Differentiation of Biosolids from Animal Faecal Material Using the 16S Ribosomal RNA Genetic Markers of Gastrointestinal Anaerobic Bacteria

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Abstract Recombinant DNA techniques were evaluated for their usefulness in distinguishing biosolids from faecal material of cow, kangaroo and sheep. It involved PCR amplification using published priming sequences, and restriction site profiling of amplified DNA across the 16S rRNA gene of anaerobic gastrointestinal bacteria, *Bacteroides spp* and *Bifidobacteria spp.* Of the three *Bacteroides spp* primer pairs, two were useful for cow faecal material though at lower annealing temperatures were also applicable to biosolids and sheep faecal material. The third primer pair was specific only for biosolids. All three primer pairs were not able to PCR-amplify *Bacteroides spp* sequences in faecal material of kangaroo. Of the three *Bifidobacteria spp* primer pairs, one was useful for sheep faecal material though at lower annealing temperature was also applicable to biosolids and cow and kangaroo faecal material. The *Bifidobacterium angulatum* specific primer pair enabled the PCR detection of anaerobes only in biosolids and in faecal material of kangaroo. The third, a *Bifidobacterium catenulatum* specific primer pair was suitable for faecal material of cow and at lower annealing temperatures was also applicable to the sample from sheep. For some primer sets, PCR amplification alone could not differentiate biosolids from other faecal samples. However, this could be resolved by digesting amplified DNA with the appropriate restriction enzymes. Overall, our evaluations show that recombinant DNA techniques have the potential to distinguish biosolids from other sources of faecal material, including that from kangaroo.

Keywords *Bacteroides spp*, *Bifidobacteria spp*, biosolids, kangaroo, 16S rRNA gene

Introduction

Biosolids are the stabilised solid waste by-product from wastewater treatment processes that are currently used throughout agriculture in Australia as a soil amendment and fertiliser replacement. It excludes animal manures, untreated septage (septic tanks), municipal solid waste and untreated wastewater sludges, hazardous wastes, industrial sludges (oil refinery and waste) and grit and screenings removed during the initial wastewater treatment process. The land application of biosolids is regulated in Australia, and elsewhere (Synagro Technologies Inc., 2002), to ensure that biosolids are applied safely and responsibly near waterbodies, so that the risk of environmental pollution is minimised. In Western Australia for example, the Water Corporation has three main

metropolitan wastewater treatment plants producing 86, 000 wet tonne of lime-amended biosolids and biosolids cake. Of these, 57, 000 wet tonne (or 73%) was applied onto agricultural land as fertiliser for the production of broad acre crops (Penney, 2006). Nutrient enrichment of waterbodies with elevated levels of nitrogen and phosphorus can lead to eutrophication and associated problems and therefore land managers need to ensure that there is minimal off-site movement of products containing these nutrients (Pritchard, 2006). Consequently, it would be of benefit to develop a monitoring strategy in areas where biosolids are applied to ensure that nutrients in biosolids are not inadvertently contaminating waterbodies. In some instances an increase in nutrient levels in waterbodies could indicate faecal contamination from biosolids but other possibilities could include run-off by inorganic fertilisers or through the grazing of livestock (Sinton, 1998) and wildlife, for example kangaroos. Therefore a distinction between biosolids and other sources of contamination needs to be established.

Despite significant advances towards developing a system of indicators for faecal source tracking, conflicting opinions remain regarding their effectiveness (Scott, 2002; Gilpin, 2003; Fogarty and Voytek, 2005). Coliforms, the traditional indicators of faecal pollution are not unique to humans (Dombek, 2000; Carson, 2001; Noble, 2006) because adventitious levels of such enterobacteriacae persist in the environment (Menaia, 1998; Bitton, 2005; Layton, 2006). Recently, attention has focussed on two new and promising markers in *Bacteroides spp* and *Bifidobacteria spp (*Bernhard and Field, 2000a, b). These anaerobes predominate the intestinal microflora of both human and animal digestive systems and together outnumber coliforms by several hundred-fold (Allsop and Stickler, 1985). The development of human-specific *Bacteroides* markers has increased the value of these potential indicators (Bernhard and Field, 2000a, b) which previously were not recognised because of difficulties in culturing anaerobes in the laboratory (Nebra, 2003). Significantly, some species are specific to humans, whereas others are found exclusively in animals (Bonjoch, 2004; Dorai-Raj, 2005). Several priming sequences on the 16S rRNA gene have been reported to be useful for the identification by PCR amplification (and variations from this technique) of both *Bacteroides spp* (Bernhard and Field, 2000a, b) and *Bifidobacteria spp* (Kaufmann, 1997; Matsuki, 1999).

Given the increasing application of biosolids in the agricultural sector, and the anticipated need for monitoring of water bodies, water quality managers are exploring the potential for *Bacteroides spp* and *Bifidobacteria spp* as biosolids source indicators. In this report, we evaluate recombinant DNA techniques for their utility at differentiating biosolids from other faecal sources including that from the kangaroo (a marsupial). In particular, we survey published primer sequences to determine their applicability to samples from regional Western Australia.

Methods

Isolation of DNA. Biosolids (Woodman Point, WWTP), cow, sheep and kangaroo faecal material were supplied by the Water Corporation, Western Australia. DNA was isolated using the QIA DNA Stool kit (QIAGEN 2004). Certified genomic DNA of reference bacteria from the ATCC (American Type Culture Collection) were used as controls and to confirm the specificity of primer pairs. They were; *Bacteroides fragilis* (*B. fragilis,* ATCC 25285), *Bacteroidesvulgatus* (*B. vulgatus,* ATCC 8482), *Bifidobacterium adolecentis* (*B. adolecentis,* ATCC 15703), *Bifidobacterium infantis* (*B. infantis,* ATCC15697), *Escherichia coli* (*E. coli,* ATCC 10798) and *Staphylococcus aureus* (*S. aureus,* ATCC 10832).

PCR Amplification. Each PCR reaction contained 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl (pH 8.3), 0.25 μM primers, 250 μM deoxynucleoside triphosphates and 0.75 Units of Taq DNA polymerase. The amplification conditions were; 95^{o}C for 2 min, then 35 cycles at 95^{o}C for 15 s,

50°C for 30 s, and 72°C for 1 min and finally, 72°C for 10 min. PCR reagent blanks (ie. without DNA) were run with each batch of amplifications. The specificity of each primer pair was also evaluated over an annealing temperature gradient of 45°C to 65°C across each DNA isolate.

Primer Pair	Faecal Indicator	Reference
Bac 32F/Bac 708R	*Bacteroides-Prevotella*	Bernhard and Field 2000b
F1/F2	*Bacteroides-Prevotella*	Menaia 1998
G1/G2	*Bacteroides-Prevotella*	Menaia 1998
lm 26/lm 3	*Bifidobacterium genus*	Kaufmann 1997
BiANG1/BiANG2	*Bifidobacterium angulatum*	Matsuki 1999
BiCATg1/BiCATg2	*Bifidobacterium catenulatum*	Matsuki 1999

Table 1: List of published primers used in this study.

Restriction enzyme digestion. Up to 10 μL of amplified DNA was digested with 5 Units of restriction enzyme in a final volume of 25 μL for 4 h at 37°C. A panel of twenty-four different restriction enzymes were screened for ability to provide informative restriction fragment patterns which are diagnostic for each host. Digests were electrophoresed on 4% agarose MS (Roche Diagnostics) and restriction fragments were sized against a DNA molecular weight ladder VI (referred to as Lane D, in Figures 1 and 2). Molecular weights below 100 bp were not reported.

Results and Discussion

PCR amplification

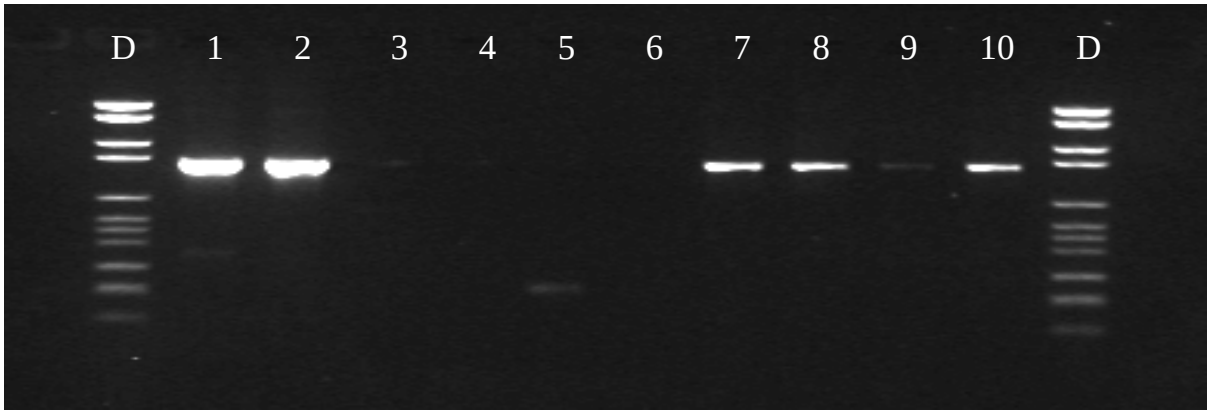

Figure 1. Specificity of PCR-amplification with F1/F2 *Bacteroides spp* primer pair. Lane 1: *B. fragilis;* Lane 2: *B. vulgatus*; Lane 3: *B. adolecentis*; Lane 4: *B. infantis*; Lane 5: *E. coli*; Lane 6: *S. aureus*; Lane 7: cow faecal material; Lane 8: biosolids; Lane 9: kangaroo faecal material; Lane 10: sheep faecal material; Lane D: DNA molecular weight ladder VI.

It can be seen in Fig. 1, that DNA of approximately 950 bp was produced when DNA isolates were PCR-amplified with the F1/F2 *Bacteroides spp* primer pair of Menaia et al. (1998). Amplified DNA of the same size was detected in the ATCC reference standards *B. fragilis* and *B. vulgatus* (Lanes 1 and 2) and biosolids (Lane 8), and faecal material of cow and sheep (Lanes 7 and 10, respectively). None was detected in Bifidobacteria (*B. adolecentis* and *B.infantis*), *E. coli* and *S. aureus* (Lanes 3-6, respectively). The poor response in kangaroo faecal material (Lane 9) suggests that the F1/F2 primer pair was not suitable for marsupials. This was not surprising given that the majority of molecular work has been done on gastrointestinal anaerobes in animals other than kangaroo. In contrast, *B. adolecentis*, *B.infantis*, biosolids, and cow, sheep and kangaroo faecal material were positive with the lm 26/lm 3 *Bifidobacterium genus* primer pair except for the *Bacteroides spp* (*B. fragilis* and *B. vulgatus*), *E. coli* and *S. aureus* (data not shown). Overall, the ATCC reference standards helped establish the specificities of the primers.

Primer Pair	Biosolids	Cow	Sheep	Kangaroo
Bac 32F/Bac 708R	+	+	+	-
F1/F2	+	+	+	-
G1/G1	+	-	-	-
lm 26/lm 3	+	+	+	+
BiANG1/BiANG2	+	-	-	+
BiCATg1/BiCATg2	-	+	+	-

Table 2. Detection of *Bacteroides spp* and *Bifidobacterium spp* in biosolids and in animal faecal material. Highlighted areas represent preferential amplification at the highest permissible annealing temperature.

The results of PCR amplification with other primer pairs are summarised in Table 2. DNA of approximately 700 base pairs (bp) was generated when DNA isolates were PCR-amplified with the Bac 32F/Bac 708R *Bacteroides spp* primer pair of Bernhard and Field (2000b) with the exception of kangaroo faecal material. Raising the annealing temperature to 61^{o}C resulted in the detection of *Bacteroides spp* but only in cow faecal material. The same was observed for the F1/F2 primer pair of Menaia et al. (1998) but at an annealing temperature of 63^{o}C. A positive response with the G1/G2 primer pair, also of Menaia et al. (1998), was recorded only in biosolids (650 bp), suggesting that these primers could be useful for detecting biosolids by PCR amplification alone. The lm 26/lm 3 *Bifidobacterium spp* primer pair of Kaufmann et al. (1997) detected anaerobes in all the samples. However, raising the annealing temperature to 61^{o}C resulted in the detection of Bifidobacteria but only in sheep faecal material. Interestingly, 3 bands were evident in biosolids and this banding pattern was not affected by increases in the annealing temperature. It contrasted with the single band obtained from PCR amplification of biosolids with other primer pairs. The BiANG1/BiANG2 primer pair of Matsuki et al. (1999) was suitable only for biosolids and kangaroo faecal material. Indeed, it was the only primer pair that could distinguish biosolids (300 bp) from kangaroo faecal material (800 bp) by direct PCR amplification. The BiCATg1/BiCATg2 primer pair, also of Matsuki et al. (1999), was suitable only for cow and sheep faecal material (1 kbp) implying its usefulness for identifying *Bifidobacterium spp* in ruminants. The BiCATg1/BiCATg2primer pair detected Bifidobacteria only in the faecal samples of cow and

sheep. Raising the annealing temperature to 61^{o}C resulted in the preferential amplification of DNA isolated from cow faecal material. Generally, and under conditions of lower stringency, the host identity of amplified DNA could not be distinguished by size alone. The only exception was with the BiANG1/BiANG2 primers that distinguished biosolids from kangaroo faecal material.

Restriction Enzyme Analysis

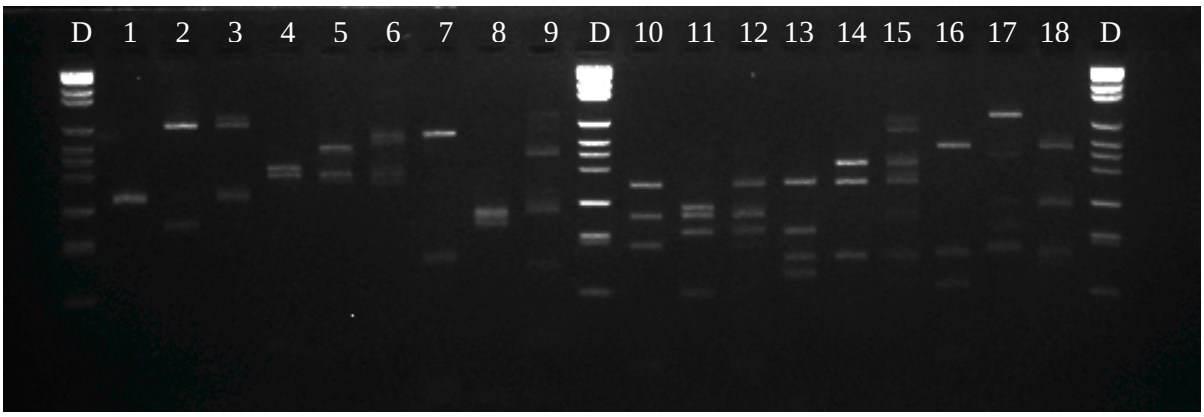

Figure 2. Distinction of biosolids from animal faecal material by restriction enzyme digestion of F1/F2 PCR amplified DNA. *Ban II*: Lanes 1-3; *Dde I*: Lanes 4-6; *Hae III*: 7-9; *Hpa II*: 10-12; *Rsa I*: 13-15; *Taq I*: Lanes 16-18. Cow faecal material: Lanes 1, 4, 7, 10, 13 and 16; biosolids: Lanes 2, 5, 8, 11, 14 and 17; sheep faecal material: Lanes 3, 6, 9, 12, 15 and 18; DNA molecular weight ladder VI: Lane D.

Up to 24 restriction enzymes were screened for ability to produce the restriction fragment patterns which enable the distinction of biosolids from other faecal sources. As can be seen in Fig. 2, restriction enzyme digestion of PCR-amplified DNA (with *Bacteroides spp* F1/F2 primer pair) produced an array of restriction fragment patterns which distinguished biosolids from other faecal samples. *Hpa II* reduced the 950 bp amplicon of biosolids into 4 restriction fragments of 150, 240, 270 and 290 bp (Lane 11). In contrast, the cow faecal material was distinguished by 3 fragments of 200, 270 and 360 bp (Lane 10). The sample of sheep faeces was characterised by 3 fragments of 250, 270 and 360 bp (Lane 12). Biosolids was also distinguished from other faecal material using *Ban II* (Lanes 1-3), *Dde I* (Lanes 4-6), *Hae III* (Lanes 7-9), *Rsa I* (Lanes 13-15) and *Taq I* (Lanes 16-18). Some partial digestion was observed but overall that did not affect the differentiation process. The utility of restriction enzymes was also empirically examined on amplified DNA of other primer pairs (data not shown), however, the F1/F2 PCR-amplified DNA offered the best resolution for distinguishing biosolids from other faecal sources.

Conclusions

The aim of this research was to assess the effectiveness of recombinant DNA techniques in distinguishing biosolids from faecal material of other sources, for example, cow, sheep and kangaroo. The use of *Bacteroides spp* and *Bifidobacterium spp* as biosolids indicators was found to be promising for the differentiation process. This was conditional however, on: (1) the use of a combination of primer pairs to minimise the risk of misidentification; (2) PCR amplification being performed at the highest permissible annealing temperature and; (3) the use of restriction enzymes that confirm the identity of the host. Kangaroo faecal material contains *Bifidobacteria spp* but

suitable primers need to be found for *Bacteroides spp*. Biosolids are applied at regional locations in Western Australia which is typified by the cohabitation of humans, poultry, livestock and wildlife (eg. kangaroo). Therefore, there is the need to develop a more accurate snapshot on the distribution of *Bacteroides spp* and *Bifidobacterium spp*. This can only be achieved by extending the sampling size, the species range and by developing measures for limiting the risks of misidentification.

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