Cluster-specific gene markers enhance *Shigella* and Enteroinvasive *Escherichia coli in silico* serotyping

Xiaomei Zhang[1], Michael Payne[1], Thanh Nguyen[1], Sandeep Kaur[1], Ruiting Lan[1]*

[1]School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, New South Wales, Australia

*Corresponding Author
Email: r.lan@unsw.edu.au
Phone: 61-2-9385 2095
Fax: 61-2-9385 1483

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Abstract

Shigella and enteroinvasive *Escherichia coli* (EIEC) cause human bacillary dysentery with similar invasion mechanisms and share similar physiological, biochemical and genetic characteristics. The ability to differentiate *Shigella* and EIEC from each other is important for clinical diagnostic and epidemiologic investigations. The existing genetic signatures may not discriminate between *Shigella* and EIEC. However, phylogenetically, *Shigella* and EIEC strains are composed of multiple clusters and are different forms of *E. coli*. In this study, we identified 10 *Shigella* clusters, 7 EIEC clusters and 53 sporadic types of EIEC by examining over 17,000 publicly available *Shigella*/EIEC genomes. We compared *Shigella* and EIEC accessory genomes to identify the cluster-specific gene markers or marker sets for the 17 clusters and 53 sporadic types. The gene markers showed 99.63% accuracy and more than 97.02% specificity. In addition, we developed a freely available *in silico* serotyping pipeline named *Shigella* EIEC Cluster Enhanced Serotype Finder (ShigEiFinder) by incorporating the cluster-specific gene markers and established *Shigella*/EIEC serotype specific O antigen genes and modification genes into typing. ShigEiFinder can process either paired end Illumina sequencing reads or assembled genomes and almost perfectly differentiated *Shigella* from EIEC with 99.70% and 99.81% cluster assignment accuracy for the assembled genomes and mapped reads respectively. ShigEiFinder was able to serotype over 59 *Shigella* serotypes and 22 EIEC serotypes and provided a high specificity with 99.40% for assembled genomes and 99.38% for mapped reads for serotyping. The cluster markers and our new serotyping tool, ShigEiFinder (https://github.com/LanLab/ShigEiFinder), will be useful for epidemiologic and diagnostic investigations.

Impact statement

The differentiation of *Shigella* strains from enteroinvasive *E. coli* (EIEC) is important for clinical diagnosis and public health epidemiologic investigations. The similarities between *Shigella* and EIEC strains make this differentiation very difficult as both share common ancestries within *E. coli*. However, *Shigella* and EIEC are phylogenetically separated into multiple clusters, making high resolution separation using cluster specific genomic markers possible. In this study, we identified 17 *Shigella* or EIEC clusters including five that were newly identified through examination of over 17,000 publicly available *Shigella* and EIEC genomes. We further identified an individual or a set of cluster-specific gene markers for each cluster using comparative genomic analysis. These markers can then be used to classify isolates into clusters and were used to develop an *in silico* pipeline, ShigEiFinder

57 (https://github.com/LanLab/ShigEiFinder) for accurate differentiation, cluster typing and
58 serotyping of *Shigella* and EIEC from Illumina sequencing reads or assembled genomes. This
59 study will have broad application from understanding the evolution of *Shigella*/EIEC to
60 diagnosis and epidemiology.
61
62 **Data summary**
63 Sequencing data have been deposited at the National Center for Biotechnology Information
64 under BioProject number PRJNA692536.
65
66
67 **Introduction**
68 *Shigella* is one of the most common etiologic agents of foodborne infections worldwide and
69 can cause diarrhea with a very low infectious dose (1, 2). The infections can vary from mild
70 diarrhea to severe bloody diarrhea referred to as bacillary dysentery. The estimated cases of
71 *Shigella* infections are 190 million with at least 210,000 deaths annually, predominantly in
72 children younger than 5 years old in developing countries (3-7). *Shigella* infections also have a
73 significantly impact on public health in developed countries although most cases are travel-
74 associated (8).
75
76 The *Shigella* genus consists of four species, *Shigella sonnei*, *Shigella flexneri*, *Shigella boydii*
77 and *Shigella dysenteriae* (9). Serological testing further classifies *Shigella* species into more
78 than 55 serotypes through the agglutination reaction of antisera to *Shigella* serotype specific O-
79 antigens (10, 11). Up to 89.6% *Shigella* infections were caused by *S. flexneri* (65.9%) and *S.*
80 *sonnei* (23.7%) globally (12, 13). The predominant serotype reported in *Shigella* infections has
81 been *S. flexneri* serotype 2a while *S. dysenteriae* serotype 1 has caused the most severe disease
82 (11, 14). Note that for brevity, in all references to *Shigella* serotypes below, *S. sonnei*, *S.*
83 *flexneri*, *S. boydii* and *S. dysenteriae* are abbreviated as SS, SF, SB and SD respectively and a
84 serotype is designated with abbreviated "species" name plus the serotype number e.g. *S.*
85 *dysenteriae* serotype 1 is abbreviated as SD1.
86
87 Enteroinvasive *Escherichia coli* (EIEC) is a pathovar of *E. coli* that causes diarrhoea with less
88 severe symptoms to *Shigella* infections in humans worldwide, particularly in developing
89 countries (8, 13, 15-18). EIEC infections in developed countries are mainly imported (19).
90 EIEC has more than 18 specific E. coli O-serotypes (19, 20). Although the incidence of EIEC

91 is low (17), EIEC serotypes have been associated with outbreaks and sporadic cases of
92 infections (20-22). In contrast to *Shigella*, EIEC infections are not notifiable in many countries
93 (23, 24).
94
95 *Shigella* and EIEC have always been considered very closely related and share several
96 characteristics (25-28). *Shigella* and EIEC are both non-motile and lack the ability of ferment
97 lactose (24). Some of EIEC O antigens are identical or similar to *Shigella* O antigens (O112ac,
98 O124, O136, O143, O152 and O164) (26, 29-31). Furthermore, *Shigella* and EIEC both carry
99 the virulence plasmid pINV, which encodes virulence genes required for invasion (32, 33) and
100 contain *ipaH* (invasion plasmid antigen H) genes with the exception of some SB13 isolates (10,
101 23, 24, 34, 35). *Shigella* and EIEC have arisen from *E. coli* in multiple independent events
102 and should be regarded as a single pathovar of E. coli (25, 26, 28, 36-38). Previous
103 phylogenetic studies suggested that *Shigella* isolates were divided into 3 clusters (C1, C2 and
104 C3) with 5 outliers (SS, SB13, SD1, SD8 and SD10) (25, 38) whereas EIEC isolates were
105 grouped into four clusters (C4, C5, C6 and C7) (26). The seven *Shigella*/EIEC clusters and 5
106 outliers of *Shigella* are within the broader non-enteroinvasive *E. coli* species except for SB13
107 which is closer to *Escherichia albertii* (39, 40). WGS-based phylogenomic studies have also
108 defined multiple alternative clusters of *Shigella* and EIEC (23, 28, 41).
109
110 The traditional biochemical test for motility and lysine decarboxylase (LDC) activity (42) and
111 molecular test for the presence of *ipaH* gene have been used to differentiate *Shigella* and EIEC
112 from non-enteroinvasive E. coli (24, 43-45). Agglutination with *Shigella*/EIEC associated
113 antiserum further classify *Shigella* or EIEC to serotype level. However, cross-reactivity, strains
114 not producing O antigens, and newly emerged *Shigella* serotypes may all prevent accurate
115 serotyping (10, 46). Serotyping by antigenic agglutination is being replaced by molecular
116 serotyping (47, 48), which can be achieved through examination of the sequences of O antigen
117 biosynthesis and modification genes (8, 24, 49-52).
118
119 Recently, PCR-based molecular detection methods targeting the gene *lacY* were developed to
120 distinguish *Shigella* from EIEC (53, 54). However, the ability of the primers described in these
121 methods to accurately differentiate between *Shigella* and EIEC was later questioned (23, 28).
122 With the uptake of whole-genome sequencing technology, several studies have identified
123 phylogenetic clade specific markers, species specific markers and EIEC lineage-specific genes
124 for discrimination between *Shigella* and EIEC and between *Shigella* species (23, 27, 28, 41, 55,

125 56). More recently, genetic markers *lacY*, *cadA*, *Ss_methylase* were used for identification of
126 *Shigella* and EIEC (10). However, these markers failed to discriminate between *Shigella* and
127 EIEC when a larger genetic diversity is considered (23, 28, 55). A Kmer-based approach can
128 identify *Shigella* isolates to the species level but misidentification was also observed (56).
129
130 In this study, we aimed to i), identify phylogenetic clusters of *Shigella* and EIEC through large
131 scale examination of publicly available genomes; ii), identify cluster-specific gene markers
132 using comparative genomic analysis of *Shigella* and EIEC accessory genomes for
133 differentiation of *Shigella* and EIEC; iii), develop a pipeline for *Shigella* and EIEC *in silico*
134 serotyping based on the cluster-specific gene markers combined with *Shigella* and EIEC
135 serotype-specific O antigen and H antigen genes. We demonstrate that these cluster-specific
136 gene markers enhance *in silico* serotyping using genomic data. We also developed an
137 automated pipeline for cluster typing and serotyping of *Shigella*/EIEC from WGS data.
138
139 **Materials and Methods**
140 **Identification of *Shigella*/EIEC isolates from NCBI database**
141 *E. coli/Shigella* isolates from the NCBI SRA (National Center for Biotechnology Information
142 Sequence Read Archive) as May of 2019 were queried. Raw reads were retrieved from ENA
143 (European Nucleotide Archive). The *ipaH* gene (GenBank accession number M32063.1) was
144 used to screen E. coli/*Shigella* reads using Salmon v0.13.0 (57). Taxonomic classification for
145 *E. coli/Shigella* was confirmed by Kraken v1.1.1 (58). Molecular serotype prediction of *ipaH*
146 negative *Shigella* isolates was performed by ShigaTyper v1.0.6 (10). Isolates that were *ipaH*
147 positive and isolates with designation of SB13 by ShigaTyper were selected as *Shigella*/EIEC
148 database.
149
150 The sequence types (STs) and ribosomal STs (rSTs) of *ipaH* gene negative E. coli (non-
151 enteroinvasive E. coli) isolates were examined. STs and rSTs for these isolates were obtained
152 from the E. coli/*Shigella* database in the Enterobase (59) as of May 2019. For STs and rSTs
153 with only one isolate, the isolates were selected. For STs and rSTs with more than one isolates,
154 one representative isolate for each ST and rST were randomly selected. In total, 12,743 ipaH
155 negative E. coli isolates representing 3,800 STs and 11,463 rSTs were selected as non-
156 enteroinvasive E. coli control database.
157
158 **Genome sequencing**

159 Whole-genome sequencing (WGS) of 31 EIEC strains used in a previous study (26) was
160 performed by Illumina NextSeq (Illumina, Scoresby, VIC, Australia). DNA libraries were
161 constructed using Nextera XT Sample preparation kit (Illumina Inc., San Diego, CA, USA) and
162 sequenced using the NextSeq sequencer (Illumina Inc.). FASTQ sequences of the strains
163 sequenced in this study were deposited in the NCBI under the BioProject (PRJNA692536).
164
165 **Genome assembly and data processing**
166 Raw reads were *de novo* assembled using SPADES v3.14.0 assembler with default settings
167 [http://bioinf.spbau.ru/spades] (60). The metrics of assembled genomes were obtained with
168 QUAST v5.0.0 (61). Three standard deviations (SD) from the mean for contig number, largest
169 contig, total length, GC, N50 and genes were used as quality filter for assembled genomes.
170
171 The STs for isolates in *Shigella*/EIEC database was checked by using mlst
172 (https://github.com/tseemann/mlst) with the *E. coli* scheme from PubMLST (62). rSTs were
173 extracted from the E. coli/*Shigella* rMLST database in Enterobase (59) as of May 2019.
174 Serotype prediction for isolates in *Shigella*/EIEC was performed by ShigaTyper v1.0.6 (10).
175 Serotyping of E. coli O and H antigens were predicted by using SerotypeFinder v2.0.1 (63).
176
177 **Selection of isolates for *Shigella*/EIEC identification dataset**
178 The selection of isolates for the identification dataset was based on the representative isolates
179 for each ST, rST and serotype of *Shigella* and EIEC in the *Shigella*/EIEC database. For STs
180 and rSTs with only one isolate, the isolate was selected. For STs and rSTs with more than one
181 isolates, one representative isolate for each ST, rST was randomly selected. A representative
182 experimentally confirmed isolate of each serotype of *Shigella* and EIEC was also randomly
183 selected. 72 ECOR strains downloaded from Enterobase (59) and 18 *E. albertii* strains were
184 used as controls for the identification dataset. The details of the identification dataset are listed
185 in Table S1. The remaining isolates in *Shigella*/EIEC database were referred as validation
186 dataset (Table S2).
187
188 The identification dataset was used to characterise the phylogenetic relationships of *Shigella*
189 and EIEC. The identification dataset was also used to identify cluster-specific genes. The
190 validation dataset was used to evaluate the performance of cluster-specific gene markers using
191 the *in-silico* serotyping pipeline.
192

193 **Phylogeny of *Shigella* and EIEC based on WGS**

194 Three phylogenetic trees including identification tree, confirmation tree and validation tree
195 were constructed by Quicktree v1.3 (64) with default parameters to identify and confirm the
196 phylogenetic clustering of *Shigella* and EIEC isolates. The phylogenetic trees were visualised
197 by Grapetree and ITOL v5 (65, 66).

198

199 The identification phylogenetic tree was generated based on isolates in the identification
200 dataset for the characterisation of clusters of *Shigella* and EIEC isolates (Fig. 1). A subset of
201 485 isolates known to represent each identified cluster from the identification dataset were then
202 selected. The subset of 485 isolates from the identification dataset and 1,872 non-
203 enteroinvasive E. coli isolates from non-enteroinvasive E. coli control dataset (2,357 isolates
204 total) were used to construct a confirmation tree. This tree was used for confirmation of the
205 phylogenetic relationships between identified *Shigella*/EIEC clusters in the identification
206 dataset and non-enteroinvasive E. coli isolates. The validation tree was generated based on
207 1,159 representative isolates from the validation dataset that were selected in the same way as
208 the identification dataset and a subset of 485 isolates from the identification dataset to assign
209 validation dataset isolates to clusters.

210

211 **Investigation of *Shigella* virulence plasmid pINV**

212 The presence of *Shigella* virulence plasmid pINV in isolates were investigated by using BWA-
213 MEM v0.7.17 (Burrows-Wheeler Aligner) (67) to align isolate raw reads onto the reference
214 sequence of pINV (68) (NC_024996.1). Mapped reads were sorted and indexed using
215 Samtools v1.9 (69). The individual gene coverage from mapping was obtained using Bedtools
216 coverage v2.27.1 (70).

217

218 **Identification of the cluster-specific gene markers**

219 Cluster-specific gene markers were identified from *Shigella*/EIEC accessory genomes. The
220 genomes from the identification dataset were annotated using PROKKA v1.13.3 (71). Pan- and
221 core-genomes were analysed by roary v3.12.0 (72) using an 80% sequence identity threshold.
222 The genes specific to each cluster were identified from the accessory genes with an in-house
223 python script. In this study, the number of genomes from a given cluster containing all specific
224 genes for that cluster was termed true positives (TP), the number of genomes from the same
225 cluster lacking any of those same genes was termed false negatives (FN). The number of

genomes from other clusters containing all of those same genes was termed false positives (FP).

The sensitivity (True positive rate, TPR) of each cluster-specific gene marker was defined as TP/(TP+FN). The specificity (True negative rate, TNR) was defined as TN/(TN+FP).

Validation of the cluster-specific gene markers

The ability of cluster-specific gene markers to assign *Shigella*/EIEC isolates was examined by using BLASTN to search against the validation dataset (Table S2) and non-enteroinvasive E. coli control database for the presence of any of the cluster-specific gene marker or a set of cluster-specific gene markers. The BLASTN thresholds were defined as 80% sequence identity and 50% gene length coverage.

Development of ShigEiFinder, an automated pipeline for molecular serotyping of *Shigella*/EIEC

ShigEiFinder was developed using paired end illumina genome sequencing reads or assembled genomes identify cluster-specific gene markers combined with *Shigella*/EIEC serotype specific O antigen genes (wzx and wzy) and modification genes (Fig. 2, Data S1). We used the same signature O and H sequences from ShigaTyper and SerotypeFinder (Data S2) (10, 63). These include *Shigella* serotype-specifc wzx/wzy genes and modification genes from ShigaTyper and E. coli O antigen and *fliC* (H antigen) genes from SerotypeFinder. *ipaH* gene and 38 virulence genes used in analysis of virulence of 59 sporadic EIEC isolates were also included in the typing reference sequences database. Seven House Keeping (HK) genes -*recA*, *purA*, *mdh*, *icd*, *gyrB*, *fumC* and *adk* downloaded from NCBI were used for contamination checking.

Raw reads were aligned to the typing reference sequences by using BWA-MEM v0.7.17 (67). The mapping length percentage and the mean mapping depth for all genes were calculated using Samtools coverage v1.10 (69). To determine whether the genes were present or absent, 50% of mapping length for all cluster-specific genes, virulence genes and O antigen genes and 10% for *ipaH* gene were used as cutoff value. The ratio of mean mapping depth to the mean mapping depth of the 7 HK genes was used to determine a contamination threshold with ratios less than 1% for *ipaH* gene and less than 10% for other genes assigned as contamination. Reads coverage mapped to particular regions of genes were checked by using samtools mpileup v1.10.

260

261 Assembled genomes were searched against the typing reference sequences using BLASTN
262 v2.9.0 (73) with 80% sequence identity and 50% gene length coverage for all genes with
263 exception of *ipaH* gene which was defined as 10% gene length coverage.
264

265 ShigEiFinder was tested with the identification dataset and validated with the *Shigella*/EIEC
266 validation dataset and non-enteroinvasive E. coli control database. The specificity defined as (1
267 - the number of non-enteroinvasive E. coli isolates being detected / the total number of non-
268 enteroinvasive E. coli isolates) * 100.
269
270

271 **Results**

272 **Screening sequenced genomes for *Shigella*/EIEC isolates**

273 We first screened available *E. coli* and *Shigella* genomes based on the presence of *ipaH* gene.
274 We examined 122,361 isolates with the species annotation of E. coli (104,256) or *Shigella*
275 (18,105) with paired end illumina sequencing reads available in NCBI SRA database. Of
276 122,361 isolates, 17,989 isolates were positive to the *ipaH* gene including 455 out of 104,256
277 E. coli isolates and 17,434 out of 18,105 *Shigella* isolates. The 17,989 *ipaH* positive *E. coli* and
278 *Shigella* genomes and 571 *ipaH* negative "*Shigella"* genomes were checked for taxonomic
279 classification and genome assembly quality. 17,320 *ipaH* positive *E. coli* and *Shigella* genomes
280 and 246 *ipaH* negative "*Shigella"* genomes passed quality filters. Among 246 *ipaH* negative
281 "*Shigella"* genomes, 11 isolates belonged to SB13 by using ShigaTyper (10) while the
282 remaining 235 isolates were classified with taxonomic identifier of E. coli by Kraken v1.1.1
283 (58) and were removed from analysis. A total of 17,331 genomes including 17,320 *ipaH*
284 positives and 11 SB13 genomes were selected to form the *Shigella*/EIEC database, which
285 contained 429 genomes with species identifier of *E. coli* and 16,902 genomes with species
286 identifier of *Shigella.*
287

288 Isolates in *Shigella*/EIEC database were typed using MLST, ShigaTyper and SerotypeFinder.
289 MLST and rMLST divided the 17,331 *Shigella*/EIEC isolates into 252 STs (73 isolates
290 untypeable by MLST) and 1,128 rSTs (3,513 isolates untypeable by rMLST). Of 16,902
291 genomes with species identifier of *Shigella*, 8,313 isolates and 8,189 isolates were typed as
292 *Shigella* and EIEC respectively by ShigaTyper while 400 isolates were untypeable. ShigaTyper

293 typed the majority of the 8,313 isolates as SF (66.82%) including 25.43% SF2a isolates,
294 followed by SS (19.69%), SB (7.22%) and SD (6.27%).
295
296 SerotypeFinder typed 293 of the 429 *E. coli* genomes into 71 *E. coli* O/H antigen types.
297 Among these 293 isolates with typable O/H antigen types, 190 isolates belonged to 22 known
298 EIEC serotypes (O28ac:H-, O28ac:H7, O29:H4, O112ac:H26, O121:H30, O124:H30,
299 O124:H24, O124:H7, O132:H7, O132:H21, O135:H30, O136:H7, O143:H26, O144:H25,
300 O152:H-, O152:H30, O164:H-, O164:H30, O167:H26, O173:H7 and 2 newly emerged EIEC
301 serotypes O96:H19 and O8:H19) (20-22). The remaining 136 of 429 genomes were O antigen
302 untypable and typed to 15 H antigen types only by SerotypeFinder, of which H16 was the
303 predominant type.
304
305 **Identification of *Shigella* and EIEC clusters**
306 *Shigella* and EIEC are known to have been derived from *E. coli* independently. To identify
307 previously defined clusters (25, 26) and any new clusters from the 17,331 *Shigella*/EIEC
308 genomes, we selected representative genomes to perform phylogenetic analysis as it was
309 impractical to construct a tree with all genomes. The selection was based on ST, rST and
310 serotype of the 17,331 *Shigella*/EIEC genomes. One isolate was selected to represent each ST,
311 rST and serotype for a total of 1,830 isolates. The selection included 252 STs, 1,128 rSTs, 59
312 *Shigella* serotypes (21 SB serotypes, 20 SF serotypes, 17 SD serotypes and SS), 22 EIEC
313 known serotypes and 31 other or partial antigen types. A further 31 in-house sequenced EIEC
314 isolates, 18 EIEC isolates used in a previous typing study (41), 72 ECOR strains and 18 *E.*
315 *albertii* strains were also included to form the identification dataset of 1,969 isolates. Details
316 are listed in Table S1. A phylogenetic tree was constructed based on the identification dataset
317 to identify the clusters (Fig. 1).
318
319 All known clusters were identified (Fig. 1) including 3 *Shigella* clusters (C1, C2, C3) and 5
320 outliers (SD1, SD8, SD10, SB13 and SS) as defined by Pupo et al (25) and 4 EIEC clusters
321 (C4, C5, C6 and C7) defined by Lan et al. (26). Each of these clusters was supported by a
322 bootstrap value of 80% or greater (Fig. S1). 1,789 isolates of the 1,879 *Shigella*/EIEC isolates
323 (1,830 isolates from the *Shigella*/EIEC database, 31 in-house sequenced EIEC isolates and 18
324 EIEC isolates from Hazen *et al.*) fell within these clusters.
325

326 Of the remaining 90 *Shigella*/EIEC unclustered isolates, 31 belonged to 5 *Shigella*/EIEC
327 serotypes including 5 SB13 isolates, 8 SB12 isolates, 2 EIEC O135:H30 isolates, 12 EIEC
328 serotype O96:H19 isolates and 4 EIEC O8:H19 isolates, while 59 isolates were sporadic EIEC
329 isolates which are described in detail in the separate section below. The 5 SB13 isolates were
330 grouped into one lineage within *E. coli* and close to known *Shigella*/EIEC clusters rather than
331 the established SB13 cluster outside *E. coli* which was within the *E. albertii* lineage. The
332 former was previously named as atypical SB13 while the latter was previously named as
333 typical SB13 (39). The 8 SB12 isolates formed one single cluster close to SD1 and atypical
334 SB13 clusters. Two EIEC O135:H30 isolates were grouped as a separate cluster close to C5.
335 Twelve isolates belonging to EIEC serotype O96:H19 and 4 isolates typed as O8:H19 were
336 clustered into two separate clusters, both of which were more closely related to SD8 than other
337 *Shigella*/EIEC clusters. Therefore, atypical SB13 and SB12 were defined as new clusters of
338 *Shigella* while EIEC O96:H19, EIEC O8:H19 and EIEC O135:H30 were defined as C8, C9
339 and C10 respectively. In total there were 10 *Shigella* clusters and 7 EIEC clusters (Table 1).
340
341 **Analysis of the 59 sporadic EIEC isolates**
342 To determine the phylogenetic relationships of the above defined clusters and the remaining 59
343 sporadic EIEC isolates within the larger non-enteroinvasive *E. coli* population a confirmation
344 tree was generated using 485 isolates representing the known clusters and 1,872 representative
345 non-*Shigella*/EIEC isolates (Fig. S2). The 59 sporadic EIEC isolates including 2 EIEC isolates
346 M2330 (O152:H51) and M2339 (O124:H7) sequenced in this study and 57 isolates were
347 interspersed among non-*Shigella*/EIEC isolates and did not form large clusters. Groups of these
348 isolates that were not previously identified were named as sporadic EIEC lineage followed by
349 their serotype. For example, M2339 (O124:H7) grouped together with one other EIEC isolate
350 with the same O and H antigens O124:H7 and were named 'sporadic EIEC lineage O124:H7'.
351 There were 53 sporadic EIEC lineages including 5 lineages with 2 or more isolates and 48
352 lineages with only one isolate. The STs, rSTs and antigen types of these 59 isolates were listed
353 in the Table S1.
354
355 Some of the sporadic EIEC isolates fell into STs containing *ipaH* negative isolates. We
356 therefore examined the presence of the pINV virulence plasmid in the sporadic EIEC isolates.
357 We selected 38 genes that are essential for virulence including 35 genes (12 *mxi* genes, 9 *spa*
358 genes, 5 *ipaA-J* genes, 6 *ipgA-F* genes as well as *acp*, *virB*, *icsB*) in the conserved entry region
359 encoding the Mxi-Spa-Ipa type III secretion system and its effectors and 3 regulator genes

360 (*virF*, *virA* and *icsA*/*virG*) (24, 33, 68) and determined the presence of pINV in the 59 sporadic
361 EIEC isolates by mapping the sequence reads onto a pINV reference sequence (68). Reads
362 from 18 non-*Shigella/EIEC* isolates that shared the same ST as one of 58 sporadic isolates
363 were positive for these genes.
364
365 The number of essential virulence genes with mapped reads in the 59 sporadic EIEC isolates
366 were analysed (Fig. S3). Those isolates containing more than 25 of the 38 essential virulence
367 genes were defined as virulence plasmid positive. While isolates containing between 13 and 25
368 were defined as intermediate and less than 13 were defined as virulence plasmid negative.
369
370 The 2 newly sequenced sporadic EIEC isolates (M2330 and M2339) were positive for the
371 virulence plasmid and of the other 57 sporadic EIEC isolates, 39 isolates were positive, 9
372 isolates were negative and 9 isolates were intermediate (Table S1). The results were compared
373 with those non-*Shigella/*EIEC isolates belonging to the same ST. The virulence plasmid was
374 absent in all non-*Shigella/*EIEC isolates while all sporadic EIEC isolates in these STs were
375 either positive or intermediate. Therefore, this analysis confirmed the sporadic isolates
376 belonged to EIEC and the STs contained both EIEC and non- EIEC isolates.
377
378 **Identification of cluster-specific gene markers**
379 In this study, cluster-specific gene markers were either a single gene present in all isolates of a
380 cluster and absent in all other isolates or a set of genes (two or more) that as a combination
381 were only found in one cluster. For the marker sets, a subset of cluster-specific gene markers
382 for a given cluster could be found in other clusters but the entire set was only found in the
383 target cluster.
384
385 Comparative genomic analysis on 1,969 accessory genomes from the identification dataset was
386 used to identify cluster-specific gene markers or marker sets. Multiple candidate cluster-
387 specific gene markers or marker sets of markers for each of 17 *Shigella*/EIEC clusters and 53
388 sporadic EIEC lineages were identified through screening the accessory genes from 1,969
389 genomes. These gene markers or marker sets were 100% sensitive to clusters but with varying
390 specificity. The cluster-specific gene markers or marker sets with the lowest FP rates were then
391 selected from candidate cluster-specific gene markers by BLASTN searches against genomes
392 in the identification dataset using 80% sequence identity and 50% gene length threshold.
393

394 Five single cluster-specific gene markers (C7, C10, SB12, SB13 and atypical SB13) and 12
395 sets of cluster-specific gene markers (C1, C2, C3, C4, C5, C6, C8, C9, SS, SD1, SD8 and
396 SD10) were selected for *Shigella*/EIEC cluster typing. The sensitivity and specificity for each
397 cluster-specific gene marker or a set of cluster-specific gene markers for the identification
398 dataset were listed in Table 2. The cluster-specific gene markers or marker sets were all 100%
399 sensitive and 100% specific with the exception of C1 (99.94% specificity), C3 (99.91%
400 specificity) and SS (99.8% specificity). A single specific gene for each of 53 sporadic EIEC
401 lineages were also selected with the exception of one lineage which has a set of 2 genes. These
402 genes were all 100% sensitive and specific for a given sporadic EIEC lineage.
403
404 All cluster-specific gene markers, 37 in total (5 single, 32 genes in 12 sets) and 54 sporadic
405 EIEC lineages specific gene markers were located on chromosome but one of C4 gene markers
406 and 5 sporadic EIEC lineages specific genes were located on plasmid by NCBI BLAST
407 searching. None of the cluster-specific gene markers were contiguous in the genomes. The
408 location of these cluster-specific gene markers was determined by BLASTN against
409 representative complete genomes of *Shigella*/EIEC containing gene features downloaded from
410 NCBI GenBank. In those cluster or sporadic lineages with no representative complete genome
411 specific gene markers were named using their cluster or sporadic EIEC lineage followed by the
412 cluster or lineage number. For example, C7 specific gene marker was named "C7 specific
413 gene".
414
415 The functional characterization of these specific gene markers were identified from RAST
416 annotation (74). For 37 cluster-specific gene markers, 22 had known functions and 15 encoded
417 hypothetical proteins with unknown functions, while 11 sporadic EIEC lineages specific gene
418 markers were identified with known functions and 43 were hypothetical proteins with
419 unknown functions. The location and functions of specific gene markers are listed in Table S3.
420
421 **Validation of cluster-specific gene markers**
422 The ability of cluster-specific gene markers to correctly assign *Shigella*/EIEC isolates was
423 evaluate with 15,501 *Shigella*/EIEC isolates in the validation dataset, 12,743 isolates from non-
424 enteroinvasive *E. coli* control database.
425
426 Using cluster-specific gene markers, 15,443 of the 15,501 (99.63%) *Shigella*/EIEC isolates
427 were assigned to clusters which included 15,337 *Shigella* isolates, 102 EIEC isolates, 4

428 sporadic EIEC isolates, and 38 (0.24%) isolates with more than one clusters. Twenty of the
429 15,501 (0.13%) *Shigella*/EIEC isolates were not assigned to any of identified clusters.
430
431 To confirm the assignment of cluster-specific gene markers, we constructed a "validation"
432 phylogenetic tree (Fig. S4) using 1,159 representative isolates from the validation dataset and a
433 subset of 485 isolates from each cluster from the identification dataset. Isolates that grouped
434 with known cluster isolates (from identification dataset) with strong bootstrap support were
435 assigned to that cluster. All 1,159 isolates were grouped into known clusters on the validation
436 phylogenetic tree. The cluster-specific gene markers assignments were entirely consistent with
437 cluster assignments by phylogenetic tree.
438
439 We tested cluster-specific gene markers with the 12,743 non-enteroinvasive *E. coli* isolates.
440 The *Shigella*/EIEC cluster-specific gene markers were highly specific with specificity varying
441 from 98.8% to 100% for cluster-specific genes and 97.02% to 100% for sporadic EIEC specific
442 genes. Details are listed in Table S4.
443
444 **Development of an automated pipeline for molecular serotyping of *Shigella*/EIEC**
445 Above results showed that cluster-specific gene markers were sensitive and specific and can
446 distinguish *Shigella* and EIEC isolates. We therefore used these genes combined with
447 established *Shigella*/EIEC serotype specific O antigen and H antigen genes to develop an
448 automated pipeline for *in silico* serotyping of *Shigella*/EIEC (Fig. 2).
449
450 The pipeline is named *Shigella* EIEC Cluster Enhanced Serotype Finder (ShigEiFinder).
451 ShigEiFinder can process either paired end Illumina sequencing reads or assembled genomes
452 (installable package: https://github.com/LanLab/ShigEiFinder, online too:
453 https://mgtdb.unsw.edu.au/ShigEiFinder/). ShigEiFinder classifies isolates into Non-
454 *Shigella*/EIEC*, Shigella* or EIEC clusters based on the presence of *ipaH* gene, number of
455 virulence genes, cluster specific genes. The "Not *Shigella*/EIEC" assignment was determined
456 by the absence of the *ipaH* gene, virulence genes (<26) and absence of cluster-specific gene
457 markers. The "*Shigella* or EIEC clusters" assignments were made based on the presence of
458 *ipaH* gene, and/or more than 25 virulence genes together with the presence of any of cluster-
459 specific gene markers or marker set, whereas the presence of *ipaH* gene and/or more than 25
460 virulence genes with absence of any of cluster-specific gene markers were assigned as
461 "*Shigella*/EIEC unclustered".

462

463 *Shigella* and EIEC isolates were differentiated and serotypes were assigned after cluster
464 assignment. ShigEiFinder predicts a serotype through examining the presence of any of
465 established *Shigella* serotype specific O antigen and modification genes and E. coli O and H
466 antigen genes that differentiate the serotypes as ShigaTyper and SerotypeFinder (10, 63). A
467 "novel serotype" is assigned if no match to known serotypes.

468

469 Two pairs of *Shigella* serotypes, SB1/SB20 and SB6/SB10, are known to be difficult to
470 differentiate as they share identical O antigen genes (10, 46, 75). ShigaTyper used a heparinase
471 gene for the differentiation of SB20 from SB1 and *wbaM* gene for the separation of SB6 from
472 SB10. We found that fragments of the heparinase and *wbaM* genes may be present in other
473 serotypes and cannot accurately differentiate SB1/SB20 and SB6/SB10. We found a SB20
474 specific gene which encoded hypothetical proteins with unknown functions and located on a
475 plasmid by comparative genomic analysis of all isolates in C1 accessory genome. The SB20
476 specific gene can reliably differentiate SB20 from SB1and also one SNP each in *wzx* and *wzy*
477 genes that can differentiate SB6 from SB10. We used these differences (Data S1) in
478 ShigEiFinder for the prediction of these serotypes.

479

480 **The accuracy and specificity of ShigEiFinder in cluster typing**

481 The accuracy of ShigEiFinder was tested with 1,969 isolates (1,969 assembled genomes and
482 1,951 Illumina reads [note no reads available for 18 EIEC isolates from NCBI) from the
483 identification dataset and 15,501 isolates from the validation dataset. The results are listed in
484 Table 3.

485

486 ShigEiFinder was able to assign 99.54% and 99.28% of the isolates in the identification dataset
487 to clusters for assembled genomes and read mapping respectively. The accuracy was 99.70%
488 and 99.81% for assembled genomes and read mapping respectively when applied to the
489 validation dataset. Discrepancies were observed between assembled genomes and read
490 mapping (Table 3). There were more isolates assigned to "*Shigella*/EIEC unclustered" in read
491 mapping, in contrast there were more isolates assigned to multiple clusters in genome
492 assemblies. The specificity of ShigEiFinder was 99.40% for assembled genomes and 99.38%
493 for read mapping when evaluated with 12,743 non-*Shigella*/EIEC *E. coli* isolates. An
494 additional 2 isolates were detected as sporadic EIEC lineages by read mapping.

495

496 **Comparison of ShigEiFinder and ShigaTyper**

497 To demonstrate ShigEiFinder for differentiation of *Shigella* from EIEC and enhancement of
498 cluster based serotyping, the comparison of read mapping results between ShigEiFinder and
499 the existing *in silico Shigella* identification pipeline ShigaTyper (10) was performed with 488
500 isolates used in ShigaTyper and 15,501 isolates from *Shigella*/EIEC validation dataset used in
501 the present study.

502

503 The 488 isolates used in ShigaTyper consisted of 23 other species, 45 *E. coli* isolates and 420
504 *Shigella* isolates. ShigEiFinder identified 23 other species isolates and 453 out of 465 *E. coli*
505 and *Shigella* isolates, in agreement with ShigaTyper assignment. ShigEiFinder also assigned
506 the remaining 3 EIEC isolates and 9 (either multiple *wzx* or no *wzx* genes found) isolates
507 untypeable by ShigaTyper to *Shigella*/EIEC clusters.

508

509 ShigEiFinder assigned 15,471 of 15,501 *Shigella*/EIEC isolates to *Shigella* or EIEC clusters
510 and then to a serotype. The accuracy of ShigEiFinder to correctly assign isolates to *Shigella* or
511 EIEC clusters was 99.81% (15,471/15,501). By contrast, ShigaTyper assigned 7,277 isolates
512 (46.95%) to *Shigella*, 7.976 isolates (51.45%) to EIEC, 177 (1.14%) isolates to multiple *wzx*
513 genes and failed to type 71 (0.46%) isolates.

514

515 The predicted serotype of 7,277 (46.96%) *Shigella* isolates by ShigaTyper agreed with the
516 results of ShigEiFinder. For 8,224 isolates typed as EIEC or untypable by ShigaTyper, 99.73%
517 (8,202/8,224) of the isolates were assigned to *Shigella* or EIEC clusters by ShigEiFinder (Table
518 4). Of these isolates, the majority belonged to SS, SD1 and SF which were erroneously
519 predicted as EIEC by ShigaTyper.

520

521

522 **Discussion**

523 *Shigella* and EIEC cause human bacillary dysentery with similar invasion mechanisms,
524 however the pathogenicity of these 2 groups varies (8, 43). The prevalence of each of the four
525 *Shigella* "species" also varies (11-13). Differentiation of *Shigella* and EIEC from each other is
526 important for epidemiologic and diagnostic investigations. However, their similar
527 physiological, biochemical and genetic characteristics make this differentiation difficult.

528

529 **Determining phylogenetic clusters for better separation of *Shigella* isolates from EIEC**

530 From a phylogenetic perspective, *Shigella* and EIEC strains consisted of multiple phylogenetic
531 lineages derived from commensal *E. coli*, which do not reflect the nomenclature of *Shigella*
532 and EIEC (23, 25, 26, 28, 38, 41). In the present study, we identified all phylogenetic clusters
533 of *Shigella* and EIEC through large scale examination of publicly available genomes.
534 Phylogenetic results demonstrated that *Shigella* isolates had at least 10 clusters while EIEC
535 isolates had at least 7 clusters. The 10 *Shigella* clusters included the 7 previously defined
536 lineages including 3 major clusters (C1, C2 and C3) and 5 outliers (SD1, SD8, SD10, SB13
537 and SS) (25) and 2 newly identified clusters (SB12 and SB13-atypical). The 7 EIEC clusters
538 consisted of 4 previously defined EIEC clusters (C4, C5, C6 and C7) (26) and 3 newly
539 identified EIEC clusters (C8 EIEC O96:H19, C9 EIEC O8:H19 and C10 EIEC O135:H30).
540
541 Our WGS-based phylogeny provided high resolution for assigning *Shigella* and EIEC isolates
542 to clusters. Several serotypes that are currently increasing in frequency (SB19, SB20, SD14,
543 SD15, SD provisional serotype 96-626) (76-79) were assigned to clusters and five new
544 clusters/outliers were identified. SB13 isolates in this study formed two known lineages. One
545 lineage was located outside of *Shigella*/EIEC clusters and represented the outlier SB13 which
546 is in fact belonging to the newly defined species *E. albertii* (25, 26, 38, 39). The second lineage
547 was with *E. coli*, and was defined as atypical SB13 previously (39). The newly identified
548 *Shigella* outlier SB12 was previously grouped into C3 based on housekeeping gene trees (25,
549 38) but was seen as outliers in two other studies (28, 56).
550
551 Newly identified clusters C8 (EIEC O96:H19) and C9 (EIEC O8:H19) represented the
552 emergence of novel EIEC serotypes. A recent study revealed that EIEC serotype O96:H19 (C8)
553 could be the result of a recent acquisition of the invasion plasmid by commensal E. coli (80).
554 The EIEC serotype O8:H19 (C9) had not been reported previously.
555
556 Apart from the 17 major and outlier clusters of *Shigella* and EIEC, the presence of 53 sporadic
557 EIEC lineages indicated greater genetic diversity than has been observed previously. Isolates
558 belonging to these sporadic EIEC groups were more closely related to non-enteroinvasive E.
559 coli isolates than to major *Shigella*/EIEC lineages. However, 41 of these isolates, representing
560 38 sporadic EIEC lineages, carried pINV. *Shigella* and EIEC both carry the *Shigella* virulence
561 plasmid pINV which is vital for virulence and distinguishes *Shigella*/EIEC from other *E. coli*
562 (24, 33, 68). Therefore, these isolates may represent recently formed EIEC lineages through
563 acquisition of the pINV. The remaining 18 isolates contained the *ipaH* gene but may or may

564 not carry pINV. It is possible that these strains carried very low copy number of pINV or the
565 pINV plasmid was lost during culture.
566
567 **Highly sensitive and specific cluster-specific gene markers for differentiation of *Shigella***
568 **and EIEC isolates**
569 Several studies have identified phylogenetic related genomic markers for discrimination of
570 *Shigella* and EIEC and between *Shigella* species (23, 27, 28, 41, 55, 56). However, these
571 phylogenetic analyses were performed only with a small number of genomes (23, 28, 55). In
572 addition, non-invasive E. coli isolates were included in some of the phylogenetic clusters
573 identified (28) which led to non-invasive E. coli isolates being identified by the markers.
574
575 We identified cluster-specific gene markers for each respective cluster which were only
576 composed of *Shigella* or EIEC isolates. Sets of cluster-specific gene markers were identified
577 for those clusters where no single suitable marker is present. The combination of genes
578 enhances the specificity of cluster-specific gene markers as demonstrated by the 100%
579 sensitivity and very high specificity in this analysis (Table 2). Genes specific to each of the 53
580 sporadic EIEC lineages were also identified and they were sensitive and specific, although it
581 should be noted that these values are based on very small sample sizes.
582
583 The cluster-specific gene markers or marker sets can be used to differentiate *Shigella*/EIEC
584 from non-enteroinvasive *E. coli* independent of *ipaH* gene. The *ipaH* gene as a molecular
585 target has been used to differentiate *Shigella* and EIEC from non-enteroinvasive E. coli (24,
586 43-45). In our study, the cluster-specific gene markers were specific to *Shigella*/EIEC with
587 98.8% to 100% specificity when evaluated on non-enteroinvasive *E. coli* control database,
588 providing confidence that the cluster-specific genes or sets are robust markers for the
589 identification of *Shigella*/EIEC. 53 sporadic EIEC lineage specific gene markers also have
590 very high specificity (97.02% to 100%) against non-enteroinvasive *E. coli* control database.
591
592 The cluster-specific gene markers or marker sets are able to assign *Shigella*/EIEC isolates to
593 correct clusters in 99.63% of cases and can clearly distinguish *Shigella* isolates from EIEC
594 when applied to the validation dataset. While ShigaTyper assigned 46.95% isolates to *Shigella*
595 and 51.45% isolates to EIEC in the same dataset we tested, leading to a large proportion of
596 isolates incorrectly assigned. The majority of the isolates predicted as EIEC by ShigaTyper
597 were SS or SD1 as they belonged to SS and SD1 specific STs and were positive to a set of SS

598 or SD1 specific gene markers and grouped into SS or SD1 cluster on our phylogenetic tree.
599 The genes used in ShigaTyper were SS specific marker Ss_methylase gene (81, 82) together
600 with SS O antigen wzx gene. However, SS specific marker Ss_methylase gene was found in
601 other *Shigella* serotypes and EIEC (10) and SS O antigen wzx gene were located on a plasmid
602 which is frequently lost (83). Similarly, the SD1 O antigen genes used in ShigaTyper were
603 plasmid-borne which may also lead to inconsistent detection (84, 85). A previous study
604 identified 6 loci to distinguish EIEC from *Shigella* (23). We searched the 6 loci against our
605 *Shigella*/EIEC database and found that some *Shigella* isolates were misidentified as EIEC
606 isolates, such as SD8 isolates incorrectly identified as EIEC subtype 13. Our cluster-specific
607 genes can differentiate SD8 isolates from EIEC with 100% accuracy. Therefore, the cluster-
608 specific gene markers marker sets described here provided nearly perfect differentiation of
609 *Shigella* from EIEC.
610
611 The cluster-specific gene markers or marker sets are able to differentiate SS and SF (with
612 exception of SF6) from SB and SD. SF and SS are the major cause of *Shigella* infections,
613 accounting for up to 89.6% annual cases (11-13). Differentiation of SS and SF isolates from
614 SB and SD is also beneficial for diagnosis and surveillance. A recent study identified "species"
615 specific markers for the detection of each of the four *Shigella* "species" and validated with only
616 one isolate per species (55). A molecular algorithm based on *Shigella* O antigen genes can
617 detect 85% of SF isolates (52). In contrast, a set of SF specific genes in our study can correctly
618 identify SF isolates with 99.62% accuracy.
619
620 The cluster-specific gene markers or marker sets can also assign *Shigella*/EIEC isolates to
621 serotype level if the cluster has single serotype such as SD1, SD8, SD10, SB13, SB12, EIEC
622 O144:H25 (C7), EIEC O96:H19 (C8), EIEC O8:H19 (C9) and EIEC O135:H30 (C10). The
623 remaining EIEC, SF, SB and SD serotypes were distributed over the major clusters C4-6, C3,
624 C1 and C2 respectively. Cluster-specific gene markers or marker sets combined with serotype
625 associated O antigen and modification genes can further identify these isolates to serotype level
626 if the cluster has multiple serotypes. Once an isolate is assigned to a cluster, only serotype
627 associated O antigen and modification genes found in that cluster need be examined. This
628 allows the elimination of ambiguous or incorrect serotype assignments that may otherwise
629 occur, increasing the overall accuracy of the method.
630
631 **Cluster-specific gene marker based ShigEiFinder can accurately type *Shigella* and EIEC**

632 To facilitate the use of cluster-specific gene markers or marker sets for typing, we developed
633 an automated pipeline, ShigEiFinder, for *in silico* molecular serotyping of *Shigella*/EIEC.
634 ShigEiFinder provided *Shigella*/ EIEC differentiation as well as serotype prediction by yielding
635 "presence or absence" of cluster-specific gene markers or marker sets combined with
636 *Shigella*/EIEC O antigen genes and modification genes in a query isolate (either reads or
637 assembled genomes). We showed 99.70% and 99.81% accuracy to assign isolates to the correct
638 clusters from 15,501 *Shigella*/EIEC isolates in validation dataset for the assembled genomes
639 and reads mapping respectively. In contrast, the existing *in silico Shigella* serotyping pipeline
640 ShigaTyper had 46.95% accuracy for reads mapping when tested with the same validation
641 dataset, with 51.45% of isolates in validation dataset being predicted as EIEC by ShigaTyper.
642
643 The genetic determinants used in ShigaTyper for differentiation of *Shigella* from EIEC and
644 identification of SS were *lacY*, *cadA*, *Ss_methylase,* SS and SD1 O antigen *wzx* genes (10). As
645 discussed above some of these genes were found to be non-specific in this study. Compared
646 with ShigaTyper, the cluster-specific gene markers used in ShigEiFinder for identification of
647 *Shigella* and EIEC provided higher discriminatory power than ShigaTyper. ShigEiFinder also
648 provided a high specificity with 99.40% for assembled genomes and 99.38% for reads
649 mapping.
650
651 ShigEiFinder can differentiate *Shigella* isolates from EIEC and distinguish SS and SF (with
652 exception of SF6) isolates from SB and SD accurately. It also can identify SD1 isolates
653 directly. ShigEiFinder was able to serotype over 59 *Shigella* serotypes and 22 EIEC serotypes.
654 Therefore, ShigEiFinder will be useful for clinical, epidemiological and diagnostic
655 investigations and the cluster-specific gene markers identified could be adapted for
656 metagenomics or culture independent typing.
657
658 **Conclusion**
659 This study analysed over 17,000 publicly available *Shigella*/EIEC genomes and identified 10
660 clusters of *Shigella*, 7 clusters of EIEC and 53 sporadic types of EIEC. Cluster-specific gene
661 markers or marker sets for the 17 major clusters and 53 sporadic types were identified and
662 found to be valuable for *in silico* typing. We additionally developed ShigEiFinder, a freely
663 available *in silico* serotyping pipeline incorporating the cluster-specific gene markers to
664 facilitate serotyping of *Shigella*/EIEC isolates using genome sequences with very high
665 specificity and sensitivity.

Authors and contributors

Conceptualization: R.L, M.P.; Investigation: X.Z., M.P., T.N., S.K.; Methodology: M.P., R.L. Writing – original draft: X.Z.; Writing – review and editing: M.P., R.L.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Data bibliography

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Abbreviations

SS, *Shigella sonnei*; SF, *Shigella flexneri*; SB, *Shigella boydii*; SD, *Shigella dysenteriae*; EIEC, Enteroinvasive *Escherichia coli*; NCBI SRA, National Center for Biotechnology Information Sequence Read Archive; ST, sequence type; rST, ribosomal ST; MLST, Multilocus sequence typing; rMLST, Ribosomal MLST; ECOR, *Escherichia coli* reference collection; WGS, whole-genome sequencing; TP, true positive; FN, false negative; FP, false positive; HK, House Keeping.

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927 **Table 1: The summary of identified *Shigella*/EIEC clusters and outliers in identification dataset**

Clusters (no of serotypes)[#]	No of isolates	No. STs	No. rSTs	Serotypes
C1 (25)	288	36	166	SB1-4, SB6, SB8, SB10, SB14, SB18, SB11[b], SB19-20[b]; SD3-7, SD9, SD11-13, SD14-15[*], SD-96-265[*]; SF6
C2 (9)	101	19	56	SB5, SB7, SB9, SB11, SB15, SB16, SB17; SD2, SD-E670-74[b]; SD2
C3 (20)	744	81	437	SF1a, SF1b, SF1c (7a), SF2a, SF2b, SF3a, SF3b, SF4a, SF4av, SF4b, SF4bv, SF5a, SF5b, SF7b, SFX, SFXv (4c), SFY, SFYv, SF novel serotype; SB-E1621-54*
C4 (9)	51	6	21	O28ac:H-, O28ac:H7, O136:H7, O164:H-, O164:H7, O29:H4, O173:H7, O124:H7, O132:H7[*]
C5 (6)	62	4	15	O121:H30, O124:H30, O164:H30, O132:H21, O152:H30, O152:H-
C6 (3)	20	2	6	O143:H26, O167:H26, O112ac:H26[b]
C7	10	1	3	O144:H25
C8[a]	12	2	1	O96:H19
C9[a]	4	1	2	O8:H19
C10 [#]	2	1	1	O135:H30
CSS	427	39	294	
CSD1	70	8	56	SD1
CSD8	7	3	3	SD8
CSD10	2	2	1	SD10
CSB12[a]	8	2	6	SB12
CSB13	7	3	3	SB13

Clusters (no of serotypes)[#]	No of isolates	No. STs	No. rSTs	Serotypes
CSB13-atypical[a]	5	3	3	SB13
Sporadic EIEC lineages[a] (53)	59	49	53	53 antigen types

928

929 [#]Numbers in parentheses are the number of serotypes within that cluster.

930 [a]: Clusters identified as new clusters in this study.

931 [b]: Serotypes were inconsistent with previous analyses.

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934 **Table 2: The sensitivity and specificity of cluster-specific genes**

Clusters	Cluster-specific genes (Single/sets)[b]	Identification dataset (1969 isolates)		
		No of isolates	Sensitivity	Specificity
C1	Set of 4 genes	288	100	99.94[a]
C2	Set of 3 genes	101	100	100
C3	Set of 3 genes	744	100	99.59[a]
C4	Set of 2 genes	51	100	100
C5	Set of 3 genes	62	100	100
C6	Set of 2 genes	20	100	100
C7	Single gene	10	100	100
C8	Set of 2 genes	12	100	100
C9	Set of 2 genes	4	100	100
C10	Single gene	2	100	100
CSS	Set of 5 genes	427	100	99.87[a]
CSD1	Set of 2 genes	70	100	100
CSD8	Single gene	7	100	100
CSD10	Single gene	2	100	100
CSB12	Single gene	8	100	100
CSB13	Single gene	7	100	100
CSB13-atypical	Single gene	5	100	100
53 Sporadic EIEC lineages	Single gene / lineage	59	100	100

935

936 [a]:The specificity of cluster-specific gene set less than 100% was due to at least one FP found in
937 that set.

938 [b]: The sequences of these genes were listed in Data S1.

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940 **Table 3: The accuracy of ShigEiFinder with identification dataset and validation dataset**

941 | ShigEiFinder assignments | Identification Dataset (n=1,969)[a] | | Validation dataset (n=15,501) | |
|---|---|---|---|---|
| | **Genomes** | **Reads mapping** | **Genomes** | **Reads mapping** |
| *Shigella*/EIEC clusters | 1871 | 1848 | 15,455 | 15,471 |
| Multiple *Shigella*/EIEC clusters | 9 | 6 | 33 | 7 |
| *Shigella*/EIEC unclustered | 0 | 8 | 13 | 23 |
| Not *Shigella*/EIEC | 89 | 89 | 0 | 0 |
| Accuracy[b] | 99.54% | 99.28% | 99.70% | 99.81% |

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949 [a]: Identification dataset has 90 non-*Shigella*/EIEC strains including 72 ECOR strains and 18 *E.albertii* strains. 1,969 assembled genomes and
950 1,951 reads (reads not available for 18 EIEC isolates downloaded from NCBI) in identification dataset. One of *E.albertii* strain was assigned as
951 SB13 which was grouped into SB13 cluster on the phylogenetic tree.

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953 [b]: The accuracy was defined as the number of *Shigella*/EIEC isolates being correctly assigned to cluster over the total number of tested.

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963 **Table 4: Discrepant assignment of 8,224 isolates by ShigEiFinder and Shigatyper**

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ShigEiFinder Assignment	ShigaTyper assignment			Total
	EIEC	Multiple wzx	Non-prediction	
SS	7,465	12	7	7,484
SF	117	61	10	188
C1 and C2 (SB/SD)	17	99	51	167
SB12	0	2	0	2
SD1	244	1	1	246
SD8	1	0	0	1
SD10	0	0	2	2
EIEC	97	0	0	97
Sporadic EIEC lineages	15	0	0	15
Multiple clusters	5	2	0	7
Shigella/EIEC unclustered	15	0	0	15
Total	7,976	177	71	8,224

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979 **Figure legends:**

980 **Figure 1: *Shigella*/EIEC cluster Identification phylogenetic tree**

981 Representative isolates from the identification dataset were used to construct the phylogenetic
982 tree by Quicktree v1.3 (64) to identify *Shigella* and EIEC clusters and visualised by
983 Grapetree's interactive mode. The dendrogram tree shows the phylogenetic relationships of
984 1879 *Shigella* and EIEC isolates represented in the identification dataset. Branch lengths are
985 log scale for clarity. The tree scales indicated the 0.2 substitutions per locus. *Shigella* and EIEC
986 clusters are coloured. Numbers in square brackets indicate the number of isolates of each
987 identified cluster. CSP is sporadic EIEC lineages.

988

989 **Figure 2: *in silico* serotyping pipeline workflow**

990 Schematic of *in silico* serotyping *Shigella* and EIEC by cluster-specific genes combined with
991 the *ipaH* gene and O antigen and modification genes and H antigen genes, implemented in
992 ShigEiFinder. Both assembled genomes and raw reads are accepted as data input.

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1006 **Supplementary Material**

1007 **Figure S1**: **Identification phylogenetic tree**

1008 An identification phylogenetic tree constructed by Quicktree v1.3 (64) and visualised by ITOL
1009 v5 shows the phylogenetic relationships of 1879 *Shigella* and EIEC isolates in identification
1010 dataset. The tree scales indicated the 0.01 substitutions per locus. *Shigella* and EIEC clusters

are colored. The internal branches are colored to represent the bootstrap values. Green color indicates the maximum bootstrap value (1). The red color shows the minimum bootstrap value (0). Each of cluster is well supported by bootstrap value. CSP is sporadic lineages.

Figure S2-A: **Confirmation phylogenetic tree**

A confirmation phylogenetic tree was constructed by Quicktree v1.3 (64) based on 2375 isolates and visualised by Grapetree's interactive mode. The tree shows the phylogenetic relationships between identified *Shigella*/EIEC clusters in identification dataset and non-enteroinvasive *E.coli* isolates. Branch lengths are log scale for clarity. The tree scales indicated the 0.1 substitutions per locus. Known *Shigella* and EIEC clusters from identification dataset are colored. Numbers in square brackets indicate the number of isolates of each identified cluster. CSP is sporadic lineages.

Figure S2-B: **Confirmation phylogenetic tree**

A confirmation phylogenetic tree constructed by Quicktree v1.3 (64) and visualised by ITOL v5 shows the phylogenetic relationships between identified *Shigella*/EIEC clusters in identification dataset and non-enteroinvasive *E.coli* isolates. The tree scales indicated the 0.01 substitutions per locus. *Shigella* and EIEC clusters are colored. The internal branches are colored to represent the bootstrap values. Green color indicates the maximum bootstrap value (1). The red color shows the minimum bootstrap value (0). Each of cluster is well supported by bootstrap value. CSP is sporadic lineages.

Figure S3: **Distribution of mapped 38 virulence genes in 59 sporadic isolates**

The presence of *Shigella* virulence plasmid pINV in 59 sporadic isolates in identification dataset was determined by the mapped 38 virulence genes. Detailed genes were described in Results "**Investigation of *Shigella* virulence plasmid pINV in 59 sporadic isolates**". Three

1037 categories were defined based on the number of virulence genes mapped to isolate. Virulence
1038 plasmid positive: > 25 genes mapped to isolate; Intermediate: 13 to 25 genes mapped to isolate;
1039 Virulence plasmid negative: less than 13 genes mapped to isolate.
1040
1041 **Figure S4 (A)**: **Validation phylogenetic tree**
1042 A validation tree was generated by Quicktree v1.3 (64) and visualised by Grapetree's
1043 interactive mode to assign representative isolates in validation dataset to clusters. Branch
1044 lengths are log scale for clarity. The tree scales indicated the 0.2 substitutions per locus.
1045 Known *Shigella* and EIEC clusters from identification dataset are colored. Numbers in square
1046 brackets indicate the number of isolates of each identified cluster. Isolates in validation dataset
1047 are colored white. The isolates are assigned to clusters if they grouped into known cluster
1048 isolates. CSP is sporadic lineages.
1049
1050 **Figure S4 (B)**: **Validation phylogenetic tree**
1051 A validation phylogenetic tree was constructed by Quicktree v1.3 (64) and visualised by ITOL
1052 v5 to assign representative isolates in validation dataset to clusters. The tree scales indicated
1053 the 0.01 substitutions per locus. *Shigella* and EIEC clusters are colored. The internal branches
1054 are colored to represent the bootstrap values. Green color indicates the maximum bootstrap
1055 value (1). The red color shows the minimum bootstrap value (0). Each of cluster is well
1056 supported by bootstrap value. Isolates that grouped with known cluster isolates (from
1057 identification dataset) with strong bootstrap support are categorised into that cluster. CSP is
1058 sporadic lineages.
1059
1060 **Table S1**: 1,969 isolates used in identification dataset
1061 **Table S2**: 15,501 isolates used in validation dataset
1062 **Table S3**: The location and function of cluster-specific genes

1063 **Table S4**: The results of cluster-specific gene markers tested with 12,743 non-enteroinvasive

1064 *E.coli* isolates

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1066 **Data S1**: Algorithms incorporated into the ShigEiFinder

1067 **Data S2**: Genetic signature O and H genes from ShigaTyper and SerotypeFinder

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1069 **Data Availability Statement**

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1071 Custom python scripts used in this study are available from the authors on request.

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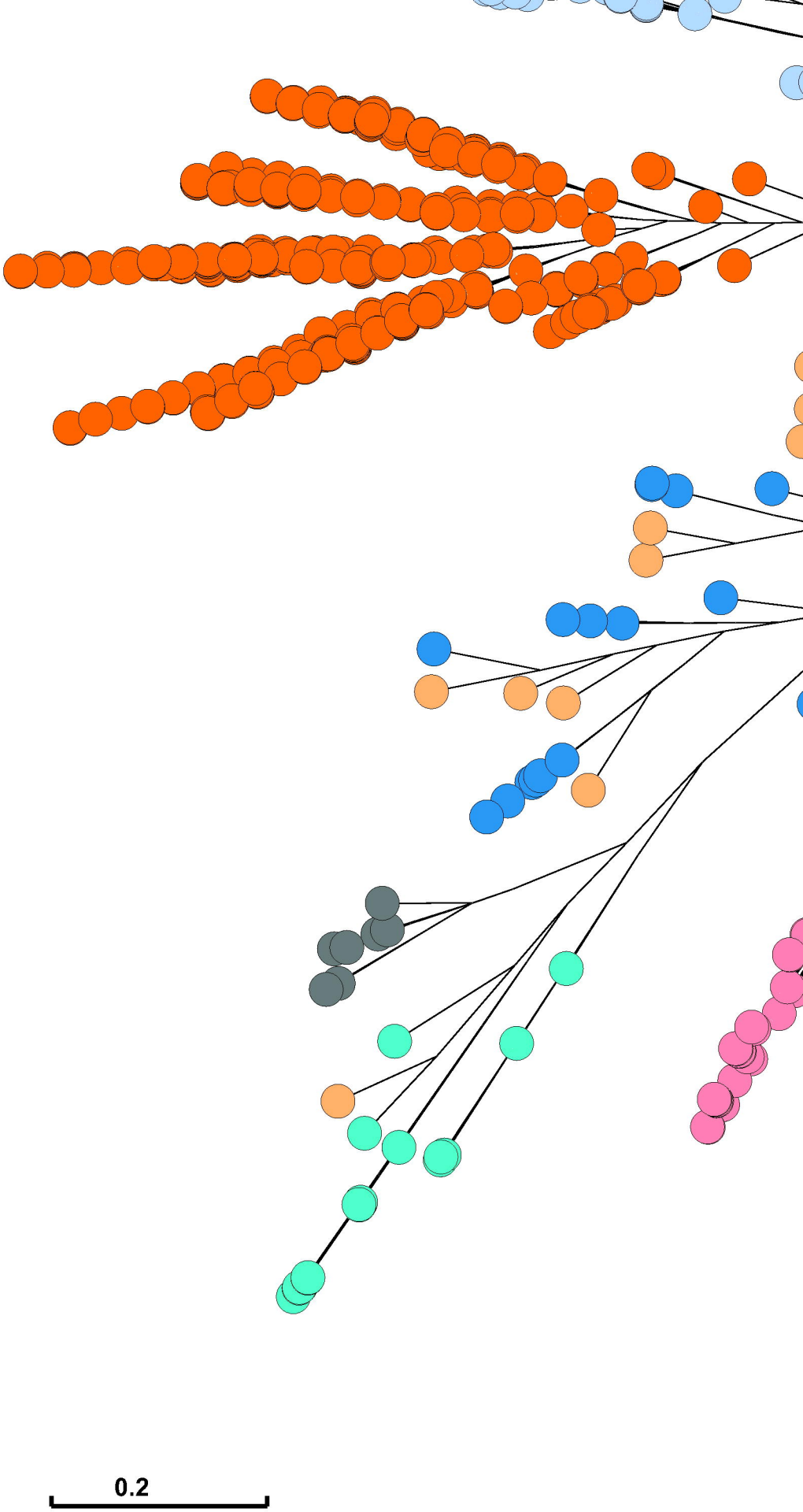
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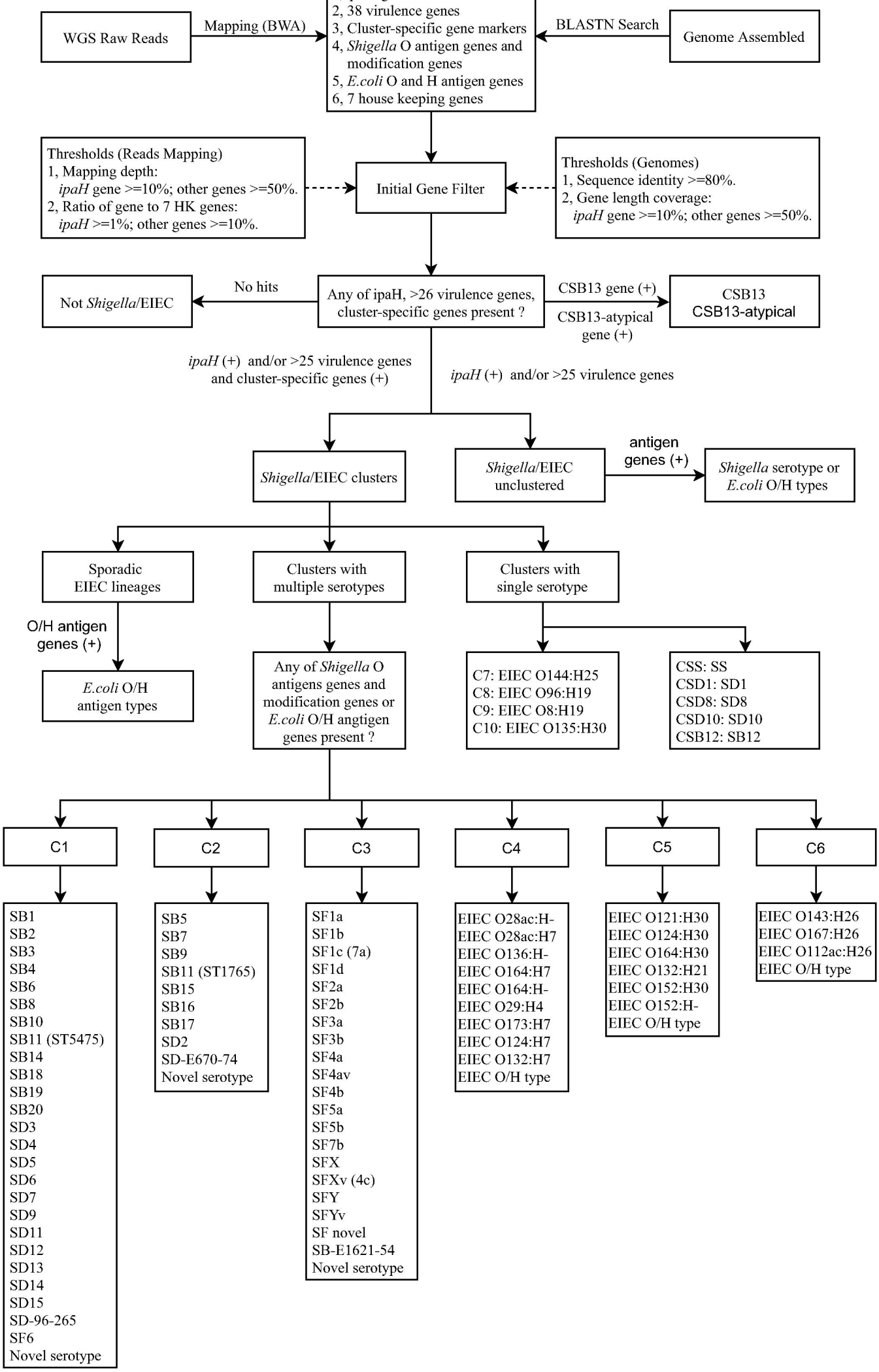
WGS Raw Reads
Mapping (BWA)
2, 38 virulence genes
3, Cluster-specific gene markers
4, Shigella O antigen genes and modification genes
5, E.coli O and H antigen genes
6, 7 house keeping genes
BLASTN Search
Genome Assembled
Thresholds (Reads Mapping)
1, Mapping depth:
ipaH gene >=10%; other genes >=50%.
2, Ratio of gene to 7 HK genes:
ipaH >=1%; other genes >=10%.
Initial Gene Filter
Thresholds (Genomes)
1, Sequence identity >=80%.
2, Gene length coverage:
ipaH gene >=10%; other genes >=50%.
Not Shigella/EIEC
No hits
Any of ipaH, >26 virulence genes, cluster-specific genes present ?
CSB13 gene (+)
CSB13-atypical gene (+)
CSB13
CSB13-atypical
ipaH (+) and/or >25 virulence genes and cluster-specific genes (+)
ipaH (+) and/or >25 virulence genes
Shigella/EIEC clusters
Shigella/EIEC unclustered
antigen genes (+)
Shigella serotype or E.coli O/H types
Sporadic EIEC lineages
Clusters with multiple serotypes
Clusters with single serotype
O/H antigen genes (+)
E.coli O/H antigen types
Any of Shigella O antigens genes and modification genes or E.coli O/H angtigen genes present ?
C7: EIEC O144:H25
C8: EIEC O96:H19
C9: EIEC O8:H19
C10: EIEC O135:H30
CSS: SS
CSD1: SD1
CSD8: SD8
CSD10: SD10
CSB12: SB12
C1
C2
C3
C4
C5
C6
SB1
SB2
SB3
SB4
SB6
SB8
SB10
SB11 (ST5475)
SB14
SB18
SB19
SB20
SD3
SD4
SD5
SD6
SD7
SD9
SD11
SD12
SD13
SD14
SD15
SD-96-265
SF6
Novel serotype
SB5
SB7
SB9
SB11 (ST1765)
SB15
SB16
SB17
SD2
SD-E670-74
Novel serotype
SF1a
SF1b
SF1c (7a)
SF1d
SF2a
SF2b
SF3a
SF3b
SF4a
SF4av
SF4b
SF5a
SF5b
SF7b
SFX
SFXv (4c)
SFY
SFYv
SF novel
SB-E1621-54
Novel serotype
EIEC O28ac:H-
EIEC O28ac:H7
EIEC O136:H-
EIEC O164:H7
EIEC O164:H-
EIEC O29:H4
EIEC O173:H7
EIEC O124:H7
EIEC O132:H7
EIEC O/H type
EIEC O121:H30
EIEC O124:H30
EIEC O164:H30
EIEC O132:H21
EIEC O152:H30
EIEC O152:H-
EIEC O/H type
EIEC O143:H26
EIEC O167:H26
EIEC O112ac:H26
EIEC O/H type