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Short Communication

Virulence Profiles among Gastrointestinal and Extraintestinal Clinical Isolates of *Plesiomonas shigelloides*

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ABSTRACT: The genus *Plesiomonas*, represented by a single species, *Plesiomonas shigelloides*, is a gram-negative bacillus associated with gastrointestinal and extraintestinal diseases in humans. In this study, 44 clinical isolates (gastrointestinal, $n = 41$; extraintestinal, $n = 3$) were genetically confirmed to be *P. shigelloides* using the *hug* gene. All 20 virulence genes were detected in the gastrointestinal isolates, ranging from 7.7% to 100%; however, only 12 genes were detected in the extra-gastrointestinal isolates, ranging from 33.3% to 100%. The *phlA* gene was significantly associated with the gastrointestinal isolates ($P = 0.0216$). The results of this study suggest that *phlA* may play a role in gastrointestinal infections. However, *pilF*, *tolC*, and *fur* were detected in both gastrointestinal and extraintestinal clinical isolates, and further investigations are warranted to elucidate their role in the pathogenesis of *P. shigelloides.*

Plesiomonas is one of the oldest established genera in the family *Enterobacteriaceae*, and is represented by a single oxidase-positive species (1). In humans, *Plesiomonas shigelloides* is mostly associated with self-limiting diarrheal disease but less frequently causes acute secretory gastroenteritis, invasive shigellosis-like disease, and cholera-like illness (1,2). Extraintestinal manifestations of *P. shigelloides* are uncommon, but cases of bacteremia, cellulitis, meningitis, and meningoencephalitis have been reported (3,4). In addition, food- and water-borne outbreaks of *Plesiomonas*-associated infections have been documented (1). Among these, *P. shigelloides* serotype O17 was isolated from several ill persons and also from implicated food (cuttlefish salad) and water (tap water), thereby supporting the enteropathogenicity of this bacterium (5). The exact role in the pathogenesis of infections caused by this organism remains unclear. With next-generation sequencing technologies, virulence traits contributing to its pathogenicity in *P. shigelloides* isolates GN7, NCTC10360, 302-73, and LS1 have been reported (6). These identified virulence traits were homologous or orthologous to those of other pathogens, and all four isolates possessed pathogenicity islands. This valuable information could assist in screening virulence factors and in understanding the pathogenic mechanisms involved in *P. shigelloides* infections. To date, no study has investigated the virulence profiles of *P. shigelloides* in gastrointestinal and extraintestinal infections. Hence, the objective of the present study was to screen 20 randomly selected virulence genes among the gastrointestinal and extraintestinal clinical isolates of *P. shigelloides* using a polymerase chain reaction (PCR) assay.

Forty-four archived *P. shigelloides* isolates that had been identified by the API 20E system were collected from the University Hospital, University of Malaya, Kuala Lumpur, and used for this study. Forty-one samples were recovered from patients with gastrointestinal infections, such as gastroenteritis, cholera-like diarrhea, dysentery, food poisoning, and diarrhea, while three samples were associated with extra-gastrointestinal diseases, such as ascending cholangitis and pyrexia of unknown origin. Genomic DNA was prepared using an adapted in-house boiling method (7). All *P. shigelloides* isolates were genetically confirmed by *hugA* gene amplification using primers (forward: 5′-AGACGCTCTTGACGTTCG -3′ and reverse: 5′-AGCAAGTTGCGCTGTTTG3-3′), and one clinical isolate was randomly chosen and confirmed by direct DNA sequencing.

Twenty virulence genes described in the literature (6) were randomly selected for screening in this study using self-designed primers (Table 1). In silico analysis showed that the sensitivity of the primers ranged from 92.6% to 100%, while the specificities were above 91%, except for *flaA* (87%). Overall, all 20 genes were successfully amplified to the expected amplicon size (Fig. 1). These genes encode for flagellar motor switch protein (*flim*), RNA polymerase sigma factor (*rpoS*), (p)

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Table 1. Primer nucleotide sequences for DNA amplification and amplicon sizes of virulence genes of *Plesiomonas shigelloides*

Gene	Upstream primer sequence (5′ to 3′)	Downstream primer sequence (5′ to 3′)	Amplicon size (bp)	Annealing temperature (°C)
flim	TGAATACGTGCACACCCT	GCATGTCGGTGTCACTCTT	461	55
rpoS	TGTCGTCAAAGAGCTGAAC	GGGTCAAACCAATTTCACT	387	55
relA	GGCGATGGTAGAGGATTTC	CATCTGACGGGTACGGAT	664	55
gmh	TGTGAAGCGTCTGAGGTG	GCTTCAACATAGCGAGAGAA	290	55
glnA1	AGCACCCTGATTATCCGTT	CCTTGATGGTCATGGTGTT	448	55
rfaD	ATTCCAAAGAGCTGCTGC	CTACCCGCAAACAGCTTAG	343	55
gale	CCGTATGGACGCAGTAAG	ACGTGCGGCTTTATCAGT	483	55
vasA	AGCGGCAATGATTTATCCA	GCAAATGACGGTGAGCTG	893	58
clpP	GCTTTAGTGCCGATGGTTGT	GCCATTCGGTAAACAGAAGC	378	58
ompA	GGGTGGTGGTATCGGTTA	ATCAGGTTTGATGCCACA	312	55
cpsA	CAATATGGCATCAACGTGC	GACCGATTTGGCTACCGTAT	357	57
flaA	CACTGAACGCAGAGCTGAAG	GAGTCTGGTTGGCCTGAGAC	447	59
phlA	GGGCATTCAAGAAGTCCAAA	TGTCAGTGCCTTGCTCAATC	482	59
hcp	TATCGAAGGCAAAACCCAAG	CCAATCAATTTTGCGGTAGG	519	59
pilF	GCTGTCGGGATGTGTCAGTA	GCATATTTGCCTTGTGAGCA	369	59
tolC	CAAGCAGTTCAGCACCGATA	GCTCGTTCGCTTTAATGGAG	419	59
lapC	CCCTCCCGTTGAATTACTGA	AAATCAGAACGCCCATGAAG	570	59
pulJ	GCCACATTGAATGCTCTTGA	ATCGCCTCCCAATTACCTTT	515	59
fur	AAGCCGGTCTGAAGGTCAC	CAGGCTTTCGTTCTGACGAC	404	59
tonB	AGCAAAACCCAAGCAAGCTA	GCGATCCAGTAGGGGAAAAC	433	59

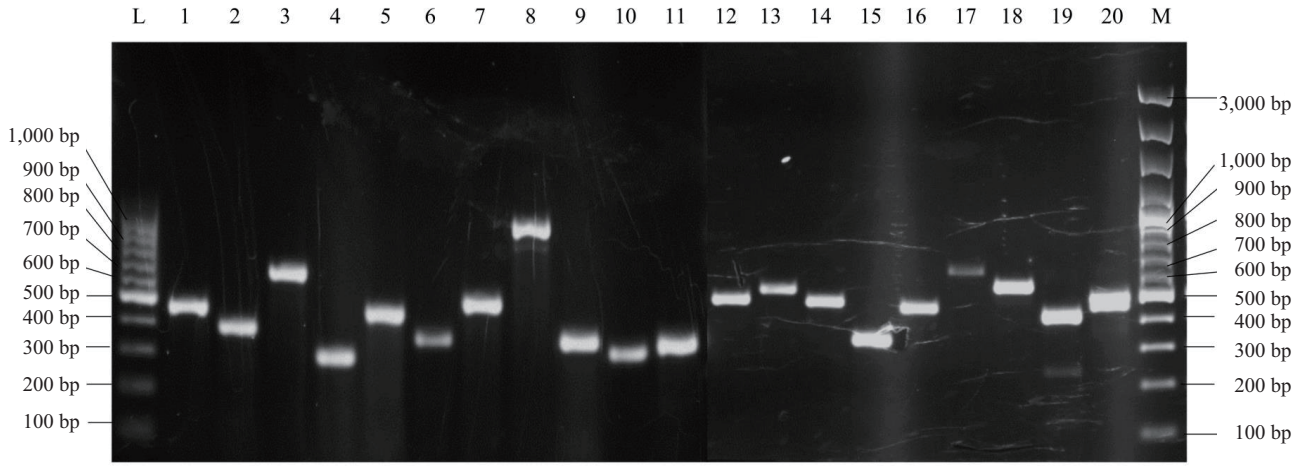
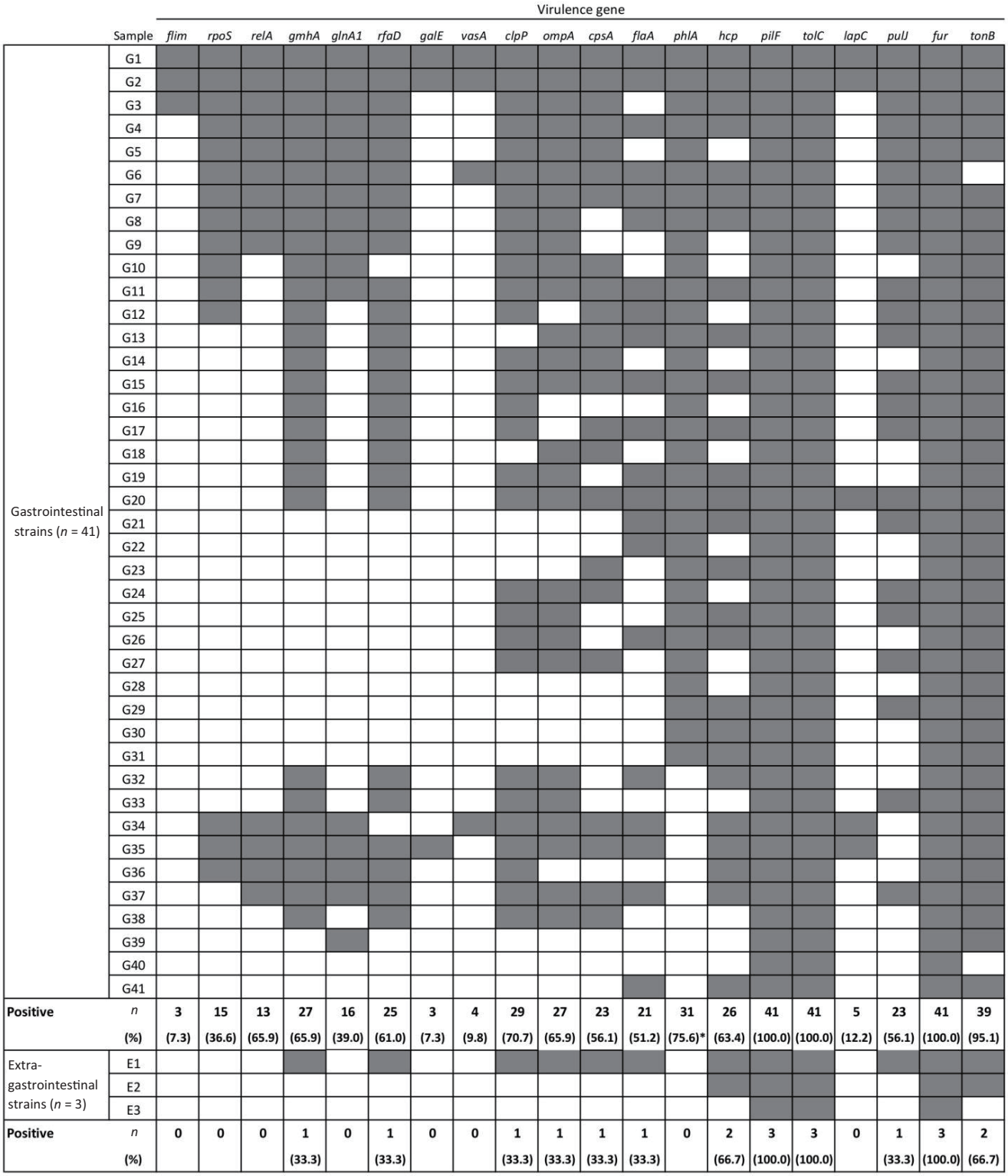


Fig. 1. Gel electrophoresis of amplified virulence gene products. Lane L: 100 bp plus molecular weight marker; lane 1: *flim* (461 bp); lane 2: *rpoS* (387 bp); lane 3: *relA* (664 bp); lane 4: *gmhA* (290 bp); lane 5: *glnA1* (448 bp); lane 6: *rfaD* (343 bp); lane 7: *galE* (483 bp); lane 8: *vasA* (893 bp); lane 9: *clpP* (378 bp); lane 10: *ompA* (312 bp); lane 11: *cpsA* (357 bp); lane 12: *phlA* (482 bp); lane 13: *hcp* (519 bp); lane 14: *flaA* (447 bp); lane 15: *pilF* (369 bp); lane 16: *tolC* (419 bp); lane 17: *lapC* (570 bp); lane 18: *pulJ* (515 bp); lane 19: *fur* (404 bp); lane 20: *tonB* (433 bp); lane M:100 bp plus molecular weight marker.

ppGpp synthetase (*relA*), phosphoheptose isomerase (*gmhA*), Glutamine synthetase type I (*glnA1*), ADP-L-glycero-D-mannoheptose 6-epimerase (*rfaD*), UDP-glucose 4-epimerase (*gale*), T6SS component TssF (*vasA*), ATP-dependent Clp protease proteolytic subunit (*clpP*), outer membrane protein (*ompA*), capsular polysaccharide, synthesis enzyme (*cps*), flagellin protein (*flaA*), hemolysin (*phlA*), T6SS component (*hcp*), Type IV pilus biogenesis protein (*pilF*), Type I secretion outer membrane (*tolC*), Type I secretion system (*lapC*), Type II secretory pathway (*pulJ*), ferric uptake regulator (*fur*), and ferric siderophore (*tonB*). The Fisher exact test with a 2 × 2 contingency table was used for data analysis, and differences at $P < 0.05$, calculated with two tails, were considered statistically significant.

Among the three *P. shigelloides* extra-gastrointestinal isolates, only 12 genes were detected, and none of them possessed the following 8 genes: *flim*, *rpoS*, *relA*,



Asterisk ∗ indicates statistical significance with *P*-value of 0.0216.
phiA - RefSeq Locus Tag of PLESHI_02232 and protein ID of EON90308.1.

Fig. 2. Heatmap of presence/absence of the 20 genes with variable pattern of virulence among clinical *Plesiomonas shigelloides*.

glnA1, *gale*, *vasA*, *phlA*, and *lapC*. In contrast, all 20 virulence genes were detected in 41 *P. shigelloides* gastrointestinal isolates with varying frequencies (7.7% to 100%) (Fig. 2), and *phlA* was significantly (P = 0.0216) associated with these gastrointestinal isolates compared to extra-gastrointestinal isolates. Few studies have examined the hemolytic activity of *P. shigelloides*, and contradictory results have been reported. Janda and Abbott (8) reported that 94% (34/36) of *P. shigelloides* produced beta-haemolysis using agar overlay and contact-dependent hemolysis assays. Salerno et al. (9) also reported that most strains in their

study demonstrated hemolytic activity; however, a low frequency (23.3%, 7/30) of hemolysin activity was observed in a study conducted by Čižnár et al. (10). In addition, Vitovec et al. (11) were unable to demonstrate hemolytic activity during mono- and co-infection with *Cryptosporidium parvum* in neonatal BALB/c mice, and no hemolytic activity was observed in human intestinal (Henle 407) cell lines (12). Collectively, it is of interest to confirm the function of the *phlA* gene in *P. shigelloides*. Nonetheless, future studies are required to elucidate its role in pathogenicity.

In addition, three virulence genes, *fur*, *phiF*, and *tolC*, were the most prevalent (100%) among both the gastrointestinal and extra-gastrointestinal isolates, suggesting that they are the most common and essential genes required for pathogenesis. Rodríguez-Rodríguez and Santos (13) reported that iron acquisition is an important trait in the virulence of *P. shigelloides*, as they observed that six environmental strains with a conserved *fur* gene expressed a high level of this gene when grown under iron-depleted conditions compared to iron-abundant conditions. *pilF* is required for pilus-type IV assembly and is present in many bacterial pathogens, including *Neisseria meningitidis* (14). Mutation of *pilF* resulted in complete loss of surface fibers and attenuated virulence in mouse models, thus confirming *that Neisseria* pili contributes to host cell adhesion and virulence, paving the way for therapeutic options for meningococcal infections. Another gene, *tolC*, exists in all gram-negative bacteria and is involved in multidrug efflux and the secretion of virulence factors by the type I protein secretion pathway. Kopping et al. (15) showed that the Δ*tolC* mutant increased the susceptibility of *Francisella tularensis* to selected antimicrobial agents and was highly attenuated for virulence in mice via intranasal and intradermal routes of infection. Altogether, *fur*, *pilF*, and *tolC* deserve further in-depth study to gain insight into their gene functions in *P. shigelloides*. In addition, degenerate primers should be considered for primer design to enhance the sensitivity and specificity of PCR (above 98–100%) in future studies.

In conclusion, our findings demonstrate that 44 clinical *P. shigelloides* strains have pathogenic potential, as all strains harbored at least three genes (*pilF*, *tolC*, and *fur*). A frequency of 75.6% of gastrointestinal isolates was found to be associated with *phlA* (P = 0.0216), suggesting that this gene may be involved in gastrointestinal diseases.

Conflict of interest None to declare.

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