Rapid identification of Enterobacter hormaechei and Enterobacter cloacae genetic cluster III

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Abstract

Aim: Enterobacter cloacae complex bacteria are of both clinical and environmental importance. Phenotypic methods are unable to distinguish between some of the species in this complex, which often renders their identification incomplete. The goal of this study was to develop molecular assays to identify Enterobacter hormaechei and Ent. cloacae genetic cluster III which are relatively frequently encountered in clinical material.

Methods and Results: The molecular assays developed in this study are qPCR technology based and served to identify both Ent. hormaechei and Ent. cloacae genetic cluster III. qPCR results were compared to hsp60 sequence analysis. Most clinical isolates were assigned to Ent. hormaechei subsp. steigerwaltii and Ent. cloacae genetic cluster III. The latter was proportionately more frequently isolated from bloodstream infections than from other material (P < 0.05).

Conclusion: The qPCR assays detecting Ent. hormaechei and Ent. cloacae genetic cluster III demonstrated high sensitivity and specificity.

Significance and Impact of the Study: The presented qPCR assays allow accurate and rapid identification of clinical isolates of the Ent. cloacae complex. The improved identifications obtained can specifically assist analysis of Ent. hormaechei and Ent. cloacae genetic cluster III in nosocomial outbreaks and can promote rapid environmental monitoring. An association was observed between Ent. cloacae cluster III and systemic infection that deserves further attention.
be under-recognition of the species (Townsend et al. 2008b). This is important because of the special impact of *C. sakazakii* in severe neonatal infections (Bowen and Braden 2006; Townsend et al. 2008a).

In-depth analysis of an *Ent. hormaechei* strain involved in a nationwide outbreak revealed it included genes related to transfer or mobile elements, as well as resistance and putative virulence genes, all of which might have contributed to a high genomic plasticity and to a high epidemicity (Paauw et al. 2009). For example, a high pathogenicity island, HPI-ICEEh1, had a functional yersiniabactin-iron-uptake capability which was comparable to the ICE element of HPI-ICEEc1 from *E. coli* ECOR31 (Paauw et al. 2010). Other studies, too, presenting lateral gene transfer between *Ent. cloacae* and other *Enterobacteriaceae* demonstrated such mobility of elements. (Paauw et al. 2006; Bryant et al. 2013; Coelho et al. 2012). These findings emphasize the evolutionary potential of ECC members for acquisition of virulence and drug resistance determinants.

Virulence-related properties are still unclear. However, a silver determinant, *sil*, was recently identified not only in a pathogenic *Ent. cloacae* strain causing an outbreak, but also in other clinical ECC isolates, especially those frequently associated with nosocomial infections (Kremer and Hoffmann 2012b). In addition, *Ent. cloacae* with resistance to antibiotics, including to carbapenems, has been reported (Hamada et al. 2013; Hamprecht et al. 2012; Osterblad et al. 2012; Satlin et al. 2012; Sonnevend et al. 2012).

In view of the significant representation of *Ent. hormaechei* and the generic cluster III in clinical isolates of the ECC, we aimed to develop rapid and convenient methods for their identification using quantitative real-time polymerase chain reaction (qPCR) assays. qPCR is regarded as a high-throughput, rapid, and cost-effective molecular platform. These features will facilitate prompt diagnosis of clinical infection caused by ECC with enhanced pathogenic potential, as well as assisting in environmental investigations.

**Materials and methods**

**Bacterial strains**

A total of 112 reference, clinical and environmental strains were included in this study. Twenty-five clinical isolates were derived from bloodstream infections. An additional 31 clinical isolates were obtained from the following sources: urine (10), wound (7), sputum (4), bile (2), abscess (1), eye (1), joint (1), peritoneal fluid (1), peritoneum (1), pleural fluid (1), rectal swab (1) and throat (1).

Environmental isolates were recovered from routine microbial screening of potable or marine water and powdered infant formula (PIF).

**DNA extraction**

DNA extraction from bacterial cultures was conducted using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI), according to manufacturer protocols. All DNA preparations were kept at −20°C until use.

**Molecular characterization**

*Enterobacter cloacae* complex clinical isolates were allocated to their genetic clusters by *hsp60* sequence analysis carried out as previously described (Hoffmann and Roggenkamp 2003).

Genomic DNA was amplified by the TaKaRa Ex Taq PCR kit (TAKARA Bio Inc., Otsu, Japan) with *Hsp60*-F and *Hsp60*-R. PCR was conducted on a Biometra thermocycler (Biometra, Goettingen, Germany) under the following conditions: initialization 7 min at 94°C, followed by 30 cycles: 30 s at 94°C, 30 s at 57°C and 60 s at 72°C. Both strands of the purified PCR product were sequenced using a commercial sequencing service (HyLabs, Rehovot, Israel). Nucleotide sequences of *hsp60* used in this study for ECC analysis were retrieved from GenBank; accession numbers are specified in parenthesis: cluster I- *Enterobacter asburiae* (AF417141), cluster II- *Enterobacter kobei* (AJ567899), cluster III (AJ543864), cluster IV (AJ543806), cluster V- *Enterobacter ludwigii* (AJ417114), cluster VI- *Ent. hormaechei* subsp. *oharae* (AJ543782), cluster VII- *Ent. hormaechei* subsp. *hormaechei* (AJ417108), cluster VIII- *Ent. hormaechei* subsp. *steigerwaltii* (AJ543908), cluster IX (AJ543820), cluster X- *Enterobacter nimipressuralis* (AJ567900), cluster XI- *Ent. cloacae cloacae clus* (ATCC 13049) and cluster XII- *Ent. cloacae* subsp. *dissolvens* (AJ417143). The GenBank accession numbers of *hs* sequenced in this study are HF955015 to HF955034 and HG764317 to HG764347. *Enterobacter cloacae* complex strains were allocated to their genetic clusters according to the *hsp60* partial sequence protocol, using ClustalW (Larkin et al. 2007).

**qPCR assay**

TaqMan primers and probes for C-III and EH qPCR assays targeting *Ent. cloacae* genetic cluster III and *Ent. hormaechei*, respectively, were designed using MEGA 4 sequence data explorer (Tamura et al. 2007) and Primer Express software ver. 2.0 (Applied Biosystems, Foster City, CA). TaqMan primers and probe for *Ent. hormaechei* EH-SNP qPCR were designed by
Agentek Ltd. (Agentek, Tel Aviv, Israel). The primers and the probes of C-III, EH and EH-SNP qPCR assays targeting hsp60 sequences of the ECC are presented in Table 1.

Duplex qPCR assays and the simplex qPCR amplification control assay were performed using the LightCycler 480 platform (Roche, Basel, Switzerland). The qPCR program included an activation step for 15 min at 95°C, followed by 35 amplification cycles: 15 s at 95°C and 25 s at 68°C. Two detection channels, FAM/VIC, were employed, and a second derivative analysis was performed with a colour compensation step. Reaction volume was 20 μl using Absolute Blue QPCR No Rox Mix (Thermo scientific, Vilnius, Lithuania); the concentrations of primers and probes (Biosearch Technologies, Novoto, CA) were 250 and 125 nmol l⁻¹, respectively. The qPCR program for simplex EH-SNP qPCR assay included an activation step for 15 min at 95°C, followed by 35 amplification cycles: 15 s at 95°C and 25 s at 65°C.

Plasmid constructs of pGEM with hsp60 cluster III and hsp60 Ent. hormaechei amplicons were developed to evaluate the assay’s amplification efficiency and limit of detection. Amplification control was performed in parallel to the hsp60 duplex reaction targeting the gfp gene as previously described (Hoffmann et al. 2006). Fifty plasmid copies were introduced to a qPCR assay in which gfp primers and probe concentration were 500 and 250 nmol l⁻¹, respectively. An inhibition effect was defined as a delay of at least three C_T compared to the control group which had no bacterial DNA samples.

Results

The molecular identification schemes of Ent. hormaechei and ECC genetic cluster III comprised of an initial duplex assay with a confirmatory test for Ent. hormaechei. Three TaqMan qPCR assays targeting the hsp60 gene were included in the study. EH qPCR and C-III qPCR formed the initial step which was a duplex assay aimed to identify both Ent. hormaechei and genetic cluster III. The monoplex qPCR EH-SNP was an additional confirmatory step identifying Ent. hormaechei by specific single nucleotide polymorphism (SNP) recognition. No amplification inhibition was detected in any of the qPCR assays.

EH and C-III qPCR characterization

Calibration curves of EH and C-III qPCR assays were determined by 10-fold serial dilutions of plasmid pGEM constructs. Linearity was demonstrated across the tested range from 1.4 × 10⁷ to 28 gene copies. Calculated efficiencies for the EH and C-III assays were 1.9 and 1.86, respectively. The limit of detection value for both assays was 6.5 copies, for which 95% of samples were positive.

Assay specificity and sensitivity

Clinical and environmental ECC isolates were assigned to their respective genetic clusters based on hsp60 gene sequences (Table 2). This revealed that apart from cluster XII-Ent. cloacae subsp. dissolvens and cluster X-Ent. nimiressuralis, all other ECC genetic groups were represented in our study.

Eight cluster III isolates were positively identified by the C-III qPCR assay, and all 104 noncluster III bacteria were found to be negative (Tables 2 and 3). In-silico probe comparison of C-III with other ECC-equivalent sequences revealed that Ent. kobei was the least different from the C-III probe sequence, varying in only two nucleotide positions. Nevertheless, the two Ent. kobei isolates, an ATCC and a clinical source isolate, were not detected as false positive by the C-III assay. Accordingly, the C-III assay performance was 100% specific and 100% sensitive.

Table 1 Primers and probes used in the study

<table>
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<th>Assay</th>
<th>Primer and probe sequences (5′–3′)</th>
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| hsp60-PCR  | Forward: GGTAGAAGAAGGCGTGAGTTGC  
Reverse: ATGCGTCGGTGATGACATCAG                                        | Hoffmann and Roggenkamp (2003)           |
| EH         | Forward: CTGGTGCACTGGTGCACCCAA  
Reverse: CGTTGAACTGGTAGTTACCCTCACC  
Probe:[FAM]-CCGTCTGTGGTGGTCAAGA-[BHQplus] | This study                               |
| C-III      | Forward: CTGGTGCACTGGTGCACCCAA  
Reverse: CGTTGAACTGGTAGTTACCCTCACC  
Probe:[CAL Fluor Orange560]-AGAGGCATCAGTACTGGTCCGA-[BHQplus] | This study                               |
| EH-SNP     | Forward: AAGCCGGTGAGACGCC  
Reverse: CAGAAGTCGATCATGGTGCCGTAT  
Probe:[FAM]-TGAAAGGGATGTAAGAGTGA-[BHQplus] | This study                               |
| Amplification control | Forward: GACCACTACCAGAGAACC  
Reverse: GAACTCCAGCCAGACCAG  
Probe:[FAM]-AGCACCAATCGTCCCCGAGCA-[BHQ-1] | Hoffmann et al. (2006)                   |
The EH qPCR assay probe was designed to identify all three *Ent. hormaechei* subspecies but completely matched the sequence of genetic cluster IX as well. All 36 *Ent. hormaechei* clinical and environmental isolates were positive by EH qPCR (Table 2). Sensitivity, therefore, was calculated to be 100%. The design of EH qPCR included the identification of cluster IX, which was confirmed with a single clinical isolate. In addition, three other bacteria, *Ent. ludwigii*, genetic cluster IV and *Ent. kobei*, were borderline positive at a C<sub>T</sub> value of 35. The probe-equivalent sequence of *Ent. ludwigii*, genetic cluster IV isolates in the study, had only one mismatch and not two, as opposed to the strain retrieved from the GenBank.

Thirty-five of 36 *Ent. hormaechei* isolates were positive by EH-SNP qPCR (Table 2). The single species unidentified by EH-SNP qPCR could not be classified definitively to one of the *Ent. hormaechei* subspecies. The EH-SNP qPCR assay had a borderline detection of five *E. coli* isolates (Table 3). Therefore, the sensitivity and specificity of *Ent. hormaechei* detection were calculated for the

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<th>Table 2 qPCRs assay results of <em>Enterobacter cloacae</em> complex species</th>
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*EH-Ent. hormaechei* qPCR assay, EH-SNP *Ent. hormaechei* SNP qPCR assay and C-III Cluster III qPCR assay.

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<th>Table 3 qPCRs assay results of non-<em>Enterobacter cloacae</em> complex species</th>
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<tr>
<td><strong>Species</strong></td>
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<tr>
<td><em>Citrobacter koseri</em></td>
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<td><em>Cronobacter sakazakii</em></td>
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<td><em>Proteus vulgaris</em></td>
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<td><em>Salmonella enterica</em> Typhimurium</td>
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<td><em>Salmonella enterica</em> Nottingham</td>
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*EH Ent. hormaechei* qPCR assay, EH-SNP Ent. hormaechei SNP qPCR assay and C-III Cluster III qPCR assay.
combined performance of both EH and Eh-SNP assays. By definition, positive identification was only determined when both assays yielded positive results. Sensitivity was calculated to be 97%, and specificity was 97%.

Most clinical Enterobacter isolates were identified as cluster III and as Ent. hormaechei. In the latter, the subspecies steigerwaltii was dominant (Fig. 1).

A different distribution pattern was revealed among clinical isolates regarding their origin (Fig. 1). While almost all ECC blood culture isolates were allocated to Ent. hormaechei or cluster III, isolates from other clinical sources were more varied. Only one of 31 isolates from a nonblood source was identified as cluster III, as opposed to 7 of 25 isolates from blood infections (Fig. 2). This difference was statistically significant ($P < 0.05$) using the Fisher exact test. The occurrence of the Ent. hormaechei subspp. steigerwaltii in bloodstream infections (15/25) was similar to that in other infections (18/31).

**Discussion**

The goal of this study was to develop molecular qPCR assays to identify Ent. hormaechei and genetic cluster III. The qPCR assays target the hsp60 gene which has been used to assign the ECC to 13 genetic clusters. Bioinformatic analysis of the qPCR C-III probe sequence revealed that there was at least a two-base difference between cluster III and other ECC genetic clusters. Although Ent. kobei harboured only a two-base difference, it was still found to be negative when empirically tested using the C-III qPCR assay.

The EH qPCR assay identified all three Ent. hormaechei subspecies, as well as genetic cluster IX. A comparison between EH probe sequences of various groups demonstrated a single-base difference between Ent. kobei and Ent. cowanii. Moreover, Ent. ludwigii and genetic cluster IV clinical isolates from this study also harboured one-base difference, which led to borderline false-positive results. Therefore, positive identification of Ent. hormaechei was concluded only when the additional confirmatory qPCR assay targeting a SNP at hsp60 gene in Ent. hormaechei was positive.

In this study, sequencing results of the clinical isolates demonstrated that Ent. hormaechei subsp. steigerwaltii and cluster III were the most prevalent, followed by Ent. hormaechei subsp. oharae. This finding is in agreement with a previous report (Morand et al. 2009). The difference in incidence of cluster III in blood isolates vs. nonblood isolates was demonstrated as statistically significant. This justifies further evaluation in large-scale clinical studies.

*Enterobacter cloacae* complex bacteria typically have a high phenotypic expression variability, which renders the identification of some of their groups challenging. The definition of these genetic clusters was made possible

![Figure 1](image1.png) **Figure 1** Distribution of Enterobacter isolates from blood stream infections and nonbloodstream infections. (■) Bloodstream infections and (▲) nonbloodstream infections.

![Figure 2](image2.png) **Figure 2** Occurrence of Enterobacter hormaechei (subsp. steigerwaltii and subsp. oharae) and Enterobacter cloacae genetic cluster III among bloodstream and nonbloodstream infections. (●) Ent. hormaechei and (■) Ent. cloacae cluster III.
through sequencing of the hsp60 gene, which is neither a fast nor a high-throughput process. qPCR assays have become an integral component of molecular clinical diagnostics and are considered fast, high-throughput and cost-effective.

The assays reported in this study can identify Ent. hormaechei and genetic cluster III quickly and accurately and are therefore potentially important in the light of the high incidence and pathogenicity of these strains and their clinical significance.

**Conflict of interest**

The authors declare no conflict of interest.

**References**


