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IDENTIFICATION OF SOURCES OF FAECAL SOURCE ISOLATES IN NINGI CREEK, AUSTRALIA

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ABSTRACT

This study was undertaken to investigate the feasibility of using antibiotic resistance patterns (ARP) for source tracking faecal contamination in surface waters, and linking faecal contamination to on-site wastewater treatment systems (OWTS). ARP's were established for a library of 1005 known *E. coli* source isolates obtained from human, domesticated animals, livestock and wild sources. Eight commonly used antibiotics at four different concentrations were used to obtain ARP's for the *E. coli* isolates. Discriminant Analysis (DA) was used to differentiate between the ARP of sources isolates, and identify the predictive ability of the library for classifying between isolates collected from human, wastewater treatment plant and on-site system sources. The source library was used to identify sources of faecal contamination in investigated surface waters and determine the significance of OWTS as a major contributor to faecal contamination. The developed ARP library was found to be adequate for discriminating human from non-human isolates, and was used to classify 144 enumerated *E. coli* isolates collected from monitored surface water locations. The resulting ARP DA indicated that a majority of the faecal contamination in more rural areas of the study catchment was non-human. However, the percentage of human isolates increased significantly in urbanised areas using on-site systems for wastewater treatment.

Keywords: On-site Systems, *E. coli*, Antibiotic Resistance, Discriminant Analysis

1 INTRODUCTION

Increasing urbanisation and changes in land use in southeast Queensland have an impact on the quality of natural watercourses. Due to the numerous non-point sources of contamination, it is critical to develop appropriate management strategies in order to reduce their impact. However, the specific sources of contamination are often difficult to identify. Additionally, the increase in urbanisation on the urban fringes of metropolitan areas has led to the reliance on on-site systems for the treatment and dispersal of sewage effluent. Numerous studies have highlighted the common failure of on-site wastewater treatment systems (OWTS) due to numerous factors, resulting in the contamination of ground and surface water resources (for example McNellie *et al.* 1994, Harris 1995, Paul *et al.* 1997, Young and Thackston 1999, Paul *et al.* 2000, Lipp *et al.* 2001, Pang *et al.* 2003). Microbiological contamination of water resources is of critical concern due to health risks, and the degradation of recreational and drinking water resources due to nutrient inputs (Hagedorn *et al.* 1999, Wiggins *et al.* 1999). In order to effectively manage the inherent risks, identification of the different sources of contamination is crucial. The most recent methods for identifying faecal contamination are based on the use of bacterial source tracking (BST) techniques.

Faecal bacteria can be emitted from various sources, including agriculture, wild and domesticated animals, urban development and sewage treatment facilities (Kelsey *et al.* 2004). Consequently, faecal coliforms are the most commonly used indicators of faecal pollution. However, the feasibility of adopting faecal coliforms as an indicator of faecal contamination is the subject of debate (Hagedorn *et al.* 1999, Meays *et al.* 2004). The presence of faecal bacteria in water resources only indicates that faecal contamination has occurred. However, it could well be that the faecal indicators may not be from one particular source, but rather from a variety of sources in the localised region. One of the most commonly suspected sources of faecal contamination of water resources are OWTS. However, due to the numerous possible sources of faecal bacteria, it has until recently been difficult to isolate on-site systems as a prominent source of faecal pollution. The use of biochemical BST techniques, such as

Antibiotic Resistance Patterns (ARP) of different sources of faecal bacteria have become more widely used (Wiggins 1996, Hagedorn *et al.* 1999, Booth *et al.* 2003, Wiggins *et al.* 2003).

ARP essentially utilises the resistance of selected faecal bacteria isolates, in this case *Escherichia coli (E. coli),* to several antibiotics at varying concentrations in order to obtain their resistance profiles. The underlying assumption of the ARP technique is that due to the increased use of antibiotics by humans and domesticated animals, isolated *E. coli* bacteria from these host sources will have higher resistance than that of wild animals (Wiggins 1996). The ARP technique requires a library of known *E. coli* isolates, from human and non-human sources. Consequently, *E. coli* from the investigated water samples are tested for their ARP and compared to the known source library and categorised according to the respective grouping of known source isolates with similar ARPs.

2 MATERIALS AND METHODS

2.1 Study Catchment and Sample Collection

The study catchment investigated was Ningi Creek catchment, located in Caboolture Shire, Queensland State, Australia. It is 72 km^2 in extent and consists of mixed landuse including urban, agricultural, pine forestry and natural bushland. At present, the catchment is experiencing significant urban development. The urbanised areas are serviced with OWTS, and their cumulative effect has become a major concern for the local government in relation to increased pollution of Ningi Creek. Twelve surface water monitoring sites were established for determining the level of faecal pollution, and for the collection of *E. coli* isolates for source discrimination. Water samples were collected on a monthly basis over a twelve month period. Figure 1 shows the locations of the monitoring sites. A total of 144 surface water samples were collected on a monthly basis over a four month period from each of the surface water monitoring locations. This sampling period was selected to allow the collection of samples during both the drier winter period following into the spring wet season.

2.2 Development of Source Library

To develop the source library of known *E. coli* isolates, faecal samples were collected from human and the primary non-human sources of faecal matter within the catchment. As one of the objectives of this study was to assess the discriminatory potential of human isolates from different sources, three human sources were identified for sampling. Five faecal samples were collected directly from human subjects (notated through this research as Human). Four additional human faecal samples were collected from OWTS (notated as Human OS), as well as from a local municipal wastewater treatment plant (Human TP). The main reason for collecting faecal samples directly from humans as well as from sewage treatment facilities was to compare the accuracy of the predictive capability of samples collected from treatment facilities to that from actual human sources. Even though the majority of *E. coli* isolates collected from wastewater treatment facilities would be of human origin, there is a possibility of cross-contamination with non-human *E. coli* isolates. Additionally, obtaining samples from public sewage treatment facilities allows extra diversification between human source isolates in the source library.

Major non-human faecal sources identified included livestock, domestic and wild animal sources observed near monitoring locations. Nineteen faecal samples were collected representing the three major sources of domesticated animals, including dogs, cats and poultry. Faecal samples from dogs and cats were collected from healthy domestic animals not undergoing antibiotic treatment. Poultry faecal samples were collected from free range poultry farms. Additionally, fourteen livestock faecal samples representing beef and dairy cows, horses and goats were obtained from agricultural farms within the catchment. Fifteen faecal samples representing five wild animal sources were collected including kangaroo, wallaby, koala, possum, and waterfowl. All these sources were observed in the catchments.

Figure 1 Ningi Creek catchment and established monitoring sites

2.3 *E. coli* Isolate Enumeration

Isolation of *E. coli* from faecal samples was achieved by adding 1.0 g of faecal matter or 1.0 mL of effluent sample to 100 mL of sterile buffered dilution water (0.0425 g L^{-1} KH_2PO_4 and 0.4055 g L^{-1} $MgCl_2$ in 100 mL distilled water) and vortexing for one minute (APHA 1999). Serial dilutions of 10^{-2} and 10^{-4} were prepared in buffered dilution water, and 1 mL, 10 mL and 90 mL of the 10^{-4} dilution were filtered for analysis. For collected water samples, volumes ranging from 0.1 mL to 100 mL were filtered to permit isolated colonies on each plate. Filtration was performed for both faecal and water samples, using 0.45 μm, 47 mm sterile gridded membranes (Millipore Corporation, Bedford, MA). The membranes were then aseptically transferred to petri-pads soaked in M-Endo medium (Millipore Corporation, Bedford, MA) and incubated at 30°C for 24 hours. Colonies were sub-cultured onto Nutrient agar plates, and then further tested for Indole reaction, (Growth in Tryptone water at 37°C for 24 hours followed by addition of Kovac's Indole Reagent) and for growth plus gas production at 44.5°C in Brilliant Green Lactose Bile Broth (BGLBB) (Eijkmann test). Those isolates with a positive reaction to both tests were recorded as confirmed thermotolerant *E. coli.*

2.4 Antibiotic Resistance Pattern (ARP) Analysis

ARP analysis was used to distinguish between known *E. coli* sources isolates and to identify the different sources of faecal contamination with the main aim of identifying human from non-human sources (Harwood *et al.* 2000; Whitlock *et al.* 2002). Antibiotic stock solutions were prepared from commercially available antibiotics (Sigma Chemical Co. St Louis) and applied to sterile trypticase soy agar (TSA) prior to pouring into sterile petri dishes containing one specific concentration of each antibiotic. The antibiotics used and their respective concentrations were; Amoxicillin (5, 10, 15 and 20 μg l^{-1}); Cephalothin (10, 25, 50 and 100 μg l^{-1}); Erythromycin (20, 50, 100 and 200 μg l^{-1}); Gentamicin (20, 40, 60 and 80 μg l^{-1}); Ofloxacin (5, 10, 15, and 20 μg l^{-1}); Chlortetracycline (20, 40, 60 and 80 μg l^{-1}); Tetracycline (20, 40, 60 and 80 μg l^{-1}); and Moxalactam (5, 10, 15 and 20 μg l^{-1}). The antibiotics were selected due to their common use in human and domesticated animals.

Isolates selected for ARP profiling were inoculated into nutrient broth and incubated for 18 hours at 37°C. Subsequent broths were diluted to 0.5 MacFarland Standard in fresh nutrient broth. The diluted isolates were placed in multipoint inoculator cups (Denley Multipoint Inoculator A400) for inoculation onto a series of 32 antibiotic plates (8 antibiotics, 4 different concentrations), plus one TSA medium blank. Plates were incubated at 37°C for 24 hours. After incubation, each plate was inspected and the relative growth was recorded. Four different ratings (1 to 4) were used to distinguish respective ARPs. An isolate received a rating of (1) for no growth; (2) for filamentous growth; (3) for restricted growth of colonies (growth of a few colonies); and (4) for full growth of colonies.

2.5 Discriminant Analysis of Antibiotic Resistance Patterns

Antibiotic resistance patterns for each of the source and unknown *E. coli* isolates (based on the 1-4 scale for growth) were input into a spreadsheet and analysed using Discriminant Analysis (DA) with StatisiXL ver1.4 software (Roberts and Withers 2004). DA is a multivariate statistical analysis technique where a data set containing *X* variables is separated into a number of pre-defined groups using linear combinations of analysed variables. This allowed analysis of their spatial relationships and identification of the respective discriminative variables for each group (Wilson 2002).

Pooling of source isolates into categorical groups were undertaken to assess the predictability of all human (pooled Human, Human OS and Human TP) versus non-human sources, and separate Human, Human OS, Human TP versus non-human isolates. The non-human category consisted of pooling the ARP of all wild, livestock and domesticated animal isolates into single individual pooled categories. The pooled category method was expected to provide higher average rates of correct classification for the source library, as has been found in past studies (Wiggins *et al.* 1999; Harwood *et al.* 2000; Booth *et al.* 2003). In addition, to assess the ability of the library to classify between different non-human sources, additional analysis was performed with pooled categories consisting of the above pooled human groups, livestock, domestic and wild animal isolates.

3 RESULTS AND DISCUSSION

Antibiotic Resistance Patterns

From the 68 faecal samples collected from known sources, a total of 1003 *E. coli* isolates were enumerated. Of these isolates, 175 were human isolates, which in turn were separated on the basis of 101 directly human, 39 from on-site systems and 35 from OWTS. Analysed ARP for known source isolates indicated distinctive patterns depending on the sources.

3.2 Discriminant Analysis (DA) of *E. coli* Antibiotic Resistance Patterns

DA for the pooled human versus non-human isolates performed exceptionally well with an Average Rate of Correct Classification (ARCC) of 90.9%, as indicated in Table 1. Both human and non-human categories showed clear discrimination between isolates, as shown in Figure 2. The correct classification rates were similar to those derived in other studies which achieved ARCC of >80% for human versus non-human pooled categories (Wiggins *et al.* 1999, Harwood *et al.* 2000, Whitlock *et al.* 2002, Booth *et al.* 2003). Both categories were classified particularly well, with incorrect classification rates of 6.8% and 9.5% for human and non-human respectively.

Table 1. Classification rates and ARCC for human Vs non-human source isolates

Source	**Number and %CC isolates classified as**		
	Non-Human	**Human**	**Correctly Classified**
Non-Human (*n = 828*)	749	79	90.5%
Human (*n = 175*)	12	163	93.1%
Average Rate Correct Class. (ARCC)			90.9%

Similarly, DA for the separated human categories (Human, Human OS and Human TP) versus non-human isolates performed equally as well as the pooled human category, obtaining a slightly lower ARCC of 90.1% as indicated in Table 2. This is slightly lower then the pooled human category, mostly as a result of the lower performance of the Human OS category. From the analysis, a distinct separation was also identified between the actual Human, Human OS and Human TP, which form a separate classification group, as shown in Figure 3.

To assess whether the source libraries developed retained enough isolates to correctly classify the unknown sources, pulled-sample cross-validations were conducted for each of the pooled human and non-human, as well as for the human, human OS, human TP, and non-human category assessments. The overall ARCC for the libraries used to reclassify randomly pulled human samples was 88.5%. For reclassifying randomly pulled non-human source samples, the ARCC for the sources libraries was 80.4%. These ARCC values were very similar to those obtained for the original source library. Hence, the ARCC's confirmed that the library was sufficiently large enough to provide adequate discrimination between human and non-human sources. Discrimination between the different human source categories achieved a slightly lower ARCC, mainly as a result of the more distinct separation between the Human OS and Human TP with the non-human sources than that compared with the human source isolates. ARCC for classification against the human sources isolates was 90.5%, 83.5% and 100.0% for Human, Human OS and Human TP respectively. However, the reclassification on pulled non-human source isolates received a lower ARCC of 79.8%. This lower ARCC for non-human sources is caused by less discrimination achievable between the unique human and non-human sources.

3.3 Classification of Unknown Source Isolates

From the samples collected, 199 unknown isolates were enumerated. Applying DA to the unknown source isolates and using the source library, the percentage of human isolates were determined. Table 3 gives the percentages of human and non-human isolates. From the DA analysis, a majority of the unknown source isolates were classified as non-human. Classification of the separated non-human sources indicated that the majority were represented by domestic sources, followed by livestock. The

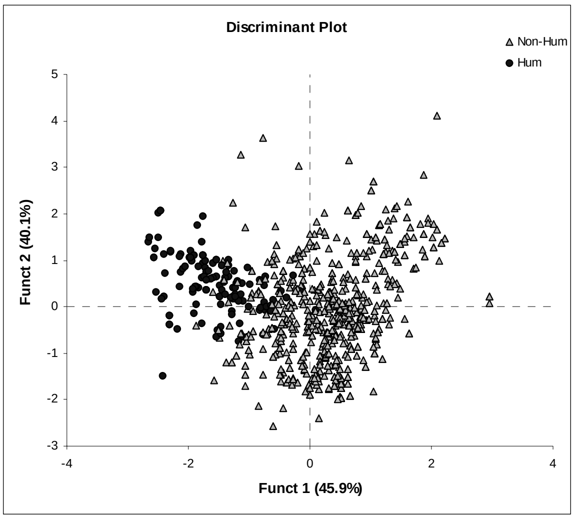

Figure 2 Discriminant analysis of source library isolates for pooled human versus non-human categories

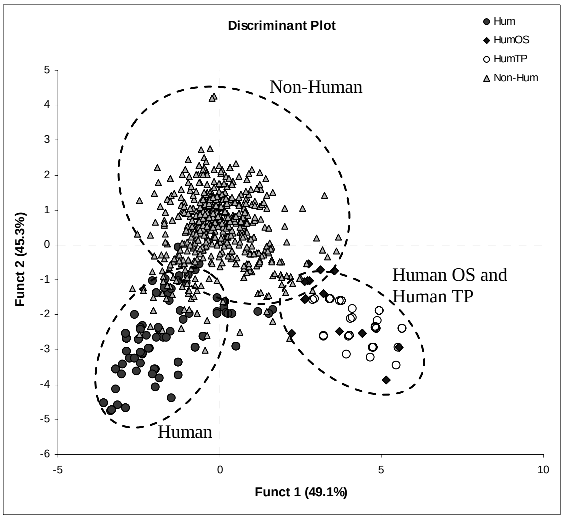

Figure 3 Discriminant analysis of source library isolates for human, human OS, human TP and non-human categories

Table 2. Classification rates and ARCC for human Vs non-human source isolates

Source	Number and %CC isolates classified as				
	Non-Human	**Human**	**Human OS**	**Human TP**	**Correctly Classified**
Non-Human (*n = 828*)	744	52	22	10	89.9%
Human (*n = 101*)	10	105	0	1	90.5%
Human OS (n = 24)	0	0	20	4	83.3%
Human TP (n = 35)	0	0	0	35	100.0%
Average Rate Correct Class. (ARCC)					90.1%

Table 3. Source identification of unknown isolates from monitored sites

Monitoring Site	No. Isolates[a]	Source Identification (%) of unknown source isolates					
		Human[b]	**Non-Human[b]**	**Human[c]**	**Domestic[c]**	**Livestock**	**Wild[c]**
Ningi Creek	*(n = 256)*						
SW1	*15*	13.33	86.67	13.33	73.33	0.00	13.33
SW2	14	21.43	78.57	14.29	50.00	7.14	28.57
SW3	19	0.00	100.00	0.00	68.42	0.00	31.58
SW4	21	4.76	95.24	4.76	61.90	0.00	33.33
SW5	10	0.00	100.00	0.00	50.00	0.00	50.00
SW6	19	0.00	100.00	0.00	78.95	0.00	21.05
SW7	17	0.00	100.00	0.00	82.35	0.00	17.65
SW8	16	6.35	93.75	6.25	68.75	6.25	18.75
SW9	29	13.79	86.21	6.90	87.50	3.45	27.59
SW10	14	7.14	92.86	0.00	92.86	0.00	7.14
SW11	14	25.00	75.00	25.00	75.00	0.00	0.00
SW12	15	25.00	75.00	12.50	62.50	0.00	25.00

[a] Unknown isolates collected from monitored sites over four months sampling period

[b] Pooled source categories for human vs non-human isolate DA

[c] Pooled source categories for human, domestic, livestock and wild isolate DA

percentage of wild isolates in the water samples increased marginally as the creek passed through the rural areas, before reducing in the downstream estuarine sections of the creek. The percentage of domestic and livestock isolates stayed fairly constant throughout the catchment, with only minor changes, with domestic isolates indicated as the most dominant source. However, as there is less discrimination potential between known domestic and livestock isolates, it can be expected that a proportion of the classified domestic isolates may actually be from livestock sources. Human source isolates decreased gradually through the catchment, before increasing after passing through the urbanised areas utilising OWTS.

Table 4. Source identification of unknown isolates using separated human sources

Monitoring Site	No. Isolates[a]	Source Identification (%) of unknown source isolates									
		Human[b]	Human OS[c]	Human TP[c]	Non-human[b]	Human[b]	Human OS[c]	Human TP[c]	Domestic[c]	Livestock[c]	Wild[c]
Ningi Creek	*(n = 256)*										
SW1	15	13.33	0.00	6.67	80.00	13.33	0.00	0.00	73.33	0.00	13.33
SW2	14	7.14	14.29	7.14	71.43	7.14	14.29	7.14	50.00	7.14	14.29
SW3	19	0.00	21.05	0.00	78.95	0.00	10.53	0.00	63.16	0.00	26.32
SW4	21	9.52	23.81	0.00	66.67	9.52	4.76	0.00	57.14	0.00	28.57
SW5	10	10.00	0.00	0.00	90.00	10.00	0.00	0.00	40.00	0.00	50.00
SW6	19	15.79	21.05	0.00	0.00	5.26	5.26	0.00	68.42	0.00	21.05
SW7	17	11.76	5.88	5.88	76.47	5.88	0.00	0.00	76.47	5.88	11.76
SW8	16	0.00	6.25	0.00	93.75	0.00	6.25	0.00	68.75	6.25	18.75
SW9	29	17.24	10.34	10.34	56.25	17.24	6.90	10.34	41.38	3.45	20.69
SW10	14	50.00	0.00	0.00	50.00	50.00	0.00	0.00	42.86	0.00	7.14
SW11	14	0.00	25.00	0.00	75.00	0.00	25.00	0.00	75.00	0.00	0.00
SW12	15	12.50	0.00	18.75	68.75	12.50	0.00	18.75	50.00	0.00	18.75

[a] Unknown isolates from collected from monitored sites over four months sampling period

[b] Pooled source categories for human, human OS, human TP vs non-human isolate DA

[c] Pooled source categories for human, human OS, human TP versus domestic, livestock and wild isolate DA

Observing the results from the classification of unknown source isolates using the separated human categories as shown in Table 4, the majority of unknown isolates were similarly categorised as wild isolates, followed by livestock. Interestingly, from the different human source categories, the majority were found to be represented by the pure human or human OS source groups, indicating the majority of human source isolates were from on-site systems in these sections of the catchment. This agrees with the types of residential development occurring in the catchment that rely solely on OWTS for the treatment of sewage.

4 CONCLUSIONS

The main purpose of this study was to discriminate between human and non-human sources, with further discrimination between different human sources of human, human OS and human TP. The results of the DA undertaken on the known source *E. coli* isolates indicated that applying ARP for the identification of human versos non-human sources of faecal contamination was feasible. To correctly classify the sources of selected isolates, developed libraries must ensure they are representative enough to provide adequate discrimination between known 'source' isolates (Wiggins 1996). It is generally recommended that a few hundred isolates for each identified source may be necessary for providing adequate discrimination between sources (Hagedorn *et al.* 1999, Wiggins *et al.* 2003). However, in the present study, it was found that a smaller source library was sufficient.

Classification of the unknown *E. coli* isolates provided two significant findings. Firstly, increasing human *E.coli* source isolates were identified in areas surrounding residential development relying on OWTS. Higher percentages of wild source isolates were identified in the less developed upstream areas, with changing percentages of source isolates between wild as the creek meandered through the catchment. However, the overall percentage contribution of wild isolates classified in the samples was quite small.

The increasing use of OWTS in rapidly urbanising areas without centralised sewage treatment facilities can cause detrimental environmental and public health impacts. However, the ability to assess microbial contamination of water resources in areas of high densities of OWTS has been difficult, as efficient means of identifying the sources of faecal pollution has been lacking. The use of ARP for identifying the various sources of faecal contamination in catchments has shown promising results, and its use for linking this contamination to OWTS has been proven to be feasible. The outcomes from this study, confirmed that ARP can be successfully utilised for assessing and identifying unknown bacteriological source contamination. However, the technique is reliant on ongoing source library development in order to increase source discrimination, as well as to ensure that changes in antibiotic resistance profiles are adequately updated.

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