2007 (Chung et al., 2021).

The genetic basis of quinolone resistance in Shigella is primarily linked to chromosomal mutations and the acquisition of resistance genes through mobile genetic elements. Mutations in

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the quinolone resistance–determining regions (QRDRs) of the gyrA and parC genes, which encode subunits of DNA gyrase and topoisomerase IV respectively, reduce the binding affinity of quinolones, thereby conferring resistance (Hirsch & Klostermeier, 2021). Moreover, plasmid-mediated resistance to quinolone (PMQR) including the expression of qnr genes, efflux pump regulators (oqxAB, qepA) and aminoglycoside acetyltransferase variants (aac(6')-Ib-cr) also increase the resistance and contribute to its horizontal transmission among bacterial populations. These genetic determinants do not only cause therapeutic problems but also increase the speed of the dissemination of resistant strains of Shigella globally, which is why the constant monitoring and the creation of those alternative treatment methods are necessary (Urban et al., 2022).

It is a complex interplay between the pathophysiological processes that makes Shigella a virulent bacteria and enables it to invade, survive and damage host gastro intestinal. The infection begins when Shigella enters intestinal epithelial cells via an M cell, and colonic epithelial cells via a typeT3SS is accountable of injecting effector proteins, including invasion plasmid antigens (Ipa proteins), to facilitate infection and cytoskeletal remodeling processes (Bliven and Lampel, 2017). Shigella can then escape the phagocytic vacuole into the cytoplasm where it replicates and disperses intercellularly by actin-based motility mediated by IcsA (VirG). VirA promotes vacuolar escape and IpaH proteins regulate host immune signaling to suppress inflammatory responses to ensure survival. Moreover, Shigella as well as certain strains produces Shiga toxins, they inhibit the production of proteins in the cells of the host and might result in consequences that are potentially fatal, such as hemolytic uremic syndrome (HUS). The severe inflammatory reaction caused by Shigella causes cells of the epithelium to die, ulcerate and disrupt the mucosal lining, causing the characteristic signs of dysentery, comprising bloody diarrhea, abdominal pain, and fever (Nasser et al., 2022).

METHODOLOGY

Study Design

This study was employed a descriptive cross-sectional design to examine Shigella in District Peshawar, KPK, Pakistan, March from to August 2025.

Sample Collection

A clinical sample, such as Stool 120, was collected from a total of 400 samples sourced from various hospitals in the Peshawar region, including Khyber Teaching Hospital, Lady Reading Hospital, Hayatabad Medical Complex, Rehman Medical Institute, and Northwest General Hospital. Subsequently, the samples were processed under aseptic conditions at the microbiology laboratory of the Sarhad Institute of Allied Health Sciences, SUIT, Peshawar.

Sample Size

Following the collection of 400 samples, Yamane's formula (n = N/N(e)2) was used to determine the sample size. The study population (2412000 people in Peshawar city), N, the sample size, and e, the expected margin error in the computation (5%, or 0.05), are among the variables employed in this formula

Selection criteria for shigellosis group

Inclusion

1) All patients, regardless of their age, were enrolled in this research study.
2) Patients presenting with signs or symptoms of gastrointestinal tract disease were included.

Exclusion

1) Patients with diseases unrelated to the gastrointestinal tract were excluded.
2) Patients that were already receiving antibiotic treatment were excluded.

Sample processing

After collecting fecal samples from patients within a span of two hours, the specimens were immediately transferred into Cary-Blair transport medium to preserve bacterial viability. As soon as the specimens were collected, they were all

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transported to the microbiology laboratory within a 24-hour period. Following their arrival, they were inoculated onto selective and differential media, which included MacConkey agar and Xylose Lysine Deoxycholate (XLD) agar. This was then followed by aerobic incubation at 37 degrees Celsius for 18–24 hours. Recultures were performed on colonies that were thought to contain Shigella. These colonies were commonly observed as pale, non-lactose fermenters on MacConkey agar and red colonies that lacked the formation of hydrogen sulfide on XLD agar. The goal of these recultures was to produce pure isolates. For the purpose of preliminary characterisation, these purified colonies were subsequently subjected to Gram staining and a series of biochemical tests.

Biochemical identification

The isolates were biochemically identified using the standard protocols outlined in Bergey's Manual of Systematic Bacteriolog.

Gram Staining

Gram staining was carried out to identify the morphological features and cell wall characteristics of the bacterial isolates. For this purpose, a thin smear of each culture was prepared on a sterile glass slide and fixed by gentle heating. The fixed smear was first treated with crystal violet, which served as the primary dye, and then with Gram's iodine, allowing the formation of a stable dye–iodine complex. To differentiate the organisms, 95% ethanol was applied briefly; this step removed the primary stain from Gram-negative bacteria while Gram-positive cells retained it. Safranin was then used as a counterstain to impart a contrasting color. Under the light microscope, the dyed slides were examined using the oil immersion objective, which had a magnification of 100 times. Gram-positive bacteria were distinguished from Gram-negative bacteria by their purple pigmentation, whereas Gram-negative bacteria have a pink appearance. The separation of the isolates was accomplished using this staining technique as the initial basis for classification.

Motility Test

Motility was tested using semi-solid motility agar prepared in glass tubes. Each tube contained medium with 0.4% agar concentration and was inoculated by a single stab with a sterile needle. After incubation at 37 °C for 24–48 hours, spreading the growth away from the stab line was interpreted as motility, while confined growth along the stab line indicated non-motile organisms.

Mannitol Fermentation

Mannitol fermentation ability was examined in glass tubes containing Mannitol Salt Agar medium. The inoculated tubes were incubated aerobically at 37 °C for 24–48 hours. A positive reaction was observed by a color change of the medium from red to yellow due to acid formation, while no color change indicated a negative result.

Triple Sugar Iron (TSI) Agar Test

For the TSI test, glass tubes containing TSI medium were inoculated by stabbing the butt and streaking the slant with a sterile inoculating needle. Tubes were incubated at 37 °C for 24 hours. Reactions were recorded as alkaline/acid (K/A), acid/acid (A/A), or alkaline/alkaline (K/K). Gas production was identified by cracks or lifting of the medium, while blackening indicated hydrogen sulfide formation.

Citrate Utilization Test

Citrate utilization was tested in glass tubes containing Simmons' citrate agar. The surface of each slant was lightly streaked with the test organism and incubated at 37 °C for up to 72 hours. A positive reaction was indicated by growth with a color change from green to blue, while absence of growth and no color change was recorded as negative.

Urease Test

Urease activity was determined in Christensen's urea agar prepared in glass tubes. Each tube was inoculated with the test isolate and incubated at 37 °C for 24–48 hours. Hydrolysis of urea released ammonia, raising the pH and producing

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a color change from light orange to pink or deep fuchsia. Tubes without color change were considered negative.

Antimicrobial susceptibility Testing

The antimicrobial susceptibility of Shigella isolates was assessed using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar, in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2024). Freshly grown colonies were suspended in sterile saline and adjusted to a 0.5 McFarland standard for uniform turbidity. The standardized suspension was then spread evenly across the agar surface with sterile cotton swabs. Antibiotic discs tested included ampicillin (10 µg), ciprofloxacin (5 µg), azithromycin (15 µg), nalidixic acid (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), and gentamicin (10 µg). The inoculated plates were incubated at 37 °C for 18–24 hours. Following incubation, the diameters of inhibition zones were measured and interpreted according to CLSI breakpoints.

Polymerase chain reaction (PCR) amplification

PCR was employed to amplify the gyrA and parC genes, along with selected virulence genes (ipaH, virA, stx1, stx2) and PMQR genes (qnrA, qnrB, qnrC, qnrD, qnrS). Amplification was carried out using an Eppendorf thermocycler. A subset of 15 samples was sequenced at Alpha Genomics Laboratory, Islamabad. Sequence alignment and comparative analysis with public databases were performed using Vector NTI Suite 9.

Data analysis

SPSS was used to carry out data analysis and Microsoft Excel to create bar charts. According to cell frequencies, statistical associations were evaluated using Fisher's exact test or the Chi - square test.

RESULTS

***Shigella* species identification**

Biochemical testing of the 120 suspected isolates confirmed their identity as *Shigella* species. The isolates consistently tested negative for urease activity and motility. They were able to ferment Mannitol but did not produce gas during the process. In Triple Sugar Iron (TSI) medium, the cultures developed an alkaline slant with an acidic butt (K/A) and showed neither gas production nor hydrogen sulfide (H_2S) formation, a reaction profile typically associated with *Shigella* species. In addition, none of the isolates utilized citrate in Simmons' citrate medium, as the medium retained its original green color without turning blue. Gram staining further demonstrated that all isolates were Gram-negative bacilli, appearing as pink-colored short rods under the microscope. The consistency of these results with the classical biochemical profile described in *Bergey's Manual of Determinative Bacteriology* provided strong confirmation of the identification of the isolates as belonging to the genus *Shigella*.

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Figure 1: Biochemical test of *Shigella;* Urease -ve, non-motile, Mannitol +ve without gas, K/A, citrate -ive.

Table 1: Shows biochemical test results for *Shigella* isolates.

Biochemical Test	Result
Urease	Negative (-)
Motility	Non motile
Mannitol	Positive (+) without gas
TSI (Triple sugar Iron)	Alkaline slant
Citrate	Negative (-)

Prevalence of Different *Shigella* Species

Out of a total of 120 confirmed *Shigella* isolates, the majority were identified as *S. sonnei*, accounting for 88 isolates (74%). *S. flexneri* represented the second most common species, with 28 isolates (24%), while *S. boydii* was the least frequent, detected in only 2 isolates (2%). These findings highlight the predominance of *S. sonnei* in the study population, followed by *S. flexneri*, with *S. boydii* occurring rarely.

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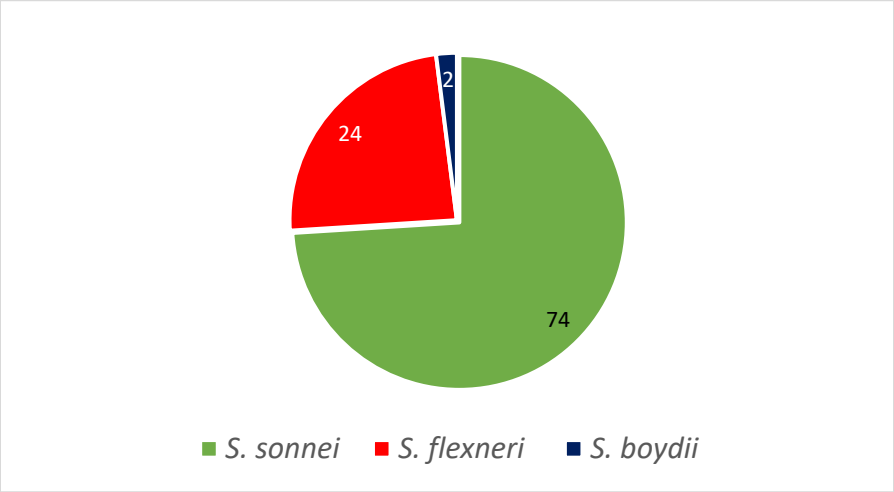

Figure 2: Proportion of *S. sonnei, S. flexneri*, and *S. boydii* in the study population.

Table 2: Distribution of serogroup for tested isolated.

Serogroup	Percentage
Shigella flexneri	24%
Shigella sonnei	74%
Shigella boydii	2%

Virulence factors

Screening of the 15 Shigella isolates for six major virulence genes (*ipaH, ial, sen, set1A, set1B, and STX*) revealed a widespread occurrence of key pathogenic markers among the strains. The *ipaH* gene was the most dominant, being detected in 95.4% of isolates, while the *ial* gene was present in 75.1% of isolates. Among the enterotoxin-associated genes, set1B was identified in 7.8% of the isolates, whereas set1A and sen were not detected in any of the strains examined. Interestingly, the Shiga toxin gene (*STX*) was detected in eight isolates of *S. sonnei*, indicating an unusual distribution of this gene beyond its typical association with *Shigella dysenteriae* type 1.

The distribution of these virulence genes varied according to serotype, suggesting heterogeneity in the pathogenic potential of the isolates. Molecular detection of virulence genes was confirmed by 1.5% agarose gel electrophoresis. In Multiplex PCR assay 1, amplification bands corresponding to ipaH and ial genes were observed, whereas set1A did not amplify in any of the isolates (M = 100 bp DNA ladder). In Multiplex PCR assay 2, amplification of ial and stx genes was recorded, while the sen gene was consistently absent across all samples (M = 100 bp DNA ladder). The distribution of virulence genes among the different Shigella serotypes revealed marked variability. The ipaH gene showed the highest prevalence, being detected in 96.6% of S. flexneri, 95.2% of S. sonnei, and 90% of S. boydii isolates. The ial gene was present in 90.8% of S. flexneri, 67.8% of S. sonnei, and in all (100%) of the S. boydii isolates examined. In contrast, the *set1B* gene exhibited a more restricted distribution, being found in 29.4% of *S. flexneri* isolates, 1.1% of *S. sonnei*, and in none of the *S. boydii* isolates. These findings highlight the heterogeneity in virulence gene carriage across *Shigella* species, which may contribute to differences in their pathogenic potential as shown in fig 4.4 and 4.5.

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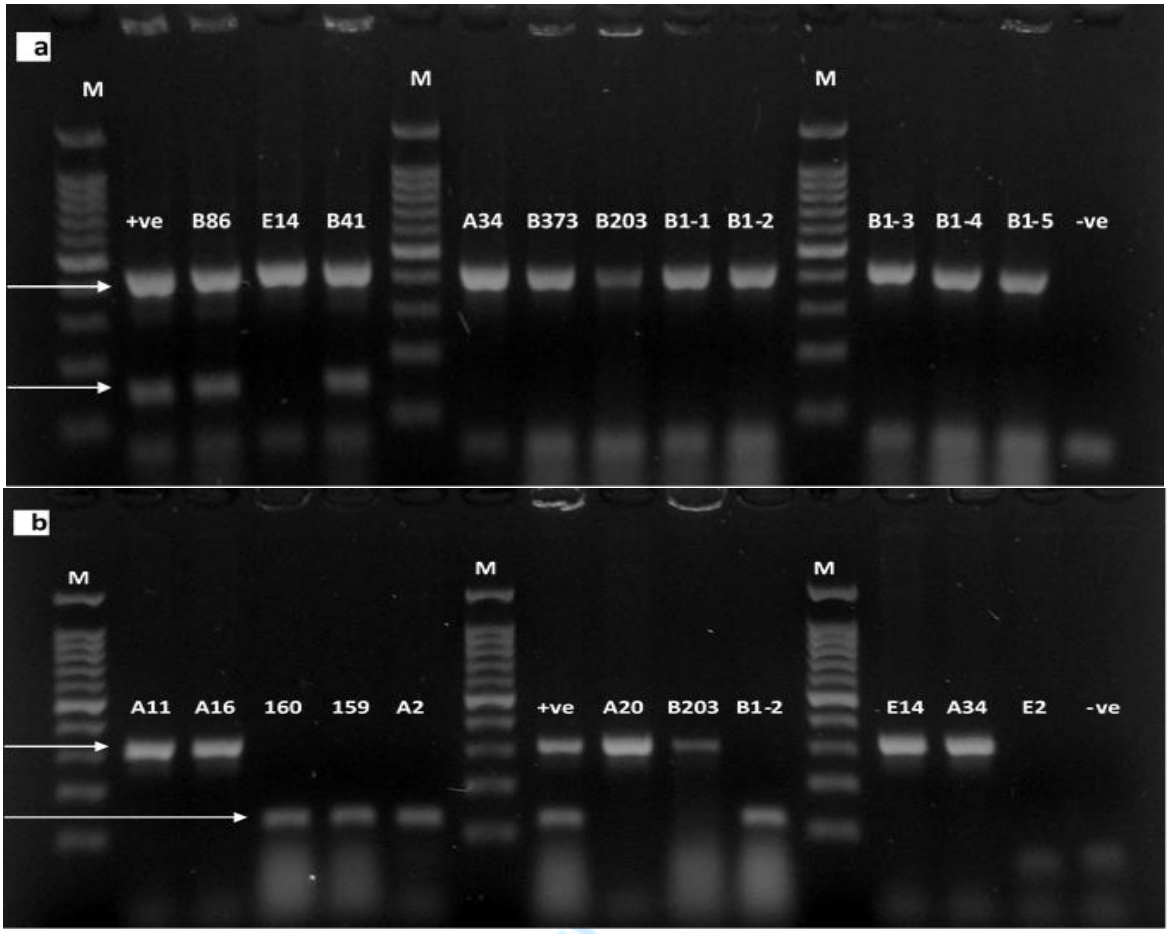

Figure 3: Showing genes Bands for *Shigella* virulence genes (M = 100 bp DNA ladder).

Distribution of virulence genes

The distribution of virulence genes among the Shigella serotypes is shown in Figures 1 and 2. The ipaH gene, a key invasion-associated marker, was detected in almost all isolates across the three serotypes. Its highest prevalence was observed in S. flexneri (96.6%), followed closely by S. sonnei (95.2%), while S. boydii exhibited a slightly lower frequency (90%). This indicates that ipaH is a conserved virulence determinant in Shigella and plays a crucial role in pathogenicity across species.For the ial gene, variation among serotypes was more evident. It was detected in all S. boydii isolates (100%) and in a majority of S. flexneri isolates (90.8%). In contrast, its prevalence was lower in S. sonnei (67.8%), suggesting serotype-specific differences in the distribution of invasion-associated genes. The complete presence of ial in S. boydii may highlight its significant role in the invasive capability of this serotype.

The distribution of the *set1B* enterotoxin gene among the examined *Shigella* serotypes showed considerable variation. The gene was completely absent in *S. boydii* (0%) and was detected at a very low frequency in *S. sonnei* (1.1%). In contrast, *S. flexneri* demonstrated a markedly higher prevalence, with *set1B* present in 29.4% of the isolates. This finding suggests that *set1B* is more commonly associated with *S. flexneri* strains compared to other *Shigella* species, indicating a possible serotype-specific role of this virulence determinant.

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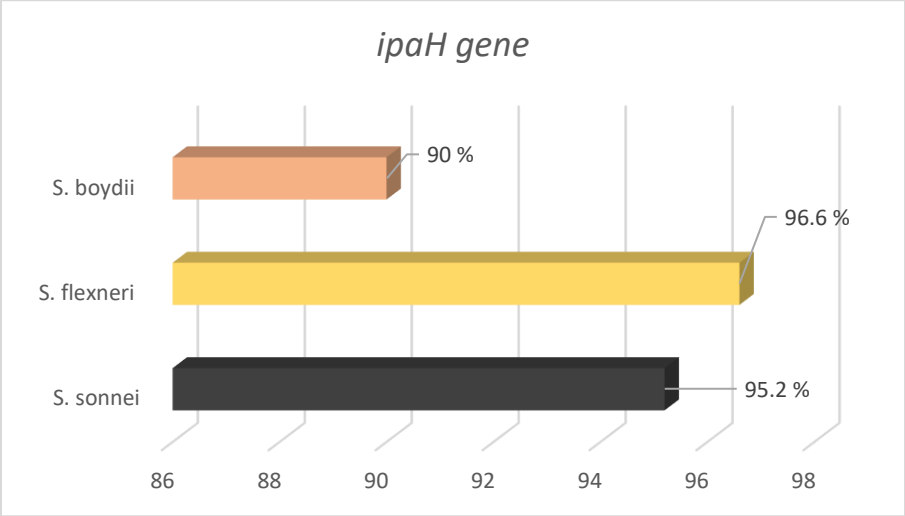

Figure 4: Prevalence of virulence genes (*ipaH*) among *Shigella* serotypes.

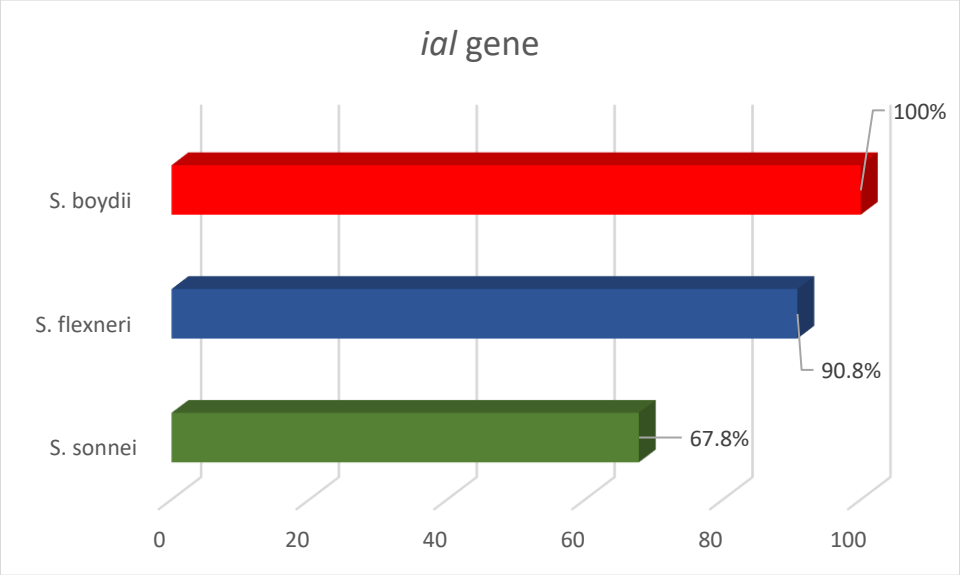

Figure 5: Prevalence of virulence genes (*ial*) among *Shigella* serotypes.

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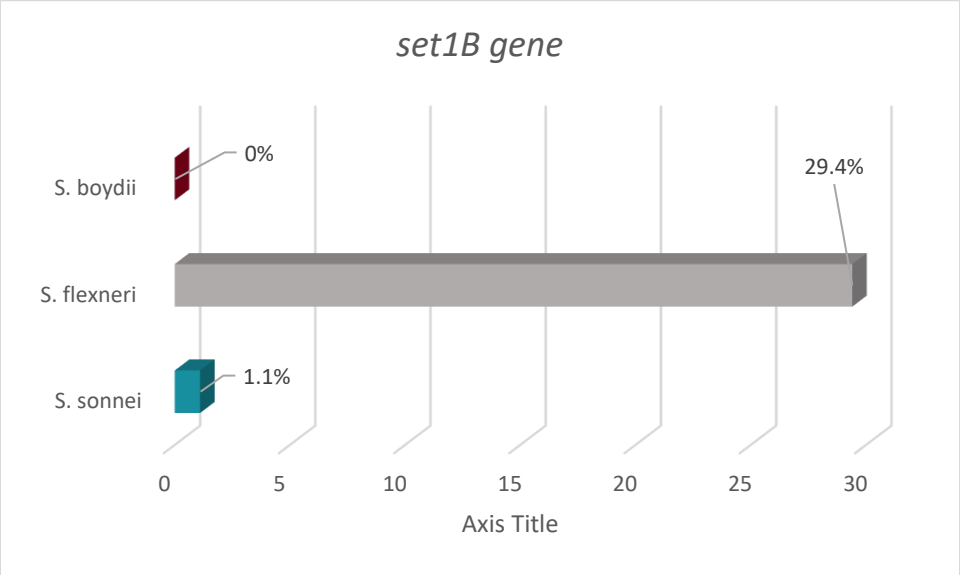

Figure 6: Prevalence of virulence genes (*set1B*) among *Shigella* serotypes.

***STX* positive samples**

A total of eight *S. sonnei* isolates were detected carrying the Shiga toxin (STX) gene, which is usually restricted to *Shigella dysenteriae* serotype 1 and certain enterohaemorrhagic *Escherichia coli* (EHEC) strains. Clinical data retrieved from the CBH archive revealed that patients infected with these strains experienced severe symptoms, indicating a high degree of virulence associated with these isolates.

Antibiotic Susceptibility Test

The antibiotic resistance profile reveals a concerning pattern of multidrug resistance among the isolates. Extremely high levels of resistance were observed against cotrimoxazole (90.4%) and tetracycline (79.5%), indicating that these agents have become largely ineffective for treatment. Moderate resistance was noted for nalidixic acid (45.8%) and ampicillin (42.2%), suggesting limited therapeutic value. In contrast, lower resistance rates were recorded for azithromycin (17.0%), chloramphenicol (15.7%), and ciprofloxacin (12.0%), which may still retain some clinical utility, though careful monitoring is required. Encouragingly, the lowest resistance was observed against gentamicin (3.0%), cefixime (3.6%), and cefotaxime (6.0%), highlighting these antibiotics as the most effective treatment options. Overall, the findings suggest that while traditional first-line drugs have lost their efficacy, newer cephalosporins and aminoglycosides remain reliable, underscoring the importance of rational antibiotic use and continuous surveillance to prevent further resistance development.

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Table 3: Antibiotic resistance profile of *Shigella* isolates.

Antibiotic	Resistance (%)
Azithromycin	17.0
Gentamicin	3.0
Nalidixic Acid	45.8
Cefixime	3.6
Cotrimoxazole	90.4
Chloramphenicol	15.7
Tetracycline	79.5
Cefotaxime	6.0
Ampicillin	42.2
Ciprofloxacin	12.0

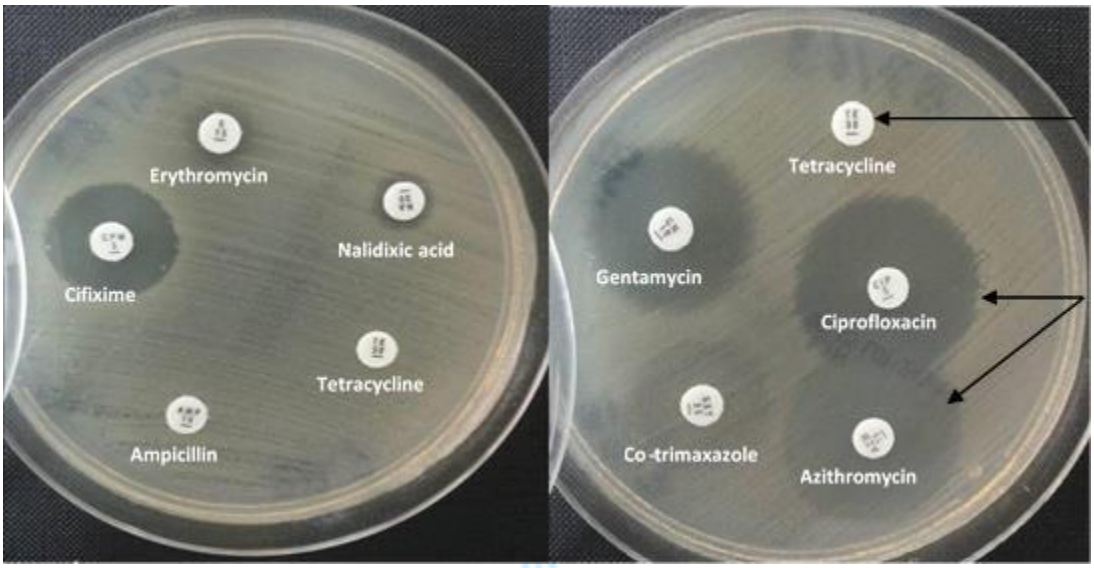

Figure 7: Antibiotic susceptibility profile of *Shigella* isolates.

Characterization of *PMQR* determinants

Out of the 83 Shigella isolates analyzed, 38 (45.8%) were positive for plasmid-mediated quinolone resistance (PMQR) genes. All PMQR-positive isolates exhibited resistance to nalidixic acid (NA), confirming the strong association between PMQR determinants and reduced susceptibility to first-generation quinolones. Furthermore, 10 isolates (26.3% of PMQR-positive) also showed resistance to ciprofloxacin (CIP), indicating that while resistance to newer fluoroquinolones remains relatively low, it is beginning to emerge in this population. The distribution of individual PMQR genes revealed that qnrA and qnrS were the most frequently detected, each present in 34 isolates (41%). The qnrD gene was also common, being identified in 30 isolates (36.1%), whereas qnrB was less frequent, detected in 15 isolates (18.1%). Importantly, the *qnr*C gene was not detected in any of the isolates, suggesting that it is either rare or absent in this geographical setting. In addition to single-gene carriage, several isolates harbored multiple PMQR genes, giving rise to distinct resistance patterns. The most common combined profiles were qnrADS, detected in 13 isolates (15.7%), and qnrABDS, observed in 11 isolates (13.3%). The presence of these multi-gene combinations highlights the potential for co-selection and horizontal transfer of resistance determinants, which may further compromise the efficacy of fluoroquinolones in clinical management. Overall, these findings indicate that

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nearly half of the Shigella isolates carry PMQR genes, with *qnrA*, *qnrS*, and *qnrD* being the predominant determinants. The strong correlation between PMQR positivity and nalidixic acid resistance, along with the emerging resistance to ciprofloxacin, underscores the clinical relevance of these genes and the need for continuous surveillance to guide treatment strategies.

DISCUSSION

Shigella species are important bacterial pathogens responsible for causing bacillary dysentery, a severe form of gastrointestinal infection. They are transmitted primarily through the fecal–oral route and are strongly associated with poor sanitation and contaminated food or water. Among the four recognized species (*S. flexneri, S. sonnei, S. boydii,* and *S. dysenteriae*), infections are most commonly reported in developing countries, where they remain a major public health concern. The pathogenicity of *Shigella* is linked to the presence of specific virulence genes that enable invasion of intestinal epithelial cells and production of toxins, leading to disease manifestations. The study identified 120 confirmed *Shigella* isolates, with *Shigella sonnei* being the most prevalent species, accounting for 74% (88 isolates), followed by *S. flexneri* at 24% (28 isolates), and S. boydii at 2% (2 isolates). This distribution aligns with global trends where *S. sonnei* is more commonly associated with shigellosis in developed regions, whereas *S. flexneri* predominates in developing countries (Nyarkoh *et al.*, 2024). Recent studies have highlighted the increasing prevalence of multidrug-resistant (MDR) *S. sonnei* strainsIn Tunisia, a 2022 outbreak demonstrated that the HC10-20662 genotype of *S. sonnei* was responsible for transmission across multiple countries, including Tunisia and parts of Europe. Likewise, data from Spain and Portugal reported that fluoroquinolone resistance in *S. sonnei* had increased to approximately 80%, reflecting the global emergence of resistant clones.The present study investigated the prevalence of six major virulence genes (*ipaH, ial, sen, set1A, set1B,* and *stx*) among 15 *Shigella* isolates. A high frequency of invasion-related genes was observed, with *ipaH* detected in 95.4% of isolates and *ial* in 75.1%. In contrast, enterotoxin genes showed much lower prevalence: *set1B* was present in only 7.8% of isolates, while *set1A* and *sen* were entirely absent. Remarkably, the *stx* gene was identified in eight *S. sonnei* isolates, an unusual finding given that this toxin is typically associated with *S. dysenteriae* type 1 and enterohemorrhagic *E. coli*.

When analyzed by serotype, the distribution of virulence genes demonstrated considerable variability. The *ipaH* gene was detected in 96.6% of *S. flexneri*, 95.2% of *S. sonnei*, and 90% of *S. boydii* isolates. The *ial* gene was present in 90.8% of *S. flexneri*, 67.8% of *S. sonnei*, and in all *S. boydii* isolates. Conversely, the *set1B* gene was more restricted, occurring in 29.4% of *S. flexneri*, 1.1% of *S. sonnei*, and absent in *S. boydii*. These findings highlight a heterogeneous distribution of virulence determinants across serotypes, which may influence the overall pathogenic potential of *Shigella*. Comparable patterns have been reported in other countries. For instance, a study conducted in Egypt found *ipaH* in 100% of *S. sonnei* isolates, while *ial* was detected in only 15.1%. The same study reported low frequencies of *set1A* (6.0%), *set1B* (1.8%), and *sen* (16.3%), with *S. flexneri* harboring more virulence genes than *S. sonnei*, suggesting a possible relationship between gene content and virulence (Sonbol et al., 2022). Similarly, research from Iran confirmed the presence of *ipaH* in all *S. sonnei* isolates, while *ial* was detected in 95.8%. Both *set1A* and *set1B* were reported in 10.9% of isolates. Importantly, that study noted associations between certain clinical symptoms and specific virulence factors, supporting the idea that gene distribution may directly influence disease outcomes (Phiri *et al.*, 2021).This study presents a detailed characterization of *Shigella* isolates, emphasizing both the distribution of virulence genes and their antibiotic resistance patterns. Interestingly, eight isolates of *Shigella sonnei* were identified as carriers of the *stx* gene, a virulence factor more commonly associated with *Shigella dysenteriae* serotype 1 and with

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enterohemorrhagic *Escherichia coli* (EHEC). Clinical records from the CBH archive indicated that these isolates were linked to severe clinical manifestations, underscoring the potential for increased virulence in *S. sonnei* strains harboring the STX gene. This finding aligns with reports from California by Zhi *et al.*, (2021), where clusters of STX-producing *S. sonnei* have been identified, leading to severe outcomes such as bloody diarrhea, though hemolytic uremic syndrome was not observed in those cases.The present study highlights an alarming trend of multidrug resistance (MDR) in *Shigella* isolates, with particularly high resistance against cotrimoxazole (90.4%) and tetracycline (79.5%), rendering these once-effective first-line agents largely obsolete. Moderate resistance to nalidixic acid (45.8%) and ampicillin (42.2%) further limits therapeutic options, while relatively lower resistance rates to azithromycin (17.0%), chloramphenicol (15.7%), and ciprofloxacin (12.0%) suggest these drugs may still retain some clinical utility, though the emergence of resistance necessitates cautious use. The lowest resistance observed against gentamicin (3.0%), cefixime (3.6%), and cefotaxime (6.0%) underscores their current effectiveness, yet global evidence warns of increasing resistance trends even to these agents. Recent studies from Iran and the UK report rising resistance to third-generation cephalosporins, ciprofloxacin, and azithromycin, with expanding MDR and XDR *S. sonnei* strains, mirroring the patterns observed in this study (Asad *et al.*, 2025). Furthermore, the detection of PMQR genes in nearly half of the isolates—particularly qnrA, qnrS, and qnrD—confirms their strong association with nalidixic acid resistance and emerging ciprofloxacin resistance. Similar findings have been documented in Iraq and Iran, where high frequencies of qnr determinants were linked to quinolone resistance and MDR phenotypes (Gonabadi *et al.*, 2024). These results collectively indicate that while some antibiotics remain effective, the increasing prevalence of MDR and plasmid-mediated resistance determinants poses serious threat to clinical management, underscoring the urgent need for continuous surveillance, rational antibiotic use, and updated treatment guidelines.2022, with a significant association between resistance and male patients Jacqueline *et al.*, (2025).

CONCLUSION

Shigella species remain a major cause of diarrhea in the Pakistani population, especially among children, and are increasingly associated with high levels of antimicrobial resistance alongside the presence of diverse virulence genes. In cases where multidrug-resistant (MDR) *Shigella* strains carry plasmid-mediated quinolone resistance (PMQR) determinants, the use of empirical antibiotic therapy may lead to adverse outcomes such as treatment failure, prolonged illness, secondary infections, and enhanced transmission through increased fecal shedding. To address this challenge, continuous surveillance, prudent antibiotic administration, and the formulation of effective therapeutic guidelines are urgently required. Molecular screening and genetic monitoring play a critical role in identifying resistant *Shigella* strains and tracking the evolution of their virulence. The findings of this study contribute to a deeper understanding of antimicrobial resistance trends and pathogenicity in *Shigella*, offering crucial evidence to support national and global public health strategies aimed at reducing the burden of pediatric dysentery and related infections.

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