ORIGINAL ARTICLE

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

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Abstract

Aims: The aim of this study was to identify the prevalence of environmentally adapted enterococci strains by analysing biochemical fingerprinting (BF) data of 3952 enterococci isolates collected over 5 years from the six catchments in Southeast Queensland, Australia.

Methods and Results: A BF method was used to type 3952 enterococci isolates from six catchments. The environmental isolates were compared with a large existing BF library comprised of 5803 enterococci isolates from 10 host groups. Environmental isolates belonged to 801 biochemical phenotypes (BPTs), of which, an average of 29.2% was specific to each catchment. When compared with the BF library, an average of 79·5% BPTs from each catchment was identical to those in the library (i.e. host-origin BPTs). The remaining 20·5% was regarded as non-host origin BPTs, as they were not in the library and constituted only 5·3% of the total isolates tested for each catchment.

Conclusions: Our data suggest that less than 5% of studied environmental strains was not identical to those in the library and seemed to be of environmental origin. From a microbial source tracking 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 were tested from both the host groups and environmental waters.

Significance and Impact of the Study: These data shed light on the importance of the size and representativeness of library–based source-tracking methods and their implications for the identification of faecal pollution in environmental waters.

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 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.
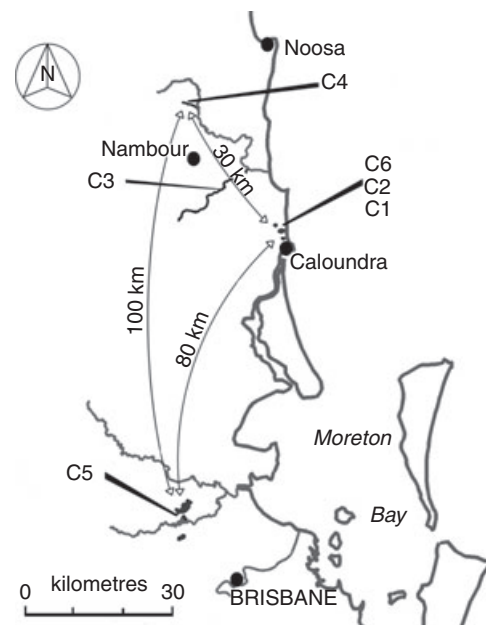
Figure 1 Location of the study catchments.

Materials and Methods

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

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

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

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 A_{620} 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 2 Number of total biochemical phenotypes (BPTs) in each catchment and comparison between catchments

Catchments	No. of total BPTs found	No. (%) of local BPTs and shared BPTs					
		C1	C2	C3	C4	C5	C6
C1	89	23 (25·9)*	10 (11·2)	14 (15·8)	4 (4·6)	16 (17·8)	22 (24·7)
C2	148	37 (25·0)	25 (16·9)*	13 (8·8)	22 (14·9)	19 (12·8)	32 (21·6)
C3	131	18 (13·7)	5 (3·80)	38 (29·0)*	33 (25·2)	17 (13·0)	20 (15·3)
C4	125	12 (9·6)	0 (0·0)	16 (12·8)	75 (60·0)*	17 (13·6)	5 (4·0)
C5	164	16 (9·7)	24 (14·6)	17 (10·5)	50 (30·5)	35 (21·3)*	22 (13·4)
C6	144	30 (20·8)	17 (11·8)	24 (16·7)	15 (10·4)	20 (13·9)	38 (26·4)*

*Local BPTs.

(i.e. 60.0%) and catchment C2 had the lowest proportion of (i.e. 16·9%) local BPTs. The remaining 70·8% BPTs from each catchment was shared among several catchments. The highest (30·5%) shared BPTs found were between C4 and C5 catchments. In contrast, none of the BPTs from C2 and C4 catchments were identical (Table 2).

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

Table 3 Number and percentage of biochemical phenotypes (BPTs) of enterococci that were not identical to the library and their occurrence among the six catchments

Catchments	No. (%) of nonidentical BPTs to the library	Representing isolates (% over total isolates tested)	No. (%) of unique BPTs	Representing isolates (% over total isolates tested)	No. (%) of shared BPTs	Representing isolates (% over total isolates tested)
C1	16 (18·0)	30 (7·1)	13 (14·6)	13 (3·10)	3 (3·40)	17 (4·00)
C2	31 (20·9)	42 (6·5)	26 (17·6)	26 (4·00)	5 (3·30)	16 (2·50)
C3	29 (22·1)	29 (3·6)	26 (19·8)	26 (3·30)	3 (2·30)	3 (0·30)
C4	21 (16·8)	21 (2·4)	18 (14·4)	18 (2·35)	3 (2·40)	3 (0·05)
C5	41 (25·0)	42 (5·1)	38 (23·2)	38 (4·65)	3 (1·80)	4 (0·45)
C6	29 (20·1)	40 (7·3)	26 (18·0)	26 (5·00)	3 (2·10)	14 (2·30)

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 landuse 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, racoon 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 5·3% 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.

Acknowledgements

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