Intimin Types Determined by Heteroduplex Mobility Assay of Intimin Gene (eae)-Positive Escherichia coli Strains

Running title: INTIMIN TYPES DETERMINED BY HMA OF eae GENE

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ABSTRACT

We developed a quick genetic approach to screen variants of the intimin gene (eae) by using a heteroduplex mobility assay (HMA) that targets its 5’ conserved region. The eae variants were categorized into 4 major HMA types and 10 minor subtypes.
Enteropathogenic (EPEC) and Shiga toxin-producing (STEC) Escherichia coli produce characteristic “attaching and effacing” lesions using intimin encoded by eae (24). The eae genes of several strains have been cloned and sequenced, and have a highly conserved 5’-terminal region, but are variable in the 3’-terminal region (13). Allele-specific PCRs targeting eae genes in the variable 3’ region have been employed to determine eae types (1, 5, 10, 27, 30, 31, 33) and subtypes in combination with restriction fragment length polymorphism (RFLP) (4, 27). Ramachandran (29) designed universal PCR primers to amplify the Int280-encoding region and identified types by RFLP. Recently, methods using real-time PCR (25) and oligonucleotide microarray (14) have been developed. Eighteen types and 9 subtypes of intimin, namely, α, α2, β1–3, γ1, γ2, δ, ε, ε2–4, ζ, η, η2, θ, τ, τ2, κ, λ, μ, ν, ξ, ο, π, ρ, and σ, have been deposited in databases.

Heteroduplex mobility analysis (HMA) for alternative method to DNA sequencing has been developed to determine variations among amplified fragments (12). HMA is a simple, rapid and inexpensive method (11, 18). In this study we demonstrated the variability of the 5' region of eae genes by HMA.

A total of 211 eae-positive strains of E. coli isolated in Thailand and
Japan were used in this study. In addition, *eae*-types—E2348/69 (O127:H6, *eae*-α) (20), H19 (O26:H11, *eae*-β) (2), PMK5 (O103:H2, *eae*-ε) (21), and EDL933 (O157:H7, *eae*-γ) (26)—were used as a control.

To categorize each strain into EPEC or STEC, we checked for *stx* genes by PCR (17) or RFLP (Denka-Seiken, Tokyo, Japan). For HMA typing, an *E. coli* culture on an agar plate was suspended in 100 µl of distilled water, boiled for 10 minutes, and used as a PCR template. The 5′-terminal region of the *eae* genes was amplified by PCR as described by Nakazawa and Itoh (23).

Amplicons were then subjected to HMA (12). Briefly, an appropriate amount of amplicon was mixed with 2 µl of the amplicon obtained from strains taken as a reference, and distilled water containing 10 mM EDTA (pH 8.0) was added to 10 µl. The mixture was denatured at 94°C for 5 min, and re-annealed at 72°C for 3 min and at 50°C for 1 hour. Heteroduplexes were separated by homemade polyacrylamide gel electrophoresis (PAGE) on a 7.5% gel with stacking gel without SDS. To test the discriminatory power of commercially available pre-cast gels, PAG-mini-“Daiichi” (Daiichi Pure Chemicals, Tokyo, Japan) and e-PAGEL (ATTO Corporation, Tokyo, Japan) was also used. Commercially available pre-cast gels gave as good a resolution as homemade gel.
The combination of the amplicons and strain KI1218 or KI1223 gave the most distinctive HMA patterns; therefore, they were used as references.

According to HMA typing, eae genes were categorized into 4 major groups, a to d, which can be sub-typed into 10 minor types (Fig. 1). O and H serotypes were determined with antisera kits (Denka-Seiken) as described by the manufacturer. Of the 211 eae-positive E. coli, 124 were distributed among 29 typable O:H serotypes (Table 1). The same serotypes generated identical HMA types; this was not observed in the case of O26:H11, whose HMA-type is associated with stx genes. All stx-positive strains are b1, whereas stx-negative strains are b1 or b2 (Table 1).

The reference strains for eae-types, α, β, γ, and ε, were typed into HMA types, a1, b1, c1, and b2, respectively (Fig. 1). Regarding the common serotype strains of references (1, 3-5, 7, 19, 27, 29, 30, 32) and this study, we compare HMA types to allele-specific eae types reported in references. With a few exceptions, the serotype strains of a1, O55:H6, O127a:H6, O142:H6, O142:H34, and O157:H45 were eae-α/α1 except O119:H6 which was typed to eae-β/β2; of b1, O15:H2, O26:H11, O119:H2, O128:H2, O153:H7 and O167:H9 were eae-β/β1; of c1, O55:H7 and O157:H7 were eae-γ/γ1; of b2, O103:H2 was eae-ε/ε1.

The PCR products of 15 strains including each HMA-type appearing in
Fig. 2 were sequenced (Greiner Japan, Tokyo). PHYLIP-style tree files were produced using CLUSTALW, DDBJ version available at http://www.ddbj.nig.ac.jp/search/ex_clustalw-j.html, and phylogenies were displayed using TREEVIEW (28). The HMA-a1 sequence of KI1318, O119:H6 (AB185253) exactly matched at positions 95-528 with the eae-β2 sequence of O119:H6 (AJ715407). According to phylogenetic analysis among α (M58154), β (AF453441) and β2, β2 was homologous to α based on its 5'-terminal region (at position 1-1600), but to β based on its 3'-terminal region (1600-end). The eae sequence of O119:H6 (AJ715407) has a mosaic structure as suggested by McGraw (22).

Fig. 2 shows a phylogram based on the 5'-terminal nucleotide sequences (95–528) of the eae gene in the databases and this study. The sequences could be clustered into five groups. The sequences of b1 differ by one to four nucleotides one another. The DNA sequences of eae-β3, eae-ε4 and eae types in cluster-x differed from HMA types by more than 5 nucleotides; the HMA types of these eae types will be analyzed in future studies. The phylogram showed (Fig. 2) good correlation with electrophoretic mobility (Fig. 1B).
universal primers. In a preliminary experiment with universal PCR primers by Ramachandran (29), several HMA types were poorly amplified using HMA.

The 5’-terminal region of eae genes is highly conserved (13); however, it contains variations that are sufficient to yield high resolution in the HMA typing method. This facilitates the subtyping of the intimin family in E. coli. To compare the discriminatory ability of typing systems, Simpson’s Index of diversity described by Hunter and Gaston (15) was applied. Simpson’s indices by Blanco (5-9) range from 0.62 (9) to 0.87 (6), 0.79 overall. Simpson’s indices by other authors (1, 4, 16, 27, 33) range from 0.66 to 0.82 and the Simpson’s index in this study was 0.81, indicating that HMA has a high discriminatory ability and is almost equal to the allele-specific PCR system developed by Blanco.

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**Nucleotide sequence accession numbers.** The sequences reported here appear in DDBJ under accession numbers AB118854-AB118869 and AB185253.
REFERENCES


Fig. 1

A) KI1218 (O153:H21, d1)

B) KI1223 (O157:H45, a1)
Fig. 2

Cluster a

- δ O86:H34
- O145:H4
- a1 KI1223 O157:H45
- μ O127:H6
- ζ O111:H9
- a3 KI1434 OUT:HNM
- ι O125:HNM
- γ O119:H6
- a1 KI1318 O119:H6
- c2 KI1609 O63:H6
- ι O2:74:HNM
- γ O157:H7
- c1 KI1231 O55:H7

Cluster b

- b1 KI1220 O128:H2
- α O103:H2
- b2 KI1221 O26:H11
- γ O80:HNM
- b1 KI1392 O26:H11
- β O15:HNM
- b1 KI1237 OUT:HNM
- b1 KI1235 O153:H7
- b1 KI1287 O103:HNM

Cluster c

- c1 KI1218 O153:H21
- θ O111:HNM
- γ O111:HNM
- d2 KI1440 OUT:H7

Cluster d

- d1 KI1218 O153:H21
- θ O111:HNM
- γ O111:HNM
- d2 KI1440 OUT:H7

Base substitutions at site
FIGURE LEGENDS

Fig. 1. HMA profile of all HMA types with HMA references.

Each of ten HMA types, a1 to d2, and 4 eae references, α, β, γ, and ε, formed heteroduplexes with (A) KI1218 strain (O153:H21, type d1) or (B) KI1223 strain (O157:H45, type a1). Heteroduplexes were separated on 7.5% homemade polyacrylamide gels and stained with ethidium bromide. Electrophoretograms are shown as negative images.


Fig. 2. Phylogram based on 5′-terminal nucleotide sequences of different intimin subtypes (nucleotide 95 to 528) and HMA types.

HMA type, name, and serotype of the strains used in this study are shown in bold-faced type. eae type and serotype of the strains deposited in the databases are shown in plain text.
The horizontal axis shows evolutionary change, and longitudinal axis is arbitrary. Scale bar indicates the number of nucleotide replacements per site.

DNA sequences with different eae genes retrieved from databases, including:

- **eae-α** (M58154), **eae-α2** (AF530555), **eae-β** (AF453441), **eae-β2** (AJ715407),
- **eae-β3** (AJ876651), **eae-γ** (Z11541), **eae-γ2** (AF025311), **eae-δ** (AJ875027),
- **eae-ε** (AF116899), **eae-ε2** (AF530554), **eae-ε3** (AJ876650), **eae-ε4** (AJ876651), **eae-ζ** (AF449417), **eae-η** (AJ308550), **eae-η2** (AJ876652),
- **eae-θ** (AF449419), **eae-ι** (AJ308551), **eae-ι2** (AF530553), **eae-κ** (U66102),
- **eae-λ** (AF530557), **eae-μ** (AJ705049), **eae-ν** (AJ705050), **eae-ζ** (AJ705051),
- **eae-ο** (AJ584841, partial), **eae-π** (AJ705052), **eae-ρ** (AJ748082), and **eae-σ** (AJ781125).
TABLE 1. Comparison of serotypes and HMA types, and the stx genes among the eae-positive Escherichia coli strains isolated from Thailand and Japan.

<table>
<thead>
<tr>
<th>HMA-type</th>
<th>Serotypes</th>
</tr>
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<tbody>
<tr>
<td>stx-negative (188)</td>
<td></td>
</tr>
<tr>
<td>a2 (5)</td>
<td>O55:H5(2), OUT:H45(3)</td>
</tr>
<tr>
<td>a3 (1)</td>
<td>OUT:HN(1)</td>
</tr>
<tr>
<td>b2 (6)</td>
<td>O26:H11(4), OR:HN(1), OUT:HUT(1)</td>
</tr>
<tr>
<td>c1 (29)</td>
<td>O55:H7(22), O55:HN(3), O157:H7(2), OUT:HN(2)</td>
</tr>
<tr>
<td>c3 (2)</td>
<td>O124:H40(1), OUT:HUT(1)</td>
</tr>
<tr>
<td>d2 (1)</td>
<td>OUT:H7(1)</td>
</tr>
<tr>
<td>stx-positive (23)</td>
<td></td>
</tr>
<tr>
<td>a2 (1)</td>
<td>OUT:H21(1)</td>
</tr>
<tr>
<td>b1 (12)</td>
<td>O26:H11(11), OUT:HN(1)</td>
</tr>
<tr>
<td>b2 (2)</td>
<td>O103:H2(1), O121:HN(1)</td>
</tr>
<tr>
<td>c1 (5)</td>
<td>O157:H7(4), O157:HN(1)</td>
</tr>
<tr>
<td>c2 (2)</td>
<td>O63:H6(1), OUT:H19(1)</td>
</tr>
<tr>
<td>d1 (1)</td>
<td>O111:HN(1)</td>
</tr>
</tbody>
</table>

1 UT: untypable, and NM: non-motile.
2 Values in parentheses indicate the number of strains isolated from each country.
3 166 eae-positive E. coli isolates from 103 patients, 56 carriers, and 7 cattle in Japan.
4 45 eae-positive E. coli isolates from patients in Thailand.