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

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Abstract

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

Plesiomonas is one of the older established genus in the family *Enterobacteriaceae* and is represented by a single oxidase-positive species (1). In humans, *P. 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). Besides, food- and water-borne outbreaks of *Plesiomonas*-associated infections have been documented (1). Among these, *P. shigelloides* isolate 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 isolate LS1 were reported (6). These identified virulence traits were homologous or orthologous to other

pathogens and all 4 isolates possessed pathogenicity islands. This valuable information could assist in the screening of virulence factors and help to understand the pathogenic mechanisms involved in *P. shigelloides* infections. Thus far, there is no study investigating virulence profiles between *P. shigelloides* responsible for gastrointestinal and extraintestinal infections. Hence, the objective of the present study was to screen 20 randomly selected virulence genes among gastrointestinal and extra-intestinal clinical isolates of *Plesiomonas shigelloides* by using a polymerase chain reaction assay.

Forty-four archived *P. shigelloides* isolates that had been identified by 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 diarrhoea, dysentery, food poisoning and diarrhoea while 3 samples were associated with extra-gastrointestinal diseases, i.e., 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 using *hugA* gene amplification using primers (Forward: 5'-AGACGCTCTTGACGTTTCG -3' and Reverse: 5'-AGCAAGTTGCGCTGTTTG3-3') and one clinical isolate was randomly chosen and confirmed by direct DNA sequencing.

Twenty virulence genes that have been described in the literature (6) were randomly selected for screening in this study using self-designed primers (Table 1). *In silico* analysis showed that primers' sensitivities range from 92.6% to 100% while specificities are above 91% except for *flaA* (87%). Overall, all 20 genes were successfully amplified with the expected amplicon sizes (Figure 1). These genes encode for flagellar motor switch protein (*flim*), RNA polymerase sigma factor (*rpoS*), (p)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 x 2 contingency table was used for data analysis and differences at $p < 0.05$ calculated with two tails were considered statistically significant.

For 3 *P. shigelloides* extra-gastrointestinal isolates, only 12 genes were detected and none of them possessed 8 genes - *flim*, *rpoS*, *relA*, *glnA1*, *gale*, *vasA*, *phlA*, *lapC*. In contrast, all 20 virulence genes were detected in 41 *P. shigelloides* gastrointestinal isolates with varying frequencies (7.7% to 100%) (Figure 2) and *phlA* was significantly ($p=0.0216$) associated with these gastrointestinal isolates when compared to extra-gastrointestinal isolate. Few studies examined hemolytic activity in *P. shigelloides* and contradict results were reported. Janda and Abbott (8) reported 94% (34/36) of *P. shigelloides* produced beta-hemolysis by using agar overlay and contact-dependent hemolysis assays. Salerno et al. (9) also reported 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). Besides, 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 in the human intestinal (Henle 407) cell lines was also observed by Maluping et al. (12). Collectively, it is of interest to confirm the function of *phlA* gene detected in *P. shigelloides*, therefore future studies are required to elucidate its role in pathogenicity.

Besides, 3 virulence genes – *fur*, *phiF* and *tolC* were the most prevalent (100%) among both gastrointestinal and extra-gastrointestinal isolates, suggesting that they are the commonest 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 6 environmental strains with a conserved *fur* gene expressed a high level of this gene when these isolates were grown under iron-depleted compared to iron-abundance conditions. The *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 the virulence in mice models, thus confirming *Neisseria* pili contributing to host cell adhesion and virulence, thus paving the way for therapeutic options for meningococcal. Another gene, *tolC* exists in all Gram-negative bacteria and is involved in multidrug efflux and secretion of virulence factors by the type I protein secretion pathway. Kopping et al. (15) showed that $\Delta tolC$ mutant increased the susceptibility of *Francisella tularensis* to selected antimicrobial agents and was highly attenuated for virulence in mice by the intranasal and intradermal routes of infection. Altogether, *fur*, *pilF* and *tolC* deserve further in-depth study to gain insights into their gene functions in *P. shigelloides*. Besides, degenerate primers should be considered for primer design in order to enhance sensitivities and specificities of PCR (above 98-100%) the future study.

In conclusion, our findings demonstrated that 44 clinical *P. shigelloides* strains pose pathogenic potential as all strains harboured at least 3 genes (*pilF*, *tolC* and *fur*). A frequency of 75.6% gastrointestinal isolates was found to be associated with *phlA* ($p=0.0216$) and this suggests that the gene may be involved in gastrointestinal diseases.

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CONFLICT OF INTEREST

The author declares that they have no conflict of interest.

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Figure Legend:

Figure 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

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

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
<i>flim</i>	TGAATACGTGCACACCCT	GCATGTCGGTGTCACTCTT	461	55 °C
<i>rpoS</i>	TGTCGTCAAAGAGCTGAAC	GGGTCAAACCAATTTCACT	387	55 °C
<i>relA</i>	GGCGATGGTAGAGGATTTC	CATCTGACGGGTACGGAT	664	55 °C
<i>gmh</i>	TGTGAAGCGTCTGAGGTG	GCTTCAACATAGCGAGAGAA	290	55 °C
<i>glnA1</i>	AGCACCCCTGATTATCCGTT	CCTTGATGGTCATGGTGTT	448	55 °C
<i>rfaD</i>	ATTCCAAAGAGCTGCTGC	CTACCCGCAAACAGCTTAG	343	55 °C
<i>gale</i>	CCGTATGGACGCAGTAAG	ACGTGCGGCTTTATCAGT	483	55 °C
<i>vasA</i>	AGCGGCAATGATTTATCCA	GCAAATGACGGTGAGCTG	893	58 °C
<i>clpP</i>	GCTTTAGTGCCGATGGTTGT	GCCATTTCGGTAAACAGAAGC	378	58 °C
<i>ompA</i>	GGGTGGTGGTATCGGTTA	ATCAGGTTTGATGCCACA	312	55 °C
<i>cpsA</i>	CAATATGGCATCAACGTGC	GACCGATTTGGCTACCGTAT	357	57 °C
<i>flaA</i>	CACTGAACGCAGAGCTGAAG	GAGTCTGGTTGGCCTGAGAC	447	59 °C
<i>phlA</i>	GGGCATTCAAGAAGTCCAAA	TGTCAGTGCCTTGCTCAATC	482	59 °C
<i>hcp</i>	TATCGAAGGCAAAACCCAAG	CCAATCAATTTTGCGGTAGG	519	59 °C
<i>pilF</i>	GCTGTCGGGATGTGTCAGTA	GCATATTTGCCTTGAGCA	369	59 °C
<i>tolC</i>	CAAGCAGTTCAGCACCGATA	GCTCGTTCGCTTTAATGGAG	419	59 °C
<i>lapC</i>	CCCTCCCGTTGAATTACTGA	AAATCAGAACGCCCATGAAG	570	59 °C
<i>pulJ</i>	GCCACATTGAATGCTCTTGA	ATCGCCTCCCAATTACCTTT	515	59 °C
<i>fur</i>	AAGCCGGTCTGAAGGTCAC	CAGGCTTTCGTTCTGACGAC	404	59 °C
<i>tonB</i>	AGCAAAACCCAAGCAAGCTA	GCGATCCAGTAGGGGAAAAC	433	59 °C

Figure 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

		Virulence gene																			
Sample		<i>flim</i>	<i>rpoS</i>	<i>relA</i>	<i>gmhA</i>	<i>glnA1</i>	<i>rfaD</i>	<i>galE</i>	<i>vasA</i>	<i>clpP</i>	<i>ompA</i>	<i>cpsA</i>	<i>flaA</i>	<i>phlA</i>	<i>hcp</i>	<i>pilF</i>	<i>tolC</i>	<i>lapC</i>	<i>pulI</i>	<i>fur</i>	<i>tonB</i>
Gastrointestinal strains (n=41)	G1																				
	G2																				
	G3																				
	G4																				
	G5																				
	G6																				
	G7																				
	G8																				
	G9																				
	G10																				
	G11																				
	G12																				
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	G34																				
	G35																				
	G36																				
	G37																				
	G38																				
	G39																				
	G40																				
	G41																				
Positive	n	3	15	13	27	16	25	3	4	29	27	23	21	31	26	41	41	5	23	41	39
	(%)	(7.3)	(36.6)	(65.9)	(65.9)	(39.0)	(61.0)	(7.3)	(9.8)	(70.7)	(65.9)	(56.1)	(51.2)	(75.6)*	(63.4)	(100.0)	(100.0)	(12.2)	(56.1)	(100.0)	(95.1)
Extra-gastrointestinal strains (n=3)	E1																				
	E2																				
	E3																				
Positive	n	0	0	0	1	0	1	0	0	1	1	1	1	0	2	3	3	0	1	3	2
	(%)				(33.3)		(33.3)			(33.3)	(33.3)	(33.3)	(33.3)		(66.7)	(100.0)	(100.0)		(33.3)	(100.0)	(66.7)

Asterisk * indicate statistical significance with p-value of 0.0216
phlA - RefSeq Locus Tag of PLESHI_02232 and protein ID of EON90308.1

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