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Review

Relationship among *Shigella* spp. and enteroinvasive *Escherichia coli* (EIEC) and their differentiation

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Abstract

Shigellosis produces inflammatory reactions and ulceration on the intestinal epithelium followed by bloody or mucoid diarrhea. It is caused by enteroinvasive *E. coli* (EIEC) as well as any species of the genus *Shigella*, namely, *S. dysenteriae, S. flexneri, S. boydii*, and *S. sonnei*. This current species designation of *Shigella* does not specify genetic similarity. *Shigella* spp. could be easily differentiated from *E. coli*, but difficulties observed for the EIEC-*Shigella* differentiation as both show similar biochemical traits and can cause dysentery using the same mode of invasion. Sequencing of multiple housekeeping genes indicates that *Shigella* has derived on several different occasions via acquisition of the transferable forms of ancestral virulence plasmids within commensal *E. coli* and form a *Shigella*-EIEC pathovar. EIEC showed lower expression of virulence genes compared to *Shigella*, hence EIEC produce less severe disease than *Shigella* spp. Conventional microbiological techniques often lead to confusing results concerning the discrimination between EIEC and *Shigella* spp. The lactose permease gene (*lac*Y) is present in all *E. coli* strains but absent in *Shigella* spp., whereas β-glucuronidase gene (*uid*A) is present in both *E. coli* and *Shigella* spp. Thus *uid*A gene and *lac*Y gene based duplex real-time PCR assay could be used for easy identification and differentiation of *Shigella* spp. from *E. coli* and in particular EIEC.

Key words: diarrhea, *E. coli*, *Shigella*, real-time PCR

Introduction

Bacillary dysentery like shigellosis, endemic throughout the world, is one of the major causes of morbidity and mortality, especially among children > 5 years of age in low and middle income countries including Bangladesh (Bardhan *et al.*, 2010; Wen *et al.*, 2012). The disease is caused by enteroinvasive *Escherichia coli* (EIEC) or any of the four species or groups of *Shigella: S. dysenteriae, S. flexneri, S. boydii*, and *S. sonnei*. In the nineties, there are about 165 million cases of *Shigella* infection annually worldwide and 1.1 million *Shigella*-related deaths, with 98% of those cases in low income countries (Kotloff *et al.*, 1999). According to a recent review on Asian countries (Bardhan *et al.*, 2010), about 125 million cases of endemic shigellosis occur per year. The study showed that the incidence of shigellosis is similar to an earlier report (Kotloff *et al.*, 1999), however, the overall mortality rate due to shigellosis has come down to ~ 0.01% and ~0.89% among the youngest age group. Although EIEC is one of the etiologic agents of diarrhea, very few epidemiologic studies have been done globally to estimate the actual disease burden due to EIEC, individual risk factors for infection or prospective reservoirs of EIEC. Adequate attention was not given to the epidemiology of EIEC, since it is often found to be rare etiological agent of diarrhea compared to other diarrhea-causing enteropathogens (Vieira *et al.*, 2007; Moreno *et al.*, 2010). Identification and differentiation of *Shigella* spp. and EIEC from environmental and clinical specimens by conventional culture and biochemical assays

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are complex and time consuming. This review focuses on phenotypic and genotypic relationship among EIEC and *Shigella* spp., and recent progress of clinical and practical research, and utility of currently available molecular methods for differentiation between *Shigella* spp. and *E. coli*, in particular emphasis on EIEC based on literature study.

Identification and Classification

The Japanese scientist Kioshi Shiga first identified *Shigella* in the 1898s. He termed it as *Bacillus* because it seemed to be related to *Bacillus coli*, which is now referred to as *E. coli* (Hale, 1991). In taxonomy, *Shigella* strains were classified into a different genus from *E. coli* in the 1940s due to their pathological significance. The genus is divided into four species and at least 54 serotypes based on their biochemical and/or the structure of the O-antigen component of LPS present on the cell wall outer membrane: *S. dysenteriae* (subgroup A, 16 serotypes), *S. flexneri* (subgroup B, 17 serotypes and sub-serotypes), *S. boydii* (subgroup C, 20 serotypes), and *S. sonnei* (subgroup D, 1 serotype) (Simmons and Romanowska, 1987; Talukder and Azmi, 2012).

On the other hand, EIEC was first reported as 'paracolon bacillus' in 1944, but it was later designated as *E. coli* O124. EIEC is associated with specific *E. coli* O-serotypes: O28ac:NM, O29:NM, O112ac:NM, O121:NM, O124:NM, O124:H30, O135:NM, O136:NM, O143:NM, O144:NM, O152:NM, O159:H2, O159:NM, O164:NM, O167:H4, O167:H5, O167:NM and O173:NM (Orskov *et al.*, 1991; Matsushita *et al.*, 1993; Nataro and Kaper, 1998; Martinez *et al.*, 1999; Gibotti *et al.*, 2004). All of the serotypes are nonmotile except few biotypes of O28ac, O29, O124, O136 and O143 (Silva *et al.*, 1980; Martinez *et al.*, 1999). Some of these EIEC-associated O antigens are identical or similar to O antigens present in *Shigella* spp., namely: O112ac, O124, O136, O143, O152, and O164 with *Shigella* O antigens of *S. dysenteriae* 2/*S. boydii* 15/*S. boydii* 1, *S. dysenteriae* 3/ provisional *Shigella* serovar 3615.53, *S. dysenteriae* 3/*S. boydii* 1, *S. boydii* 8, provisional *Shigella* serovar 3341:55, and *S. dysenteriae* 3, respectively (Cheasty and Rowe, 1983; Landersjo *et al.*, 1996; Linnerborg *et al.*, 1999). In fact, differentiation between *Shigella* and EIEC strains with the same serotype is often difficult due to their nearly identical physio-biochemical traits. EIEC strains can be differentiated from typical members of commensal *E. coli* strains by the Sereny test and/or the identification of bacterial invasion-associated proteins or genes via specific tests. However, these methods are not routinely used for laboratory diagnosis and EIEC strains are only provisionally identified by O serotyping with commercially available antisera in diagnostic laboratories (Beutin *et al.*, 1997). Where a typable isolate additionally carries a serotype occurring in both EIEC and *Shigella* spp., a taxonomic classification could be at best possible on the basis of genes responsible for the higher metabolic activity of EIEC (Ewing, 1986; Kibbee *et al.*, 2013).

Phenotypic and Genotypic Relationship

Although *Shigella* and *E. coli* are closely related, *E. coli* always show some different physio-biochemical properties than *Shigella.* More than 80% of *E. coli* are prototrophic, motile, able to decarboxylate lysine and ferment many sugars, produce gas from D-glucose and indole positive, whereas *Shigella* are auxotrophic, obligate pathogens, non-motile, unable to decarboxylate lysine, ferment few sugars and never produce gas from D-glucose, except *Shigella flexneri* 6, *S. boydii* 13 and *S. dysenteriae* 3 (Clayton and Warren, 1929; Downie *et al.*, 1933; Stypulkowska, 1964; Rowe *et al.*, 1975; Silva *et al.*, 1980; Toledo and Trabulsi, 1983; Baumann and Schubert, 1984; Ewing, 1986; Scheutz F, 2005). *Shigella* spp. are unable to ferment salicine and hydrolyze esculine (Bopp *et al.*, 2003) . Of four *Shigella* spp., only *S. sonnei* is able to ferment lactose slowly and can be mucate positive (Goodman and Pickett, 1966). However, some EIEC strains have remarkable phenotypic and genotypic similarity with *Shigella* species (Farmer *et al.*, 1985; Lan *et al.*, 2004). They are usually nonmotile, lactose negative and lysine-decarboxylase negative except for a few serotypes, which are the Sereny test negative and motile (Farmer *et al.*, 1985). The few biochemical properties that enable differentiation of *E. coli* and *Shigella* spp. are mucate fermentation and acetate utilization. EIEC may be positive for one or both of the properties, in contrast *Shigella* strains are negative for both and more than 90% of other *E. coli* strains are positive for both with very few exceptions (Bopp *et al.*, 2003; Lan *et al.*, 2004).

It was reported that *Shigella* and *E. coli* show 80-90% similarity at the nucleotide level, while other *Escherichia* spp. have a much lower degree of similarity and are genetically distant (Brenner *et al.*, 1972). Multilocus enzyme electrophoresis and ribotyping analyses revealed that *Shigella* genotypes are interspersed within *E. coli* genotypes, irrespective of their nomen-species (Rolland *et al.*, 1998). Likewise, a high degree of relatedness between *Shigella* and *E. coli* is found based on 16S rDNA analysis (Christensen *et al.*, 1998). DNA-DNA re-association studies, sequencing of numerous other housekeeping genes, virulence genes and complete genome sequencing showed that EIEC and *Shigella* spp. formed a distinctive *E. coli* pathovar (Brenner *et al.*, 1972; Brenner, 1973; Lan *et al.*, 2004). However, the discrimination between EIEC and *Shigella* spp. is necessary as both exhibit a number of clinical differences. For example, *Shigella* spp. can cause haemolytic uraemic syndrome (HUS), a clinical syndrome characterized by progressive renal failure associated with microangiopathic (nonimmune, Coombs-negative) hemolytic anemia and thrombocytopenia, whereas EIEC is not known to cause HUS (Johnson, 2000).

Genetic Structure of *Shigella*

Each of the *Shigella* genomes includes a single circular chromosome and /or a circular virulence plasmid (VP) that harbors conserved primary virulence determinants (Sasakawa *et al.*, 1992). The whole-genome sequencing of all four *Shigella* spp. and *E. coli* revealed that they share a fundamental core genome of approximately 3 Mb (Lukjancenko *et al.*, 2010). The *Shigella* chromosome has more than 200 pseudogenes, 300~700 copies IS-elements, numerous deletions, insertions, translocations and inversions. Although, *Shigella* chromosomes share most of their genes with *E. coli* K12 strain MG1655, bacteriophage-mediated horizontal gene transfer is the main contributor for the massive diversity of putative virulence genes (Venkatesan *et al.*, 2001). *Shigella* spp. became highly virulent pathogens with distinct clinical and epidemiological features via gaining and loss of gene functions in adaptation and convergent evolution, by means of bacteriophage integration, IS-mediated transposition and formation of pseudogenes (Yang *et al.*, 2005; Peng *et al.*, 2006).

Phylogenetic Relationship

The sequencing of multiple housekeeping genes indicates that *Shigella* has risen on several different occasions from several independent ancestors by acquisition of the transferable forms of ancestral VPs within the group of nonpathogenic *E. coli.* Based on the analysis of sequence variation in eight housekeeping genes of *Shigella*, Pupo *et al*. proposed that *Shigella* strains fall into three main clusters and four outliers (Pupo *et al.*, 2000). In 2007, Yang *et al.* showed a similar phylogenetic tree using the DNA sequences of 23 housekeeping genes, which corroborated the credibility of the previous conclusions (Yang *et al.*, 2007). The most striking features are that each cluster includes strains from different *Shigella* species. The phylogenic tree shows that most of the *Shigella* strains belong to three clusters (C1, C2 and C3) leaving *S. sonnei* (SS), *S. dysenteriae* (D) serotype 1, 8, 10 and *S. boydii* serotype 13 (B13) as outliers. Cluster 1 can be sub-clustered to SC1, SC2, SC3, and a minor branch consisting of only *S. dysenteriae* 7. Subcluster 1 contains only D strains (D3-4, D6, D9, D11-13), SC2 contains mostly B strains (B1, B3, B6, B8, B10, and B18) but also D5, and SC3 contains three B strains (B2, B4, and B14) and F6. Cluster 2 is mainly composed of B strains (B5, B7, B9, B11, and B15-17) and D2. Cluster 3 consists of mostly *S. flexneri* (F) strains (F1a, F1b, F2a, F2b, F3, F4a, F4b, F5, Fx, and Fy) and B12. The most profound observation is that each of the five clusters/sub clusters contains strains mostly from only one serogroup indicates that serological classification is highly correlated with the genotypes and continues to be useful in epidemiologic and diagnostic investigations. In the phylogenetic tree, B13 is distant from all the *E. coli*/*Shigella* strains, which is consistent with a recent report that B13 and *E. albertii* strains form a distinct lineage of enteric pathogens that had separated from an *E. coli*-like ancestor about 28 million years ago (Hyma *et al.*, 2005). MLST analysis revealed that EIEC strains grouped into four clusters with one outlier strain (which was found in *Shigella* cluster 2), indicating the independent derivation of EIEC several times (Lan *et al.*, 2004). In comparison of EIEC with *Shigella* clusters, the authors showed that EIEC clusters have diverged less than *Shigella* clusters, although *Shigella*-EIEC forms one single pathovar of *E. coli* (Lan *et al.*, 2004).

Pupo *et al*. proposed that the three main clusters of *Shigella* had independently evolved from multiple *E. coli* ancestors with diverse genetic backgrounds 35,000-270,000 years ago (Pupo *et al.*, 2000). This indicated that dysentery existed long before civilization and was one of the early infectious diseases of human. *S. sonnei* had developed as a human-pathogenic clone of *E. coli* approximately 10,000 years ago (Shepherd *et al.*, 2000). The descent times are relatively recent when one takes in account that a major nonpathogenic *E. coli* cluster diverged from other bacteria 8-22 million years ago. These data are probably no coincidence because pathogenesis of *Shigella* is based on surviving in the intestinal epithelial cells of humans only-a perfect host-adaptation (Pupo *et al.*, 2000). Sequence variations in the clusters of *Shigella* and EIEC indicated that EIEC might have arisen from *E. coli* ancestors after *Shigella* developed. Based on this derivation of EIEC, two hypotheses about EIEC in relation to *Shigella* were stated. First, EIEC strains are in an intermediate stage and are a potential precursor of 'full-blown' *Shigella* strains. Second, like *Shigella,* EIEC is a distinct group of organisms that is adapted to human hosts, but is better equipped to survive outside the host (Lan *et al.*, 2004).

The critical step for *Shigella* creation is the acquisition of the antecedent forms of the VP, which is a non self-transferable large single-copy plasmid of 180-230 kb (Hale *et al.*, 1983). This VP is essential for invasiveness, cell survival and apoptosis of Macrophages (Harris *et al.*, 1982; Sansonetti *et al.*, 1982b; Sansonetti *et al.*, 1983). The virulence associated genes on the pINV are probably acquired horizontally from another unrelated genus, because the A+T content of the nucleotides of these genes is 75%, while the A+T content of all *Shigella* and *E. coli* genomes is 50% (Adler *et al.*, 1989; Hale, 1991). Based on the analysis of three virulence genes (*ipg*D, *mxi*A, and *mxi*C) that are located on the invasion region of VP in *Shigella* and EIEC strains, two forms of VPs (pINVA and pINVB) were found (Lan *et al.*, 2001; Yang *et al.*, 2007). Lan *et al*. extensively studied 32 EIEC strains and found that all but two EIEC strains have the pINV A form (Lan *et al.*, 2004). *S. sonnei* has only pINVB and other serotypes have mixed form. The acquisition of the VP in an ancestral *E. coli* strain preceded the diversification by radiation of all *Shigella* and EIEC groups. The DNA sequence indicated that a 31-kb entry region of VP encodes components of the Mxi [membrane

excretion of Ipa]-Spa[surface presentation of invasion plasmid antigens] TTSS [type three secretion system] apparatus, substrates of this apparatus (IpaA-D [invasion plasmid gene]), their dedicated chaperones (IpgA, IpgC, IpgE and Spa15) and two transcriptional activators (VirB and MxiE) (Buchrieser *et al.*, 2000). Outside of the entry region, there are i) *vir*G gene, encoding outer membrane protein (VirG), responsible for bacterial movement within the cytoplasm of infected cells, ii) v*ir*F gene, encoding a transcriptional activator (VirF), controls expression of *ics*A and *vir*B, and iii) the *sep*A gene encodes a secreted serine protease of the autotransporter family. Moreover, the virulence plasmid also contains two copies of the *shet*2 gene encoding a putative enterotoxin, and genes encoding several secreted proteins (VirA, IpaH4.5, IpaH7.8, IpaH9.8) and six uncharacterized protein designated (outer *Shigella* proteins): OspB, OspC1, OspD1, OspE1, OspF, and OspG (Harris *et al.*, 1982; Sansonetti *et al.*, 1982a; Sansonetti *et al.*, 1983). The plasmid encoded proteins are directly involved in the entry into host epithelial cells. With the acquisition of the pINV, *Shigella* and EIEC were able to live in the human intestinal epithelial cells. For the invasion and maintaining in the host, *Shigella* and EIEC need a combined expression of genes located on the pINV and chromosome (Sansonetti *et al.*, 1982b; Maurelli *et al.*, 1998).

The *Shigella* genome has adapted to the acquisition of invasion plasmid by multiple different events, such as: (i) controlling at promoter level, (ii) mutations within genes, (iii) the suppression or over expression of certain genes, or (iv) deletion of anti-virulence genes which is called "black hole" to evolve toward a pathogenic lifestyle (Maurelli *et al.*, 1998). For example, the loss of *cad*A gene is a black hole in EIEC and *Shigella*. CadA encodes for lysine decarboxylase activity (LDC), which is present in almost all non-enteroinvasive *E. coli*. Cadaverine produced by lysine decarboxylase has been shown to attenuate the bacteria's ability to induce polymorphonuclear leucocytes transepithelial migration. Because of the inhibiting influence of cadaverin on the virulence of *Shigella*, LDC activity was lost by genome deletion (Maurelli *et al.*, 1998). Consequently, LDC is a biochemical trait which can be used to differentiate between other *E. coli* vs. *Shigella* and EIEC, but not between EIEC and *Shigella*.

Pathogenesis Process

The illness caused by *Shigella* or EIEC is characterized by the destruction of the colonic epithelium caused by the inflammatory response induced upon invasion of the mucosa by bacteria (Parsot, 2005). It is well established that the disease induced by EIEC is generally less severe than *Shigella* does (DuPont *et al.*, 1989; Moreno *et al.*, 2009; Bando *et al.*, 2010; Moreno *et al.*, 2012). Recombination techniques and the sequencing of the invasion plasmid and chromosomal genes associated with virulence, gave insight of the precise mechanism of infection by *Shigella*. First, the bacteria in the intestinal lumen invade the colon by transcytosis through microfold cells (M-cells) of the Follicle-Associated Epithelium (FAE) to reach the underlying submucosa (Croxen and Finlay, 2010). The disruption of tight junctions and the damage that is caused by inflammation also give *Shigella* entry to the submucosa. Although *Shigella* phagocytosed by resident macrophages, it can escape from the phagosome, and caspase-1-dependent inflammation activation resulting ultimate release from macrophages. After cell death, the bacteria, released in the submucosa, invade epithelial cells by endocytosis. During the invasion of the epithelial cells, *ipa*BCD and *mxi*AB genes of the ipa-mxi-spa island on the VP are brought to expression (Sansonetti *et al.*, 1981; Sansonetti *et al.*, 1982b; Buysse *et al.*, 1987; Venkatesan *et al.*, 2001; Moreno *et al.*, 2009; Croxen and Finlay, 2010). IpaD is believed to play a role in attaching to host cell membranes, and subsequently IpaB plays a role in the endocytic uptake of the bacteria. The roles of the other known virulence genes associated with invasion of the cell have yet to be discovered. Once internalized, the phagocytic vacuole is quickly lysed by the invading bacterium, thereby allowing its escape into the host cell cytoplasm, where it nucleates and assembles an F-actin comet at one of its poles (Bernardini *et al.*, 1989). This result in the bacterium moving inside epithelial cells and passing from cell to cell, thereby is causing a very efficient process of intracellular colonization. *Shigella* actin based motility is mediated by a single outer membrane protein, IcsA (VirG) (Lett *et al.*, 1989). Glycine-rich repeats in the amino terminal end of IcsA (VirG) bind neuronal Wiskoff-Aldrich Syndrome Protein (N-WASP) (Suzuki *et al.*, 1998), a member of the WASP family of Cdc-42-dependent mediators of actin nucleation via the Arp 2/3 complex. Formation of a complex between IcsA, N-WASP, and Arp 2/3 at the bacterial surface is sufficient to cause actin nucleation/polymerization in the presence of actin monomers (Egile *et al.*, 1999). Motile intracellular *Shigella* then involve components of the cell intermediate junction to form a protrusion that is internalized by the adjacent cell, thus causing cell-to-cell spread (Sansonetti *et al.*, 1994). Invasion of epithelial cells by *Shigella* stimulates the release of proinflammatory cytokines and chemokines, such as IL-8 attacks polymorphonuclear leukocytes (PMN) to the infection site and their transmigration through the epithelium, which results in major tissue destruction and inflammation.

Difference in Pathogenecity and Virulence Genes Expression

EIEC produce less severe disease than *S. flexneri* (Moreno *et al.*, 2009; Bando *et al.*, 2010; Moreno *et al.*, 2012). An inoculum of 10^6 EIEC cells is sufficient for infection, whereas as low as 10^2 *Shigella* cells can cause successful infection (DuPont *et al.*, 1971). *S. flexneri* induced

keratoconjunctivitis quickly and more severely than the EIEC strains. One of the hypotheses for this phenomenon is the increased efficiency of *Shigella* in spreading through epithelial cells compared to EIEC. (Moreno *et al.*, 2009). Moreover, it is noteworthy that *Shigella* and EIEC showed significant differences in the expression of regulatory and pathogenic genes (*ics*A, *ics*B, *ipa*A-D, *vir*F and *vir*B). It was found that *S. flexneri* expresses pathogenic genes at significantly higher levels than that of EIEC (Moreno *et al.*, 2009; Bardhan *et al.*, 2010). The *vir*F and *vir*B genes act in a regulatory cascade to trigger virulence genes transcription following the receipt of specific environmental signals by the bacterium (Adler *et al.*, 1989; Prosseda *et al.*, 1998). The VirF protein initiates the transcription of the *vir*B regulatory gene, and the product of this gene (VirB) in turn activates the promoters of the structural virulence genes (Porter and Dorman, 2002). Among the studied genes, only the *vir*F gene was more expressed by EIEC than by *S. flexneri*. All the other genes were less expressed in EIEC. The lower expression of these genes might lead to significant differences in virulence between EIEC and *Shigella*, leading to a weaken dissemination capacity of EIEC. These data also corroborated the differences in the mechanism by which EIEC and *S. flexneri* manipulate the host intestinal cells, and suggest that their genes respond specifically to the environment of the host cell milieu, resulting in different disease outcomes (Moreno *et al.*, 2009). Regarding the immune response related to dendritic cells (DCs), the innate immune response upon EIEC infection are preserved although DCs fail to activate naive T lymphocytes (Moreno *et al.*, 2012). Moreover, EIEC showed a late killing effect in J774 macrophage cultures in compare to *S. flexneri* (Bando *et al.*, 2010). This data could explain why EIEC takes longer time than *Shigella* species to cause diarrhea.

Current Approaches to Differentiate *Shigella* spp. and EIEC

The above described characteristics suggest that *Shigella*/EIEC could be differentiated genetically from typical *E. coli* by targeting marker genes. But designing a rapid, sensitive and reliable molecular technique for identification and differentiation between EIEC and *Shigella* spp. is very difficult due to their close biochemical similarity. Till now few molecular methods have been described for identification of the members of *Shigella*-EIEC pathover from other typical *E. coli*. For example, apyrase-based colorimetric test (Sankaran *et al.*, 2009), loop-mediated isothermal amplification method targeting the *ipa*H gene (Song *et al.*, 2005), PCR-ELISA (Sethabutr *et al.*, 2000), *Ipa*C and *Ipa*H gene -specific ELISA (Oberhelman *et al.*, 1993; Pal *et al.*, 1997), large invasive plasmid (120-140 MDa) analysis based method (Ud-Din *et al.*, 2010), colony blotting using 2.5 kb *Hind*III fragment of invasion plasmid (Small and Falkow, 1988). Moreover, PCR based assay targeting- IS630-probes (Houng *et al.*, 1997), *vir*F gene (Wang and Chen, 2012), *ipa*H gene (Thiem *et al.*, 2004) and IS1 region (Hsu *et al.*, 2007), multiplex PCR (Antikainen *et al.*, 2009; Fujioka *et al.*, 2013) and singleplex real-time PCR (Liu *et al.*, 2013) have been reported previously to detect the presence or absence of *Shigella*/EIEC. Most recently, Ojha *et al.* developed a pentaplex PCR which is able to detect and differentiate among *Shigella* spp. (Ojha *et al.*, 2013). Unfortunately, this method is also unable to differentiate EIEC from *Shigella* spp.

Lactose fermentation is the biochemical hallmark of *E. coli* which is exploited extensively for its detection by conventional culture methods (Ito *et al.*, 1991; Stoebel, 2005). The *lac*Y gene, a gene encoding lactose permease, is present in different members of the family Enterobacteriaceae like *E. coli, Enterobacter cloacae*, *Citrobacter freundii* or *Kluyvera ascorbata,* while the b-glucuronidase gene (*uid*A), which encodes the beta-glucuronidase enzyme is present in *E. coli* and *Shigella* spp. (Horakova *et al.*, 2008), Horakova *et al.* reported that the *lac*Y gene is a putative genetic marker for differentiation of *Shigella* spp. from *E. coli* (Horakova *et al.*, 2008). They developed a conventional multiplex PCR, which seemed to work well to differentiate *Shigella* from *E. coli* but not for EIEC-*Shigella* differentiation. Additionally, this conventional PCR failed to differentiate EIEC from *Shigella* due to presence of similar sized non-specific amplicons for *Shigella* spp. In consequence, Pavlovic M *et al.* developed a simple, rapid, reliable and specific probe-based duplex real-time PCR assay specific for the genes *uid*A and *lac*Y to minimize the risk of detection of nonspecific targets (Pavlovic *et al.*, 2011). They successfully differentiated ninety-six isolates including 11 EIEC isolates of different serotypes and at least three representatives of each *Shigella* species correctly. All the tested *Shigella* and *E. coli* including EIEC isolates were positive for the *uid*A gene. Additionally, all *E. coli* isolates were positive for the *lac*Y gene, whereas none of the tested *Shigella* isolate harbored the *lac*Y gene. Even cross reacting serotypes of EIEC (O112ac, O124 and O152) were clearly differentiated from *Shigella* as EIEC by the duplex real-time PCR. The selectivity of the *lac*Y-*uid*A duplex real-time PCR was 100%.

Conclusion

In summary, *Shigella* and EIEC can be differentiated from commensal *E. coli* by testing for presence of the *ipa*H-gene. Since *Shigella* and EIEC have similar physiobiochemical characteristics, conventional identification systems will identify members of the *Shigella*-EIEC pathovar as either *E. coli* or *Shigella*. Conventional cultural techniques often lead to confusing results concerning the discrimination of EIEC and *Shigella* spp. The duplex real-time PCR assay, which is simple, rapid, reliable and specific, can be used for differentiation of *Shigella* spp. from *E. coli* and in particular EIEC.

References

Adler B, Sasakawa C, Tobe T, Makino S, Komatsu K, Yoshikawa M (1989) A dual transcriptional activation system for the 230 kb plasmid genes coding for virulence-associated antigens of *Shigella flexneri*. Mol Microbiol 3:627-635.

Antikainen J, Tarkka E, Haukka K, Siitonen A, Vaara M, Kirveskari J (2009) New 16-plex PCR method for rapid detection of diarrheagenic *Escherichia coli* directly from stool samples. Eur J Clin Microbiol 28:899-908.

Bando SY, Moreno AC, Albuquerque JA, Amhaz JM, Moreira-Filho CA, Martinez MB (2010) Expression of bacterial virulence factors and cytokines during in vitro macrophage infection by enteroinvasive *Escherichia coli* and *Shigella flexneri*: a comparative study. Mem Inst Oswaldo Cruz 105:786-791.

Bardhan P, Faruque ASG, Naheed A, Sack DA (2010) Decreasing shigellosis-related deaths without *Shigella* spp.-specific interventions, Asia. Emerg Infect Dis 16:1718-1723.

Baumann P, Schubert R (1984) Vibrionaceae. *In*: Krieg NR, Holt JG (eds). Bergey's manual of systematic bacteriology Williams and Wilkins, Hagerstown, MD pp 440-445.

Bernardini ML, Mounier J, d'Hauteville H, Coquis-Rondon M, Sansonetti PJ (1989) Identification of *ics*A, a plasmid locus of *Shigella flexneri* that governs bacterial intra- and intercellular spread through interaction with F-actin. Proc Natl Acad Sci USA 86:3867-3871.

Beutin L, Gleier K, Kontny I, Echeverria P, Scheutz F (1997) Origin and characteristics of enteroinvasive strains of *Escherichia coli* (EIEC) isolated in Germany. Epidemiol Infect 118:199-205.

Bopp CA, Brenner FW, Fields PI, Wells JG, Strockbine NA. *Escherichia*, *Shigella*, and *Salmonella*. In: Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH, editors. Manual of clinical microbiology, 8th ed. Washington: ASM Press; 2003. p. 654-671.

Brenner DJ (1973) Deoxyribonucleic acid reassociation in the taxonomy of enteric bacteria. Int J Syst Bacteriol 23:298-307.

Brenner DJ, Fanning GR, Steigerwalt AG, Orskov I, Orskov F (1972) Polynucleotide sequence relatedness among three groups of pathogenic *Escherichia coli* strains. Infect Immun 6:308-315.

Buchrieser C, Glaser P, Rusniok C, Nedjari H, d'Hauteville H, Kunst F, Sansonetti P, Parsot C (2000) The virulence plasmid pWR100 and the repertoire of proteins secreted by the type III secretion apparatus of *Shigella flexneri*. Mol Microbiol 38:760-771.

Buysse JM, Stover CK, Oaks EV, Venkatesan M, Kopecko DJ (1987) Molecular cloning of invasion plasmid antigen (*ipa*) genes from *Shigella flexneri*: analysis of *ipa* gene products and genetic mapping. J Bacteriol 169:2561-2569.

Cheasty T, Rowe B (1983) Antigenic relationships between the enteroinvasive *Escherichia coli* O antigens O28ac, O112ac, O124, O136, O143, O144, O152, and O164 and *Shigella* O antigens. J Clin Microbiol 17:681-684.

Christensen H, Nordentoft S, Olsen JE (1998) Phylogenetic relationships of *Salmonella* based on rRNA sequences. Int J Syst Bacteriol 48:605-610.

Clayton F, Warren S (1929) An unusual Bacillus recovered from cases presenting symptoms of dysentery. J Hyg 28:355-362.

Croxen MA, Finlay BB (2010) Molecular mechanisms of *Escherichia coli* pathogenicity. Nat Rev Microbiol 8:26-38.

Downie A, Wade E, Young J (1933) An organism resembling the Newcastle type of dysentery bacillus associated with cases of dysentry. J Hyg 33:196-203.

DuPont HL, Formal SB, Hornick RB, Snyder MJ, Libonati JP, Sheahan DG, LaBrec EH, Kalas JP (1971) Pathogenesis of *Escherichia coli* diarrhea. N Engl J Med 285:1-9.

DuPont HL, Levine MM, Hornick RB, Formal SB (1989) Inoculum size in shigellosis and implications for expected mode of transmission. J Infect Dis 159:1126-1128.

Egile C, Loisel TP, Laurent V, Li R, Pantaloni D, Sansonetti PJ, Carlier MF (1999) Activation of the CDC42 effector N-WASP by the *Shigella flexneri* IcsA protein promotes actin nucleation by Arp2/3 complex and bacterial actin-based motility. J Cell Biol 146:1319-1332.

Ewing WH (1986) The genus *Escherichia*:Edwards & Ewing's identification of enterobacteriaceaes, Elsevier Science Publishing Co. Inc. New York.

Farmer JJ, Davis BR, Hickman-Brenner FW, McWhorter A, Huntley-Carter GP, Asbury MA, Riddle C, Wathen-Grady HG, Elias C, Fanning GR (1985) Biochemical identification of new species and biogroups of enterobacteriaceae isolated from clinical specimens. J Clin Microbiol 21:46-76.

Fujioka M, Otomo Y, Ahsan CR (2013) A novel single-step multiplex polymerase chain reaction assay for the detection of diarrheagenic *Escherichia coli*. J Microbiol Meth 92:289-292.

Gibotti A, Tanaka TL, Oliveira VR, Taddei CR, Martinez MB (2004) Molecular characterization of enteroinvasive *Escherichia coli* ipa genes by PCR-RFLP analysis. Braz J Microbiol 35:74-80.

Goodman RE, Pickett MJ (1966) Delayed lactose fermentation by enterobacteriaceae. J Bacteriol 92:318-327.

Hale TL (1991) Genetic basis of virulence in *Shigella* species. Microbiol Rev 55:206-224.

Hale TL, Sansonetti PJ, Schad PA, Austin S, Formal SB (1983) Characterization of virulence plasmids and plasmid-associated outer membrane proteins in *Shigella flexneri*, *Shigella sonnei*, and *Escherichia coli*. Infect Immun 40:340-350.

Harris JR, Wachsmuth IK, Davis BR, Cohen ML (1982) High-molecular-weight plasmid correlates with *Escherichia coli* enteroinvasiveness. Infect Immun 37:1295-1298.

Horakova K, Mlejnkova H, Mlejnek P (2008) Specific detection of *Escherichia coli* isolated from water samples using polymerase chain reaction targeting four genes: cytochrome bd complex, lactose permease, beta-D-glucuronidase, and beta-D-galactosidase. J Appl Microbiol 105:970-976.

Houng HS, Sethabutr O, Echeverria P (1997) A simple polymerase chain reaction technique to detect and differentiate *Shigella* and enteroinvasive *Escherichia coli* in human feces. Diagn Microbiol Infect Dis 28:19-25.

Hsu WB, Wang JH, Chen PC, Lu YS, Chen JH (2007) Detecting low concentrations of *Shigella sonnei* in environmental water samples by PCR. FEMS Microbiol Lett 270:291-298.

Hyma KE, Lacher DW, Nelson AM, Bumbaugh AC, Janda JM, Strockbine NA, Young VB, Whittam TS (2005) Evolutionary genetics of a new pathogenic Escherichia species: *Escherichia albertii* and related *Shigella boydii* strains. J Bacteriol 187:619-628.

Ito H, Kido N, Arakawa Y, Ohta M, Sugiyama T, Kato N (1991) Possible mechanisms underlying the slow lactose fermentation phenotype in *Shigella* spp. Appl Environ Microbiol 57:2912-2917.

Johnson JR (2000) *Shigella* and *Escherichia coli* at the crossroads: machiavellian masqueraders or taxonomic treachery? J Med Microbiol 49:583-585.

Kibbee R, Linklater N, Ormeci B (2013) Eliminating false positives in a qPCR assay for the detection of the *uid*A gene in *Escherichia coli*. J Water Health 11:382-386.

Kotloff KL, Winickoff JP, Ivanoff B, Clemens JD, Swerdlow DL, Sansonetti PJ, Adak GK, Levine MM (1999) Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies. Bull World Health Organ 77:651-666.

Lan R, Alles MC, Donohoe K, Martinez MB, Reeves PR (2004) Molecular evolutionary relationships of enteroinvasive *Escherichia coli* and *Shigella* spp. Infect Immun 72:5080-5088.

Lan R, Lumb B, Ryan D, Reeves PR (2001) Molecular evolution of large virulence plasmid in *Shigella* clones and enteroinvasive *Escherichia coli*. Infect Immun 69:6303-6309.

Landersjo C, Weintraub A, Widmalm G (1996) Structure determination of the O-antigen polysaccharide from the enteroinvasive *Escherichia coli* (EIEC) O143 by component analysis and NMR spectroscopy. Carbohydr Res 291:209-216.

Lett MC, Sasakawa C, Okada N, Sakai T, Makino S, Yamada M, Komatsu K, Yoshikawa M (1989) virG, a plasmid-coded virulence gene of *Shigella flexneri*: identification of the virG protein and determination of the complete coding sequence. J Bacteriol 171:353-359.

Linnerborg M, Weintraub A, Widmalm G (1999) Structural studies of the O-antigen polysaccharide from the enteroinvasive *Escherichia coli* O164 cross-reacting with *Shigella dysenteriae* type 3. Eur J Biochem 266:460-466.

Liu J, Gratz J, Amour C, Kibiki G, Becker S, Janaki L, Verweij JJ, Taniuchi M, Sobuz SU, Haque R, Haverstick DM, Houpt ER (2013) A laboratory-developed TaqMan Array Card for simultaneous detection of 19 enteropathogens. J Clin Microbiol 51:472-480.

Lukjancenko O, Wassenaar TM, Ussery DW (2010) Comparison of 61 sequenced *Escherichia coli* genomes. Microb Ecol 60:708-720.

Martinez MB, Whittan TS, McGraw EA, Rodrigues J, Trabulsi LR (1999) Clonal relationship among invasive and non-invasive strains of enteroinvasive *Escherichia coli* serogroups. FEMS Microbiol Lett 172:145-151.

Matsushita S, Yamada S, Kai A, Kudoh Y (1993) Invasive strains of *Escherichia coli* belonging to serotype O121: NM. J Clin Microbiol 31:3034-3035.

Maurelli AT, Fernandez RE, Bloch CA, Rode CK, Fasano A (1998) "Black holes" and bacterial pathogenicity: a large genomic deletion that enhances the virulence of *Shigella* spp. and enteroinvasive *Escherichia coli*. Proc Natl Acad Sci USA 95:3943-3948.

Moreno AC, Ferreira KS, Ferreira LG, Almeida SR, Martinez MB (2012) Recognition of enteroinvasive *Escherichia coli* and *Shigella flexneri* by dendritic cells: distinct dendritic cell activation states. Mem Inst Oswaldo Cruz 107:138-141.

Moreno AC, Ferreira LG, Martinez MB (2009) Enteroinvasive *Escherichia coli* vs. *Shigella flexneri*: how different patterns of gene expression affect virulence. FEMS Microbiol Lett 301:156-163.

Moreno AC, Filho AF, Gomes Tdo A, Ramos ST, Montemor LP, Tavares VC, Filho Ldos S, Irino K, Martinez MB (2010) Etiology of childhood diarrhea in the northeast of Brazil: significant emergent diarrheal pathogens. Diagn Microbiol Infect Dis 66:50-57.

Nataro JP, Kaper JB (1998) Diarrheagenic *Escherichia coli*. Clin Microbiol Rev 11:142-201.

Oberhelman RA, Kopecko DJ, Venkatesan MM, Salazar-Lindo E, Gotuzzo E, Yi A, Chea-Woo E, Ruiz R, Fernandez-Prada C, Leon-Barua R (1993) Evaluation of alkaline phosphatase-labelled *ipa*H probe for diagnosis of *Shigella* infections. J Clin Microbiol 31:2101-2104.

Ojha SC, Yean Yean C, Ismail A, Banga Singh K-K (2013) A pentaplex PCR assay for the detection and differentiation of *Shigella* species. BioMed Res Int 2013.

Orskov I, Wachsmuth IK, Taylor DN, Echeverria P, Rowe B, Sakazaki R, Orskov F (1991) Two new *Escherichia coli* O groups: O172 from *Shigella*-like toxin II-producing strains (EHEC) and O173 from enteroinvasive *E. coli* (EIEC). APMIS 99:30-32.

Pal T, Al-Sweih NA, Herpay M, Chugh TD (1997) Identification of enteroinvasive *Escherichia coli* and *Shigella* strains in pediatric patients by an *Ipa*C-specific enzyme-linked immunosorbent assay. J Clin Microbiol 35:1757-1760.

Parsot C (2005) *Shigella* spp. and enteroinvasive *Escherichia coli* pathogenicity factors. FEMS Microbiol Lett 252:11-18.

Pavlovic M, Luze A, Konrad R, Berger A, Sing A, Busch U, Huber I (2011) Development of a duplex real-time PCR for differentiation between *E. coli* and *Shigella* spp. J Appl Microbiol 110:1245-1251.

Peng J, Zhang X, Yang J, Wang J, Yang E, Bin W, Wei C, Sun M, Jin Q (2006) The use of comparative genomic hybridization to characterize genome dynamics and diversity among the serotypes of *Shigella*. BMC Genomics 7:218.

Porter ME, Dorman CJ (2002) In vivo DNA-binding and oligomerization properties of the *Shigella flexneri* AraC-like transcriptional regulator VirF as identified by random and site-specific mutagenesis. J Bacteriol 184:531-539.

Prosseda G, Fradiani P, Di Lorenzo M, Falconi M, Micheli G, Casalino M, Nicoletti M, Colonna B (1998) A role for H-NS in the regulation of the *vir*F gene of *Shigella* and enteroinvasive *Escherichia coli*. Res Microbiol 149:15-25.

Pupo GM, Lan R, Reeves PR (2000) Multiple independent origins of *Shigella* clones of *Escherichia coli* and convergent evolution of many of their characteristics. Proc Natl Acad Sci USA 97:10567-10572.

Rolland K, Lambert-Zechovsky N, Picard B, Denamur E (1998) *Shigella* and enteroinvasive *Escherichia coli* strains are derived from distinct ancestral strains of *E. coli*. Microbiology 144:2667-2672.

Rowe B, Gross RJ, Van Oye E (1975) An organism differing from *Shigella boydii* 13 only in its ability to produce gas from glucose. Int J Syst Bacteriol 25:301-303.

Sankaran K, Banerjee S, Pavankumar AR, Jesudason M, Reissbrodt R, Williams pH (2009) Apyrase-based colorimetric test for detection of *Shigella* and enteroinvasive *Escherichia coli* in stool. Diagn Microbiol Infect Dis 63:243-250.

Sansonetti PJ, d'Hauteville H, Formal SB, Toucas M (1982a) Plasmid-mediated invasiveness of "*Shigella*-like" *Escherichia coli*. Ann Microbiol (Paris) 133:351-355.

Sansonetti PJ, Hale TL, Dammin GJ, Kapfer C, Collins HH, Jr., Formal SB (1983) Alterations in the pathogenicity of *Escherichia coli* K-12 after transfer of plasmid and chromosomal genes from *Shigella flexneri*. Infect Immun 39:1392-1402.

Sansonetti PJ, Kopecko DJ, Formal SB (1981) *Shigella sonnei* plasmids: evidence that a large plasmid is necessary for virulence. Infect Immun 34:75-83.

Sansonetti PJ, Kopecko DJ, Formal SB (1982b) Involvement of a plasmid in the invasive ability of *Shigella flexneri*. Infect Immun 35:852-860.

Sansonetti PJ, Mounier J, Prevost MC, Mege RM (1994) Cadherin expression is required for the spread of *Shigella flexneri* between epithelial cells. Cell 76:829-839.

Sasakawa C, Buysse JM, Watanabe H (1992) The large virulence plasmid of *Shigella*. Curr Top Microbiol Immunol 180:21-44.

Scheutz F SN, (2005) Genus I. Escherichia. In: Bergey's Manual of Systematic Bacteriology, Springer, USA.

Sethabutr O, Venkatesan M, Yam S, Pang LW, Smoak BL, Sang WK, Echeverria P, Taylor DN, Isenbarger DW (2000) Detection of PCR products of the *ipa*H gene from *Shigella* and enteroinvasive *Escherichia coli* by enzyme linked immunosorbent assay. Diagn Microbiol Infect Dis 37:11-16.

Shepherd JG, Wang L, Reeves PR (2000) Comparison of O-antigen gene clusters of *Escherichia coli* (*Shigella*) *Sonnei* and *Plesiomonas shigelloides* O17: Sonnei gained its current plasmid-borne O-antigen genes from *P. shigelloides* in a recent event. Infect Immun 68:6056-6061.

Silva RM, Toledo MR, Trabulsi LR (1980) Biochemical and cultural characteristics of invasive *Escherichia coli*. J Clin Microbiol 11:441-444.

Simmons DA, Romanowska E (1987) Structure and biology of *Shigella flexneri* O antigens. J Med Microbiol 23:289-302.

Small PL, Falkow S (1988) Identification of regions on a 230-kilobase plasmid from enteroinvasive *Escherichia coli* that are required for entry into HEp-2 cells. Infect Immun 56:225-229.

Song T, Toma C, Nakasone N, Iwanaga M (2005) Sensitive and rapid detection of *Shigella* and enteroinvasive *Escherichia coli* by a loop-mediated isothermal amplification method. FEMS Microbiol Lett 243:259-263.

Stoebel DM (2005) Lack of evidence for horizontal transfer of the lac operon into *Escherichia coli*. Mol Biol Evol 22:683-690.

Stypulkowska H (1964) Atypical strain of *Shigella dysenteriae* 3 isolated in Poland. Med Dosw Mikrobiol 16:147-154.

Suzuki T, Miki H, Takenawa T, Sasakawa C (1998) Neural Wiskott-Aldrich Syndrome Protein is implicated in the actin-based motility of *Shigella flexneri*. EMBO J 17:2767-2776.

Talukder KA, Azmi IJ (2012) Population genetics and molecular epidemiology of *Shigella* species. *In:* Faruque SM (eds). Foodborne and waterborne bacterial pathogens epidemiology, evolution and molecular biology. Caister Academic Press, pp 63-76.

Thiem VD, Sethabutr O, Von Seidlein L, Van Tung T, Chien BT, Lee H, Houng HS, Hale TL, Clemens JD, Mason C (2004) Detection of *Shigella* by a PCR assay targeting the *ipa*H gene suggests increased prevalence of shigellosis in Nha Trang, Vietnam. J Clin Microbiol 42:2031-2035.

Toledo MR, Trabulsi LR (1983) Correlation between biochemical and serological characteristics of *Escherichia coli* and results of the Sereny test. J Clin Microbiol 17:419-421.

Ud-Din AIMS, Azmi IJ, Dutta DK, Wahid SUH, Iqbal MS, Faroque ASG, Endtz HP, Cravioto A, Talukder KA (2010) Plasmid profiling facilitates the detection of invasive *Shigella* and enteroinvasive *E. coli* directly from enriched stool culture broth of diarrheal patients. The 13th Annual Scientific Conference (ASCON XIII), icddr,b Dhaka, Bangladesh, pp 256.

Venkatesan MM, Goldberg MB, Rose DJ, Grotbeck EJ, Burland V, Blattner FR (2001) Complete DNA sequence and analysis of the large virulence plasmid of *Shigella flexneri*. Infect Immun 69:3271-3285.

Vieira N, Bates SJ, Solberg OD, Ponce K, Howsmon R, Cevallos W, Trueba G, Riley L, Eisenberg JN (2007) High prevalence of enteroinvasive *Escherichia coli* isolated in a remote region of northern coastal Ecuador. Am J Trop Med Hyg 76:528-532.

Wang SJ, Chen JH (2012) A rapid and specific PCR method for the detection of *Shigella* spp. in spiked samples. J Food Drug Anal 20:59-65.

Wen X, Wu Y, Bian F, Sun Y, Zheng X, Zhang Y, Jiang C, Yuan G (2012) High prevalence of atypical class 1 integrons and class 2 integrons in multi-drug resistance *Shigella flexneri* isolated from China. Afr J Microbiol Res 6:6987-6993.

Yang F, Yang J, Zhang X, Chen L, Jiang Y, Yan Y, Tang X, Wang J, Xiong Z, Dong J, Xue Y, Zhu Y, Xu X, Sun L, Chen S, Nie H, Peng J, Xu J, Wang Y, Yuan Z, Wen Y, Yao Z, Shen Y, Qiang B, Hou Y, Yu J, Jin Q (2005) Genome dynamics and diversity of *Shigella* species, the etiologic agents of bacillary dysentery. Nucleic Acids Res 33:6445-6458.

Yang J, Nie H, Chen L, Zhang X, Yang F, Xu X, Zhu Y, Yu J, Jin Q (2007) Revisiting the molecular evolutionary history of *Shigella* spp. J Mol Evol 64:71-79.