Phenotypic variations of enterococci in surface waters: analysis of biochemical fingerprinting data from multi-catchments

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Introduction

Protection of surface waters from faecal pollution is one of the more challenging tasks for water quality managers. Faecal pollution is traditionally assessed by monitoring faecal indicator bacteria, such as faecal coliforms, *Escherichia coli* and enterococci (U.S. Environmental Protection Agency (US EPA) 2003). These indicators are abundant in the intestine of warm-blooded animals and their presence in natural waters indicates faecal pollution and the presence of potential enteric pathogens (Dufour 1984). For improved management of water quality and mitigation of public health risks, it is imperative to distinguish between various non-point sources (NPS) of faecal pollution. However, monitoring the concentrations of faecal indicator(s) in natural waters does not provide sufficient information regarding their sources. In view of this, genotypic and phenotypic microbial source tracking (MST) methods have been developed (Bernhard and Field 2000; Carson *et al.* 2001; Hagedorn *et al.* 2003; Wiggins *et al.* 2003; Ahmed *et al.* 2005b). The majority of these methods require the creation of a known source...
library (i.e. library-based methods) of target strains, such as E. coli or enterococci from the suspected sources. This approach is based on the hypothesis that phenotypic or genotypic attributes of target strains are host-specific (Scott et al. 2002; Stoeckel and Harwood 2007). The most commonly used library-based methods include antibiotic resistance analysis (ARA) (Parveen et al. 1997), carbon source utilization (CSU) (Hagedorn et al. 2003), biochemical fingerprinting (BF) (Ahmed et al. 2005b), repetitive extragenic palindromic (rep) PCR (Dombek et al. 2000), and ribotyping (Scott et al. 2004). The assembled library is then used to identify, and in some cases quantify, the unknown environmental strains of the same target by various multivariate statistical analyses. Despite some initial success of these methods, questions have arisen regarding their utility in terms of size and representativeness of the library (Wiggins et al. 2003), temporal and geographical stability of the library (Gordon 2001; Hartel et al. 2002), host specificity (Gordon 2001), statistical analyses (Ritter et al. 2003), accuracy (Stoeckel et al. 2004) and associated costs (US EPA 2005). In addition, it has also been reported that environments could be dominated by environmental strains of indicator bacteria (i.e. environmentally adapted strains) which differ from genotypes or phenotypes found in the faeces of warm-blooded animals (Gordon et al. 2002; McLellan 2004; Anderson et al. 2005). It has been hypothesized that the presence of such environmentally adapted strains could be a significant limiting factor for the usefulness of library-based methods because of the possibilities that these strains may evolve and become the dominant population of target indicators in the environment. Consequently, it would be difficult to identify their sources by using a known source library. However, a recent study used pulsed-field gel electrophoresis (PFGE) and enterobacteria repetitive intergenic consensus (ERIC) PCR to fingerprint 555 E. coli isolates from two lakes in central Texas, USA, and reported that environmentally adapted strains of E. coli did not appear to dominate the E. coli populations within that limited geographical region (Casarez et al. 2007).

We previously reported the development of a large host-specific BF library based on a stringent sampling protocol from 10 host groups (Ahmed et al. 2005b) and its use to identify the likely sources of faecal pollution in several catchments in the Southeast Queensland, Australia (Ahmed et al. 2005b, 2006, 2007). The aim of this study was to analyse our existing data, collected over 5 years from these catchments, in order to identify the prevalence of environmentally adapted enterococci strains and their implications for library-based source-tracking methods.

Prevalence of environmentally adapted enterococci strains

Study catchments

Between 2003 and 2007, a total of 137 water samples were collected from the six catchments (i.e. C1 to C6; Fig. 1) located within 100 km radius in the Southeast Queensland, Australia (Table 1). Three (C1, C2 and C3) of these six catchments are extensively used for recreational activities and their surrounding areas are serviced by centralized sewage treatment plants (STP). All three catchments are subject to tidal inundation, and the entrance periodically closes following the formation of sand bars owing to tidal wave action. Once closed, the entrance will only be reopened by storm run-off following heavy rainfall events. Cattle, chickens, dogs, horses, kangaroos and waterfowls were identified as major sources of faecal pollution into these catchments (Ahmed et al. 2006). The fourth catchment (C4) is characterized by urban development with high density of septic systems in close proximity to natural waterways. Apart from the septic systems, the other major sources of faecal pollution included cattle, chickens, dogs, horses and waterfowls (Ahmed et al. 2005a). The fifth catchment (C5) is characterized by rich, fertile soils, with abundant summer rainfall with an average of 1600 mm annually. The major land uses include native vegetation, grazing, sugar cane production and horticulture crops, such as grapes and pineapples. The catchment is rural urban ranging from peri-urban agricultural producers to established residents. Cattle, dogs, horses, septic
systems and waterfowls were identified as major sources of faecal inputs into this catchment. The sixth catchment (C6) is characterized by undulating landscape with allotments featuring typical suburban houses, serviced by septic systems. The other possible sources of faecal pollution included horses, dogs and waterfowls (Ahmed et al. 2007).

Water sample processing and isolation of enterococci

The membrane filtration method was used to process all the water samples for bacterial enumeration and isolation of enterococci. Appropriate serial dilutions were made and filtered through 0.45 μm pore size (47 mm diameter) nitrocellulose membranes (Advantec, Tokyo, Japan) and placed on m-enterococcus agar plates (Difco, Detroit, USA). The plates were then incubated at 37°C for 48 h followed by testing for esculin hydrolysis using bile esculin agar (BEA) (Oxoid, London, UK) and incubated at 45°C for 1 h to confirm their identification as enterococci (Manero and Blanch 1999).

BF procedure

In total, 3952 enterococci isolates from 137 water samples were biochemically fingerprinted using PhP-RF plates (PhPlate system, PhPlate AB, Stockholm, Sweden). The 11 substrates have been described before (Iversen et al. 2002). From each water sample, up to 40 colonies (where possible) of enterococci were randomly selected and suspended into the first well of each row containing only 350 μl of growth medium. Aliquots of 25 μl of bacterial suspension were transferred into each of the other 11 wells containing 150 μl of growth medium. Plates were then incubated at 37°C, and A620 was measured at 16, 40 and 64 h using a micro plate reader (Lab-systems Multiskan, Helsinki, Finland).

The BF data were transferred to a computer connected to the reader and multiplied by 10 to give scores ranging from 0 to 30 for each reaction. After the final reading, the mean value for all three readings at different time intervals was calculated for each isolate giving scores ranging from 0 to 30 (BF) and similarities between isolates were determined as correlation coefficients after pair-wise comparisons of the isolates as previously described (Kühn et al. 1995). All data handling, including optical readings, calculations of correlations and coefficients, diversity indexes, and clustering and printing dendrograms was performed using the PhPlate software version 4001 (PhPlate AB).

BF library

In order to investigate whether environmental enterococci isolates are identical to the isolates found in the gut of warm-blooded animals, an existing multi-catchment metabolic fingerprinting library developed for this region was employed (Ahmed et al. 2006). This library consists of data from 5803 isolates from 10 host groups, including cattle, chickens, deer, dogs, ducks, horses, humans (via sewage and septic tanks), kangaroos, pigs and sheep (Ahmed et al. 2006).

Results

Comparison of BPTs among the six catchments

In all, 801 BPTs (total BPTs) of enterococci were found among the six catchments (Table 2). The number of total BPTs found in each catchment ranged between 89 (at C1) and 164 (at C5). When the total BPTs from all catchments were compared with each other, it was found that an average of 29.2% of total BPTs was specific (i.e. local BPTs) to each catchment. Catchment C4 had the highest

### Table 1 Number of water samples and enterococci isolates tested from the six catchments (i.e. C1–C6) in Southeast Queensland, Australia, between July 2003 and February 2007

<table>
<thead>
<tr>
<th>Catchments</th>
<th>Major sources of faecal pollution</th>
<th>Sampling period</th>
<th>No. of water samples tested</th>
<th>No. of isolates tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>Cattle, kangaroos, waterfowls</td>
<td>April 2005</td>
<td>14</td>
<td>423</td>
</tr>
<tr>
<td>C2</td>
<td>Cattle, horses, dogs, waterfowls</td>
<td>March 2005</td>
<td>28</td>
<td>649</td>
</tr>
<tr>
<td>C3</td>
<td>Cattle, chickens, dogs, horses, septic systems, waterfowls</td>
<td>July 2003–August 2004</td>
<td>22</td>
<td>781</td>
</tr>
<tr>
<td>C4</td>
<td>Cattle, dogs, horses, septic systems, waterfowls</td>
<td>May–June 2006</td>
<td>32</td>
<td>765</td>
</tr>
<tr>
<td>C5</td>
<td>Horses, dogs, septic systems, waterfowls</td>
<td>January–November 2006, February 2007</td>
<td>21</td>
<td>817</td>
</tr>
<tr>
<td>C6</td>
<td>Chickens, dogs, waterfowls</td>
<td>April 2005</td>
<td>20</td>
<td>517</td>
</tr>
</tbody>
</table>
Prevalence of environmentally adapted enterococci strains

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Table 2 Number of total biochemical phenotypes (BPTs) in each catchment and comparison between catchments

<table>
<thead>
<tr>
<th>Catchments</th>
<th>No. (%) of BPTs found</th>
<th>No. (%) of local BPTs and shared BPTs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C1</td>
</tr>
<tr>
<td>C1</td>
<td>89</td>
<td>23 (25.9)*</td>
</tr>
<tr>
<td>C2</td>
<td>148</td>
<td>37 (25.0)</td>
</tr>
<tr>
<td>C3</td>
<td>131</td>
<td>18 (13.7)</td>
</tr>
<tr>
<td>C4</td>
<td>125</td>
<td>12 (9.6)</td>
</tr>
<tr>
<td>C5</td>
<td>164</td>
<td>16 (9.7)</td>
</tr>
<tr>
<td>C6</td>
<td>144</td>
<td>30 (20.8)</td>
</tr>
</tbody>
</table>

*Local BPTs.

Comparison of total environmental BPTs with the library

To identify whether the total BPTs of enterococci found in all catchments reflect (i.e. degree of similarities) the enterococci BPTs found in the gut of warm-blooded animals, all BPTs from each catchment were compared separately with the existing library of enterococci BPTs. On average, 79.5% of the total BPTs from each catchment was identical to the library (i.e. host origin BPTs). The remaining 20.5% of the total BPTs from each catchment was not identical to any of those in the library (i.e. non-host origin BPTs) to the library and constituted only 5.3% (ranging between 2.4% and 7.3% in different catchments) of the total isolates tested for each catchment (Table 3).

In order to identify the occurrence and prevalence of these non-host origin BPTs among the six catchments, they were further compared with each other. On average, 18% (ranging between 14.4% and 23% in different catchments) of these was unique BPTs and constituted only 3.7% (ranging between 2.3% and 5%) of the total isolates tested for each catchment. The remaining non-host origin BPTs were shared between two or more catchments and constituted only 1.6% of the total isolates tested.

Discussion

Several questions have arisen regarding the utility of conventional faecal indicators, and their suitability for library-based MST methods (Gordon 2001; Gordon et al. 2002). One of the assumptions of MST is that the clonal composition of target strains within the host is stable over time. Strains which occur only once in a host group at a single sampling occasion are referred to as ‘transient’, while those occurring multiple times are referred to as ‘resident’ (Caugant et al. 1981). For MST purposes, the resident populations within host groups should be stable over time, and if they are not stable, the library needs to be updated regularly by adding more strains from the host groups if being utilized in environmental studies. Another important issue which has received less attention is the temporal stability of the target strains (U.S. Environmental Protection Agency 2005). For instance, E. coli electrophoretic types (ET) from a septic tank (i.e. secondary habitat) have been shown to differ from the human faeces of that particular household (i.e. primary habitat) (Gordon et al. 2002). Furthermore, researchers have
repeatedly observed that environments are dominated by environmental strains of indicators. These differ from genotypes or phenotypes found in faeces from warm-blooded animals, even when the environments in question are heavily contaminated with faecal matter (McLellan 2004; Anderson et al. 2005).

A comparison of the total BPTs among catchments showed that an average of 29% of these total BPTs was specific to each catchment. These BPTs were regarded as local BPTs. The presence of such local BPTs is not surprising considering that each catchment is characterized by different land use patterns and NPS of faecal pollution. Despite the fact that our data were collected over 5 years and the studied catchments were within a radius of 100 km, almost 71% of the total BPTs from each catchment was identical (i.e. shared) to others. This finding suggests some level of temporal stability of enterococci BPT among different catchments within a limited geographical area. A recent study which investigated the diversity of *E. coli* in surface waters by ERIC PCR genotyping method, reported similar temporal stability of *E. coli* in two catchments located approximately 30 km apart from each other (Casarez et al. 2007). On the other hand, it has also been reported that the phenotypic libraries (i.e. ARA and BF) are stable up to 1 year (Wiggins et al. 2003; Ahmed et al. 2006).

A comparison of the total BPTs from each catchment with the existing BF library (Ahmed et al. 2006) indicated that a high percentage (on average 79.5%) of BPTs from surface waters were in fact identical to those found in the faeces of warm-blooded animals. This library was developed using a stringent sampling protocol in order to capture the phenotypic diversity of enterococci from 10 host groups (representing more than 250 farms and 380 animals). This library was 'decloned' (i.e. identical strains were removed from the analysis) by cross-referencing BPTs among host groups. In this manner, the BPTs were categorized into two distinct types (i.e. unique and shared). The library constituted a total of 630 BPTs (i.e. unique and shared). The unique patterns were used to identify single host contribution, and shared patterns among animal host groups were used to identify animal host contribution. It has been previously shown to be stable and representative and was used to demonstrate the evidence of septic system failure and to identify the dominant sources of faecal pollution in various catchments in the study region (Ahmed et al. 2005a, 2006, 2007). The presence of such high level of total BPTs in our library therefore suggests that a majority of the enterococci BPTs found in these catchments were indeed of host origin. Similar results have been reported by Casarez et al. (2007), who compared the genotypic library of 883 *E. coli* strains from domestic sewage and animals with 555 *E. coli* strains from two lakes, and found that 79.5% of their isolates was of host origin.

It has to be noted that, the existing library was initially developed in 2003 (Ahmed et al. 2005b) and over time, new BPTs were added to that from multiple catchments. Interestingly, a large number of the total BPTs (i.e. ≥80%) obtained from the catchments C4 (in 2006) and C5 (in 2007) were identical to those in the library, indicating not only high temporal stability of the enterococci BPTs, but also high representativeness of the library. Only 20.5% of the total BPTs from each catchment did not exist in the library (non-host origin BPTs). It is possible that these BPTs may have originated from wild animals, such as possum, raccoon or wild birds, which were not included in our library. Another possible explanation is that some of these could be environmentally adapted BPTs given that they were not present in the library. It is difficult to interpret such findings as none of the libraries reported in the literature is complete.

Non-host origin BPTs constituted only 53% of the total isolates tested, and yet smaller portion (i.e. 3-7% of the total isolates) was unique to each catchment, suggesting that environmentally adapted enterococci isolates (if any) do not seem to have a limiting impact on the performance of library-based methods for detecting the sources of faecal pollution. In this respect, the size and representativeness of the library are crucial factors.

It can be argued that the lack of temporal stability and the occurrence of environmentally adapted genotypes of faecal indicators as reported in the literature could be related to the discriminatory power of the typing method. This conclusion is based on the fact that these studies used highly discriminatory genotypic methods, such as rep PCR, ribotyping and multilocus enzyme electrophoresis (MLEE) (Gordon et al. 2002; Hartel et al. 2003; McLellan 2004). From an MST context, where indicator bacteria are subjected to both temporal and environmental variations over years, it is conceivable that the use of such a highly discriminatory method should always be associated with testing a large number of isolates from both the host groups and the environments (Casarez et al. 2007). On the other hand, low discriminatory ability of a typing method would reveal more shared patterns among host groups if the library is developed from a large number of isolates. It has also to be noted that the shared bacterial patterns have significant implications on the specificity of the library. The specificity of a library decreases with the increased shared patterns.

In conclusion, our data suggest that less than 5% of the studied environmental strains was not identical to those in the library and seemed to be of environmental origin, yet a smaller portion of these are unique to a catchment. However, their presence in host groups could...
not be ruled out as the library used in the study cannot represent every host phenotype in the catchments. Nonetheless, from an MST context, such low level of environmentally adapted strains can have a minimal impact on the performance of the library-based methods if a large number of isolates are tested from both the host groups and environmental waters. However, it has to be noted that the successful application of the library-based methods also depends on the representativeness of the library and the discriminatory ability of the typing method used. These factors must be considered before development and a subsequent use of a library should be made to identify the sources of faecal pollution in environmental waters.

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References


