Plesiomonas shigelloides Seventy Years of Systematics and Taxonomy in Perspective of the Present-Day Diagnostic Demands

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Abstract: The pathogen Plesiomonas shigelloides was discovered first in 1947 and christened C27 paracolon. Ever since then, it has suffered several taxonomic transitions. Although, throughout the world, the bacterium is highly recognized as public health threat both in human and veterinary medicines particularly in immunocompromised health, its studies and diagnostic development could be termed lingering among similar pathogens. The limitations of the existing taxonomic and diagnostic procedures were fully acknowledged. Emphasis was placed on the need for development of novel and rapid culture-independent diagnostic probes/protocols for its strain-specific (including pathogenic and nonpathogenic strains) characterization to enhance infection managements and possibility of having a unique identification scheme that is all-encompassing without bias towards some strains. Techniques with eminent congruence, specificity and sensitivity in characterizing its diverse strains were also advocated.

Key words: Plesiomonas shigelloides, public health, systematics, infection, diagnosis

INTRODUCTION

The Plesiomonas belongs to the family Enterobacteriaceae and has the facultative fermentative anaerobe Plesiomonas shigelloides, a gram-negative polar flagellate bacillus as its only member (Kaszowska et al., 2015). The genus has suffered several translations from one taxonomic group to another over the years. Briefly, the earliest description of the organism came from Ferguson and Henderson (1947) and was named C27 paracolon. Later, a four biotyping scheme emerged based on the research by Schmid from fermentative characteristics of lactose, duleitol and salicain, followed by placement into genus Pseudomonas by Bader (1954). Its characteristic cytochrome oxidase and polar flagellation saw it into genus Aeromonas, in the Vibrionaceae family (Ewing et al., 1961). Finally, the researches by Hals and Schubert (1962), Martinez-Murcia et al. (1992), Ruimy et al. (1994), Huys and Swings (1999) and Garrity et al. (2001) chronologically saw it to the present genus Plesiomonas in the Enterobacteriaceae as the solely oxidase-positive member. Although, with the advent of different high throughput molecular techniques such as rRNA (16S and 23S) sequence-based techniques that placed P. shigelloides closely to Proteaceae via phylogenetic alignment, the taxonomic name remainstill today (Martinez-Murcia et al., 1992). A summary of P. shigelloides taxonomic transitions is presented in Table 1.

Morpho-phenotypically, P. shigelloides is a long straight motile rod, either polarly or peritrichously flagellated (2-8 flagella) with fermentative ability under facultative anaerobic conditions (Inoue et al., 1991). A typical plesionomarad cell is 0.70-1.0 μm by 2.10-3.00 μm sized (Gonzalez, 2003). Transmission electron microscopy of plesionomarad cells have shown the possession of inclusion bodies that composed polyphosphates granules (Ogawa and Amano, 1987; Pastian and Bromel, 1984). Fermentative characteristics of P. shigelloides ranged from acid but non-gas production from glucose, inositol, ornithine to lysine decarboxylation. It is an active producer of β-galactosidase, phenylalanine deaminase, elastase (hydrolyze elastin), arginine dihydrolase, ornithine decarboxylase, trehalase, lysine decarboxylase, cytochrome oxidase, maltase, chitinase and DNase (hydrolyze chitin) but unable to produced starch.

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Table 1: Summary of *P. Shigelloides* taxonomic transition over the year

<table>
<thead>
<tr>
<th>Taxonomic name/transition</th>
<th>Reasons or characteristics for placement</th>
<th>References</th>
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<tbody>
<tr>
<td>C27</td>
<td>Motile enteric, anaerogenic, paracolon, amphiobutirous organism, <em>Enterobacteriacae</em>, somatic antigen similar to <em>Shigella sonnei</em></td>
<td>Ferguson and Henderson (1947)</td>
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<tr>
<td><em>Pseudomonas shigelloides</em></td>
<td>Isolated from faces, gram-negative rods have somatic antigen like <em>Shigella sonnei</em>, Polar or lophotrichous flagellation, anaerogenic glucose fermenting, non-lactose fermenting, nitrate reducer (nitrate to nitrite)</td>
<td>Schmid (1954)</td>
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<tr>
<td><em>Pseudomonas michiganii</em></td>
<td>Lophotrichous flagellated gram-negative bacilli; and based on geographic pedigree where Ferguson and Henderson isolated this bacterium</td>
<td>Sakazaki et al. (1959)</td>
</tr>
<tr>
<td><em>Aeromonas shigelloides</em></td>
<td>Sugar fermentative ability compared with oxidative activity, cytochrome oxidase positivity</td>
<td>Hugh and Leifson (1953), Culy and Hadley (1957)</td>
</tr>
<tr>
<td><em>Pseudomonas, aeromonas or vibrio</em></td>
<td>Based on gram-negative bacteria polar flagella differentiation</td>
<td>Ewing and Johnson (1960), Ewing et al. (1961), Shevan et al. (1954), Shevan et al. (1960), Shevan (1963)</td>
</tr>
<tr>
<td><em>Pseudomonas shigelloides</em>, <em>Pseudomonadaceae</em> in the tribe <em>Pseudomonadaeae</em>, <em>Plesiomonas</em> (proposed)</td>
<td>Based on flagellation and morpho-phenotypical backdrops, only glucose-fermenting anaerobe in the family</td>
<td>Hubs and Schubert (1962)</td>
</tr>
<tr>
<td><em>Fergusonia</em> (proposed)</td>
<td>Based on comparative studies with aeromonas and vibrio</td>
<td>Sabuld and Veron (1963)</td>
</tr>
<tr>
<td>Genus <em>Plesiomonas</em> (shigelloides) placement in vibrioaceae,</td>
<td>Based on studies on Aeromonas and C27 strains, vibriostatic agent sensitivity (01289) of many strains, lower GC content compared to Aeromonas but closer or within range reported for vibrio</td>
<td>Eddy and Carpenter (1964), Veron (1965)</td>
</tr>
<tr>
<td>Vibrio shigelloides (Bader) comb. nov. (proposed) Placement in the family enterobacteraceae proposed</td>
<td>Based on comparative publication analysis</td>
<td>Hendrie et al. (1971)</td>
</tr>
<tr>
<td>Placement in the family <em>Enterobacteraciaceae</em> proposed</td>
<td>Phylogenetic closer interrelatedness with members (specifically, genus Proteus) of the family <em>Enterobacteriacaeceae</em> compared to Aeromonadaceae established by sequencing of 16S rDNA</td>
<td>Martinez-Murcia et al. (1992)</td>
</tr>
<tr>
<td>Placement in the family <em>Enterobacteraciaceae</em> was further strengthened</td>
<td>Comparative phylogenetic analysis of rRNA (small-subunit) sequences, of genera Aeromonas Vibrio, Plesiomonas and photobacterium.</td>
<td>Ruiny et al. (1994)</td>
</tr>
<tr>
<td>Placement in the family <em>Enterobacteraciaceae</em> was further strengthened</td>
<td>Based on distinctive falls out of <em>P. shigelloides</em> from the Aeromonas main cluster via fluorescent Amplified Fragment Length Polymorphism (FAFLP) discriminatory genotyping of Aeromonas spp</td>
<td>Garry et al. (2001)</td>
</tr>
<tr>
<td><em>Plesiomonas</em> reclassified to the family <em>Enterobacteraciaceae</em> as the only oxidase-positive member</td>
<td>Based on the molecular features derived from 16S rDNA, small-subunit rRNA sequencing and FAFLP (Martinez-Murcia et al., 1992; Ruiny et al., 1994; Hays and Swings, 1999)</td>
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Hydrolases, gelatinase and urease (Kelly and Kain, 1991; Santos et al., 1999; Ramaiah et al. 2000; Farmer, 1995; Stock, 2004).

*P. shigelloides* has long associated history with freshwater and its resources, marine and seafood, pets and livestock (cows, cats, dogs, monkeys) and aquatic settings in general (Levin, 2008; Kaszowska et al., 2016; Janda et al., 2016). Hence, it’s easily disseminated and contracted from such vehicular beings and media. It is also known to cross-react with *Shigella sonnei* antigenically (Sayeed et al., 1992). Therefore, detection and classification of *P. shigelloides* through antigenic reaction can result into false positive reactions and thus, it is unreliable.

Identification scheme for *P. shigelloides*: There are different identification approaches for *P. shigelloides* depending on the purpose of investigation. In general, classical microbiological techniques or cultrunomics (biochemical, metabolic and serological test, hybridization method and rapid kit systems), genomic typing, MALDI-TOF MS methods/metabolic (proteomic) characterization, PCR and sequencing techniques have been devised for identification and characterization of *P. shigelloides* from different sources.

Cultrunomics identification: For the traditional microbiological procedures, various selective and/or differential media have been applied for *P. shigelloides* isolation with possibility of visual identification from other bacteria based on coloured colony formation. Isolation of *P. shigelloides* using MacConkey agar/Sorbitol-MacConkey Agar are common and usually produced translucent colonies (Holmberg and Farmer,
1984; Rolston and Hopfer 1984; Morris et al. 2011; Novoa et al. 2016). However, the use of MacConkey agar for P. shigellaeodes could not achieved differential purpose because the organism has lactose-fermenting and non-lactose-fermenting strains (Gravenitz, 1980). Investigators such as Pitariangi et al. (1982), Penn et al. (1982), Rolston and Hopfer (1984), Kony et al. (2007) also used Hektoen enteric agar for P. shigellaeodes isolation. Obi et al. (2007) used Xylose-deoxycholate citrate agar for its isolation. In most cases, a battery of media, biochemical and metabolic test panels are used for its isolation and characterisation. Thus, make it laborious and time-consuming.

For the most suitable differential/selective recovery of P. shigellaeodes, the three-meritorious media employed include Inositol Brilliant Green Bile Salts Agar (IBGBA) (Schubert, 1977; Gravenitz and Bucher, 1983), Plesiomonas Agar (PA) (Miller and Koburger, 1985) and Plesiomonas differential agar (PDAGer) (Huq et al., 1991). These achieved higher recovery of P. shigellaeodes at 42–44°C incubation with resultant whitish-pinkish with red halo on PDAGer) colouration in contrast to colourless Aeromonad colonies (Gravenitz and Bucher, 1983; Huq et al., 1991; Jeppesen, 1995). Compositional (g L⁻¹), IBGBA is made up of beef extract 5.0 g, peptone 10.0 g, NaCl 5.0 g, brilliant green 0.00033 g, bile salt mixture 8.5 g, neutral red 0.025 g, agar 13.5 g and mero-inositol 10.0 g at a pH 7.2 (Schubert, 1977). PA is composed of NaCl 5.0 g, peptone 1.0 g, yeast extract 2.0 g, arabinose 5.0 g, mannitol 7.5 g, inositol 1.0 g, bile salts No.3 1.0 g, lysine 2.0 g, agar 15.0 g and phenol red 0.08 g at a pH 7.4 (Miller and Koburger, 1985). Whereas, PDAGer composed Beef extract 7.5 g, Peptone 7.5 g, NaCl 5.0 g, bile salt mixture 8.5 g, Meso-inositol 10.0 g, brilliant green 0.00033 g, agar 13.5 g and neutral red 0.025 g at a pH 7.4 (Huq et al., 1991). Due to the mannitol or arabinose component, contaminating mannitol and arabinose-fermenters usually produce red colonies and thus plesiomonal colonies are easily identified based on inositol fermentative ability with lysine decarboxylation (Levin, 2008). Sample enrichment is also practiced using bile peptone broth, alkaline peptone water, tetrathionate broth and tetrathionate-iodine broth with different recovery levels (Freund et al., 1988; Rahim and Kay, 1988; Damme and Vandepitte, 1980).

Rapid biochemical characterization of P. shigellaeodes using commercial kits platforms for instance TTE-AS and Analytical Profile Index (API) 20E are available. These are principled on unique sugar fermentative and protein utilizing capability, mediated through enzyme production, detectable through acid or chromogenic indicators (Krovaee et al., 2000). In general, the biochemical and metabolic tests are dependent on the enzyme and metabolite indicators. P. shigellaeodes enzyme indicators include production of β-galactosidase, decarboxylase, arginine dihydrolase, oxidase and ornithine decarboxylase but do not produce urease and cryptophan deaminase (Gravenitz, 1980, 1985). Whereas, the metabolic indicators include positive reactions to indole formation, glucose, inositol and negative reaction to acetoin (acetyl methylcarbinol), mannitol, galactin, sorbitol, saccharose, rhamnose, melibiose, L (+) arabinose and amygdalin (Gravenitz, 1980, 1985).

Other biochemical tools for P. shigellaeodes identification include Vitek system®2 (Biomerieux), MicroScan (Walkaway), MicroScan® WalkAway® plus, AutoSCAN®4 System and AutoScan 4 adopting manual or automated processes (Shi, 2013; Jun et al., 2011). Recovery of P. shigellaeodes from blood specimen by using BacAlert system detected with NC30 Microscan panel system is also available (Auxiliadora et al., 2010).

**Serologic classification and serodiagnostic procedures:**

Clonal diversities of P. shigellaeodes are usually reported in literature based on serodiagnostic techniques. This is grounded on the O-somatic and H-flagella antigenic reactions using Plesiomonas specific anti-sera. Shimada and Sakazaki (1978) introduced 30-O-somatic and 11-H-flagella antigenic typing of P. shigellaeodes which later undergone further improvement through the researches of Aldova (1994) and Aldova and Schubert (1996). With serological identification, P. shigellaeodes has been largely and successfully delineated into unique strains having 102 O-somatic and 51 H-flagellar antigenic serotypes (Aldova and Shimada, 2000). In their investigations, Gonzalez et al. (2004), observed country based, transcontinental and trans-source sero-clonality among 73 plesiomonal strains studied. Although, there were heterologous sero-distribution among strains derived from a nation and between other countries, homoe-serotypes (similar serotypes) exist in the 57 strains studied which include two O11:H2, four O22/H3, two O35:H11, two O52:H3 and two O90:H6 strains among Finnish and Cuban isolates from humans and cats and three O23:H1a1b strains among Italian and Slovakian environmental isolates. This further buttressed the public health threats that could arise from zoontic carriers such as household pets and transmissibility of P. shigellaeodes as previously reported in animal (snake) to human.
(Davis 2nd et al., 1978; Arai et al., 1980). Shared or common P. shigelloides serotypes were also observed in pets such as cats and dogs (Gauthier, 2014). Freshwater fish isolates were also found to be of the same serotypes cultured from diarrhoeic human patient (Tsukamoto et al., 1978).

The demerits with serodiagnosis or sero-classification of P. shigelloides include firstly, the presence of non-agglutinating strains within the species (Gonzalez et al., 2004). These strains may not be captured using serodiagnostic approach or sero-classification. Secondly, the test procedures are also laborious and time consuming, because its antisera are usually produced in suitable animals over a period spanning about two to several weeks (Oviassogie and Elchaise, 2006). Thirdly, P. shigelloides exhibit cross reaction with Shigella species leading to false-positive result (Ferguson and Henderson, 1947; Batta et al., 1998). Kollarova and Aimin (2001) also reported cross reaction of antisera/antibody produced by whole cell P. shigelloides immunization of rabbit with several antigens from many of Enterobacteriaceae and Vibrionaceae members in crossed immunoelectrophoretic procedures. Finally, Plesiomonas specific anti-sera are noncommercially available to the scientific communities and laboratories.

Matrix-Assisted Laser-Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) and NMR spectroscopy: The MALDI-TOF MS techniques are mainly used in the proteomics, structural and molecular characterization of P. shigelloides lipopolysaccharide (LPS) and serotype (strain) delineation. LPS known as endotoxin, is one of the main virulence arsenal for the characteristic pathogenicity of P. shigelloides (Kaszowska et al., 2016; Okawa et al., 2004) and plays a central function in plesiomonal infections including septic shock and sepsis (Alexander and Rietschel, 2001). P. shigelloides LPS is a tripartite amphiphilic, immunodominant and structural molecules of the external membrane, consists hydrophobe (aquarephobe) lipid A, core Oligosaccharide (OQS) and O-Specific Polysaccharide (O-PS) (O-antigen) (Aquilini et al., 2013; Kaszowska et al., 2013a, b). The molecular proteomic or MALDI-TOF MS fingerprinting of P. shigelloides is established on the diagnostic strain specificity of the O-PS side chain of the LPS, manifested as unique variable chemical units and associated genetic variability in the wb cluster genes involved in the O-antigens biosynthesis among the strains (Kaszowska et al., 2013a, b, Cattoff and Karibian, 2003; Raetz and Whitfield, 2002). Although, MALDI-TOF MS has been a very useful technique in P. shigelloides characterisation but, there is no congruence between its sero-strains and MALDI-TOF MS clusters (Kolinska et al., 2010).

Studies on LPS fingerprinting of P. shigelloides include O-PSs of P. shigelloides 22074 and P. shigelloides 12254 (Linnerborg et al., 1995), core OGS and structural unit of P. shigelloides 113/92 O-antigen LPS (Czaja et al., 2000; Niedziela et al., 2002). LPS molecules of P. shigelloides 144/92 (Niedziela et al., 2002) and P. shigelloides 113/92 (Niedziela et al., 2002), P. shigelloides 054 lipid A and structure and LPS biological activity (Lukasiewicz et al., 2006a, b), P. shigelloides serotype O1 core OGS with O-specific chains substituent structures (Pieretti et al., 2008, 2010), P. shigelloides 110/92 O-PS (Maciejewksa et al., 2009, 2013) and P. shigelloides AM36565 O-PS (Sawan et al., 2012), P. shigelloides serotype O17 core OGS structure with single repeating O-specific PS substituent unit. Other reports involve P. shigelloides 302-7 O1-antigen LPS pathogenicity function (Aquilini et al., 2013), structure of pseudaminic acid containing O-antigen of P. shigelloides serotype O36 (Kaszowska et al., 2016), core OGS and lipid A structure of P. shigelloides PCM 2231 (Lukasiewicz et al., 2006a, b; Maciejewksa et al., 2013), Structure of α-d-Lenose-containing semi-rough LPS of P. shigelloides CNCTC 39/89 (Kaszowska et al., 2013a, b), P. shigelloides 024:H8 LPS core OGS and repeating unit structures (Lundqvist et al., 2015).

MATERIALS AND METHODS

Hybridization method: Hybridization reaction is also exploited in the identification of P. shigelloides to subspecies level. This is usually carried out in arrays adapted in form of microplate system (hybridization method). Firstly, the pathogen is identified to species morpho-physiologically and metabolically (culturomics) and then DNA-DNA hybridization is applied to delineate it to strains. For illustration, Sugita et al. (1993) carried ecological studies of P. shigelloides in freshwater and freshwater fish and recovered seventy-four strains through microplate hybridization technique. One inherent limitation of hybridization procedures in characterization of P. shigelloides is the possible existence of cross-hybridizing and nonhybridizing strains based on the hybridization probes employed. This is because of the extremely high homologous recombination rates of P. shigelloides (Salem et al., 2007). Also, the
bacterium is highly diverse in nucleotide sequence with Simpson’s index of 99.7% and differ with an average of 1.49% in nucleotides between strains (Salerno et al. 2007). The characteristic plesiomonad extremely high recombination rate has been linked to lack of connection between its serotypes (O: H antigenic-types) and genomic background (Salerno et al., 2007). Thus, hybridization method is unsuitable for identification and could be misleading and output inaccurate results in *P. shigelloides* characterization.

**Genomic typing:** Strain delineation of plesiomonad isolates were early achieved through genomic techniques since strain-specific PCR techniques are not yet available. The common genotyping procedures include Repetitive Extragenic Palindromic (REP)-PCR, Random Amplified Polymorphic DNA PCR (RAPD-PCR), Pulsed Field Gel Electrophoresis (PFGE), (GTG)5-PCR, Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) and BOX-PCR (Welsh and McClelland, 1990; Williams et al., 1990; Olive and Bean, 1999; Shigematsu et al., 2000; Gu et al., 2006, Gonzalez et al., 2011). In practice, these techniques amplify molecular variations in the genomes of plesiomonad strains and subsequent electrophoretic separation of resultant amplicons, usually produced characteristic band profiles distinctly with varied strains. Table 2 shows a summary of genotyping primers and PCR thermal programs for possible delineation of plesiomonad isolates into stains. Genotyping of plesiomonad isolates of fish, freshwater and clinical origins were carried using RAPD by Gu et al. (2006). Gonzalez et al. (2011) delineated 24 plesiomonad isolates into 17, 19, 21, 22 genotypes with ERIC-PCR, REP-PCR, RAPD and PFGE, respectively. The existence of iso-genotypes (same clones) based on genotyping were also reported among plesiomonad strains from human and animal sources (Gonzalez et al., 2011). This phenomenon depicts possibility of bidirectional zoonotic transmissibility. The differences in the sensitivity of the genotyping procedures call for a more reliable technique. Furthermore, a hybrid system comprising two or more genotyping tools/primers could yield a more robust sensitivity in delineation of the bacterium. Thus, forms a worthy candidate for future quest and investigation. The disadvantages with genotyping of *P. shigelloides* using the above methods include:

- The methods lack specificity and could be adopted for any bacterium
- It requires an initial culuturoemics

**Polymerase Chain Reaction (PCR):** The available traditional PCR for *P. shigelloides* is only suitable for the genus identification. Strain-specific primers are not yet available for plesiomonad strains. Gonzalez et al. (2000) pioneered the development of PCR-based characterization of *P. shigelloides*. The primers and PCR thermal profile for confirmation of *P. shigelloides* isolates are presented in Table 3. The PS23FW3 and PS23RV3 were designed to amplify the 23S rRNA gene that is specific to plesiomonad species. They generate a 284-bp PCR amplicon that is identical to the base sequence 506-1189 in its rRNA (23S gene) (Gonzalez et al., 2000). The disadvantage with these primers is the associated production of DNA dimers (Gu and Levin, 2006). The PS-F and PS-R were devised to target rRNA (23S gene) of *P. shigelloides* and yield a 628 bp PCR product. The PS-F and PS23RV3 were fashioned for competitive PCR, aimed at quantitative investigation of *P. shigelloides*. The pair produces a 500-bp PCR amplicon homologous to 690-1189 base sequence of the 23S rRNA (Gu and Levin, 2006, 2007, 2008). PS-F and PS23RV3 pair are highly specific for detection of plesiomonad isolates compared to the other primers (Gu and Levin, 2006, 2007, 2008). The Hybrid primer was constructed based on nucleotide sequence 843-862 of the plesiomonad rRNA (23S gene) (Gonzalez et al., 2000; Gu and Levin, 2007, 2006, 2008).
RESULTS AND DISCUSSION

Sequencing techniques: Various sequencing platforms are available for microbial characterization and identification. Mainly, in the search for accurate, rapid and early detection, better studies and diagnosis of *P. shigelloides*, sequencing techniques are gaining attention. The sequencing of plesiomonad small-subunit rRNA and 16 s rDNA were employed in phylogenetic analyses that led to the establishment of its taxonomic closer link/relatedness to the enterobacteria (Martinez-Murcia et al., 1992; Ruiny et al., 1994) and reclassification into the Enterobacteriaceae (Garrity et al., 2001). Chida et al. (2000) determined the O-antigen 017 serotype of *P. shigelloides* through sequencing. The genes associated with *P. shigelloides* LPS core biosynthesis was also studied by Aquilina et al. (2014) via proteomic and DNA sequencing techniques. The whole-genome sequencing analysis of *P. shigelloides* 302-73 was presented by Pique et al. (2013). The whole-genome sequencing techniques have been suggested could probably be an exact and rapid diagnostic protocol for suitable detection of *P. shigelloides* in outbreaks and its virulence factors (Pique et al., 2013).

The sequencing technique remains the easiest way to rapidly characterize and possibly delineate pathogenic *P. shigelloides* from non-pathogenic strains. Since, cell cultures and animal models currently employed in the establishment of plesiomonad pathogenicity and pathogenesis are laborious and unsuitable for rapid/emergency diagnosis. The sequencing techniques although provide rapid and accurate platforms for *P. shigelloides* detection and characterization, they are only available in reference laboratories and unavailable for routine laboratory diagnosis especially in the regions where *P. shigelloides* is endemic.

CONCLUSION

Therefore, this review aims at reporting an overview of the *P. shigelloides* systematics and diagnostic development over the seven-decades and identified possible future directions and initiatives for sound identification/classification and diagnosis.

RECOMMENDATIONS

*P. shigelloides* is often associated with infectious conditions such as gastroenteritis and extraintestinal infections that have fatal severity and acute death, there is urgent attentions for rapid identification scheme that is unbiased, relatively cheap with short turn-around time and adaptable in the developing world and risk zones. Development of rapid and extremely accurate culture-independent diagnostic probes/protocols for its strain-specific (including pathogenic and non-pathogenic strains) characterization to boost treatment options require research focus.

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