Differentiation of Enteroinvasive *Escherichia coli* from *Shigella* using multiplex-PCR assay

Rajat Dhakal, PhD
Centre for Infectious Diseases and Microbiology – Public Health, ICPMR

Number of notifications for Shigellosis, Australia, 2001-2016

Source: National Notifiable Diseases Surveillance System, Australia
Are all cases of reported Shigellosis really caused by *Shigella*?

1. Phylogenetically close species
   Both cause dysentery with similar mechanism and symptoms

2. Biochemical and serological methods for *Shigella* are limited to specific pheno- and serotypes (Pavlovic et al. 2011)
3. Also cross reactivity with EIEC may occur in *Shigella* serotyping assay (Liu et al. 2008)

---

The debate is ongoing..

---

**Letter to the Editor**

*Enteroinvasive Escherichia coli* May Account for Uncultured *Shigella*

Dear Sir:

Even for cultured isolates, sometimes presumptive *Shigella* can’t be serotyped or may show the biochemical properties like *E. coli*, but yet may be reported as *Shigella*.
Previous attempt to differentiate EIEC and *Shigella*

- Pavlovic et al. 2011 used PCR for β-glucuronidase (*uidA*) gene and lactose permease (*lacY*) gene to differentiate them

- Primers used in Pavlovic et al. 2011 may not accurately distinguish EIEC and *Shigella* (Pettengill et al. 2015)

- EIEC and *Shigella* differentiation issue is still unsolved

**Laboratory identification of *Shigella***

1. Faecal Sample
2. Culture independent testing (*ipaH* PCR)
3. *ipaH* +ve
4. Subculturing (2 days)
5. Multiplex PCR on DNA from boiled culture and identification of EIEC (Less than 3 hours)
6. AIM of our study
7. Serotyping and biochemical assays (1-2 days)
8. *Shigella* serotype
Materials and Methods

*In silico* analysis for searching differentiating markers

In house sequenced 4 EIEC and 18 *Shigella* Genomes

31 EIEC Genomes From NCBI-SRA
Selection of markers from EIEC genomes

24 candidate loci

Primer Blast

Minimum number of loci among which at least two is present in every EIECs used
Could primers be designed?

6 loci selected
18 loci excluded

Primer design and testing in silico

6 primer sets were designed
• Loci 1–Primer sets 1
• Loci 2–Primer sets 2
• Loci 3–Primer sets 3
• Loci 4–Primer sets 4
• Loci 5–Primer sets 5
• Loci 6–Primer sets 6

Presence/absence of loci was evaluated in Shigella and non-invasive E. coli
## Development of multiplex PCR assay

<table>
<thead>
<tr>
<th>Multiplex PCR system A</th>
<th>Multiplex PCR system B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer set 1</td>
<td>Primer set 4</td>
</tr>
<tr>
<td>Primer set 2</td>
<td>Primer set 5</td>
</tr>
<tr>
<td>Primer set 3</td>
<td>Primer set 6</td>
</tr>
</tbody>
</table>

6 EIEC and 6 *Shigella* lab isolates optimised

<table>
<thead>
<tr>
<th>Test interpretation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>IpaH</em> positive, presence of two or more bands in gel</td>
<td>EIEC</td>
</tr>
<tr>
<td><em>IpaH</em> positive, presence of one or no band in gel</td>
<td><em>Shigella</em></td>
</tr>
</tbody>
</table>

6 more EIEC lab isolates, 48 *Shigella* lab isolates, 55 EIEC SRA records were tested

## Results
Percentages of isolates bearing specific loci among overall genomes used

- **EIECs**
  - Locus 1: 10.58%
  - Locus 2: 2.88%
  - Locus 3: 11.54%
  - Locus 4: 44.23%
  - Locus 5: 8.46%
  - Locus 6: 32.69%
  - None: 0%

- **Shigella**
  - Locus 1: 0%
  - Locus 2: 0.14%
  - Locus 3: 0.05%
  - Locus 4: 0%
  - Locus 5: 0%
  - Locus 6: 99.99%
  - None: 0%

- **Non-invasive E. coli**
  - Locus 1: 0.27%
  - Locus 2: 0.01%
  - Locus 3: 0.01%
  - Locus 4: 0.00%
  - Locus 5: 0.00%
  - Locus 6: 99.98%
  - None: 0%

Serotypes of 18 experimental *Shigella* used for designing the assay
Serotypes of 35 EIECs used for designing the assay

Sequence types of 35 EIEC used for designing the assay
Number of *Shigella* and non-invasive *E. coli* used from NCBI-SRA for evaluating loci presence/absence

![Pie chart showing distribution of Shigella and non-invasive E. coli](chart)

- S. sonnei: 3038
- S. flexneri: 2416
- S. dysenteriae: 13147
- S. boydii: 488
- Non-invasive E. coli: 490

Serotypes of 12 experimental EIECs used for optimising and testing the assay

![Bar chart showing serotypes](chart)

- O136:H9
- O136:H-
- O28ac:H-
- O152:H-
- O124:H-
- O167
- O143:H-
- O144:H-
- O144

12-Jul-17
Multiplex PCR analysis in agarose gel

Ladder, Multiplex A or Multiplex B system

<table>
<thead>
<tr>
<th>Lane #</th>
<th>L</th>
<th>A</th>
<th>B</th>
<th>A</th>
<th>B</th>
<th>A</th>
<th>B</th>
<th>A</th>
<th>B</th>
<th>A</th>
<th>B</th>
<th>A</th>
<th>B</th>
<th>L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

EIEC positive for two or more bands
Shigella negative for all bands

The limit of detection for all the bands were $10^5$ cells/ml.

Analysis of 55 isolates used for testing the assay

- Out of total 55, 52 genomes showed the presence of two or more than two markers.
- Rest 3 EIEC genomes were those which were in minor clusters in previous phylogenetic studies.
Serotypes of 55 EIECs used for testing the assay

Sequence types of 55 EIECs used for testing the assay
Conclusion

- A multiplex PCR assay has been developed.
- This method can differentiate diverse EIEC from diverse *Shigella* from the culture provided that the culture is *ipaH* positive.
- Can be performed in parallel with or before serotyping *Shigella*.
- Can save cost and time (i.e. about 2 days) of serotyping if EIEC is positive and provides confidence in calling *Shigella*.
- Future directions: development of culture independent testing using the developed markers
Acknowledgments

Pathogen Genomics Unit
A/Prof Vitali Sintchenko
Dr. Qinning Wang
Mr. Peter Howard
All staffs in CIDM Whole Genome Sequencing lab,
Enteric reference lab, Pathology West
University of New South wales
A/Prof Ruiting Lan