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Monitoring bacterial indicators and pathogens in cattle feedlot waste by real-time PCR

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ABSTRACT

Quantitative microbial health risk assessment requires accurate enumeration of pathogens in hazard-containing matrices as part of the risk characterization process. As part of a risk management-oriented study of cattle feedlot waste contaminants, we investigated the utility of quantitative real-time PCR (qPCR) for surveying the microbial constituents of different faecal wastes. The abundance of *Escherichia coli* and enterococci were first estimated in five cattle feedlot waste types from five localities. Bacteria were quantified using two culture methods and compared to the number of genome copies detected by qPCR targeted at *E. coli* and *Enterococcus faecalis*. Bacterial numbers detected in the different wastes (fresh faeces, pen manure, aged manure, composted manure, carcass manure compost) ranged from 10^{-7} to 10^{2} g^{-1} (dry weight). Both indicator groups were detected by qPCR with a comparable sensitivity to culture methods across this range. qPCR measurements of *E. coli* and *E. faecalis* correlated well with MPN and spread plate data. As a second comparison, we inoculated green fluorescent protein (GFP) labeled reference bacteria into manure samples. GFP labeled *E. coli* and *Listeria monocytogenes* were detected by qPCR in concentrations corresponding to between 18% and 71% of the initial bacterial numbers, compared to only 2.5–16% by plating. Our results supported our selection of qPCR as a fast, accurate and reliable system for surveying the presence and abundance of pathogens in cattle waste.

1. Introduction

Quantitative Microbial Risk Assessment (QMRA) is being used increasingly to inform water and waste management (Fewtrell et al., 2001; Haas et al., 1999). Its application depends on reliable quantification of pathogen numbers in source material and measurement of the effectiveness of barriers such as chemical disinfection and sunlight by studying the behavior of model microorganisms such as faecal indicators.

Traditionally, estimation of risks posed by pathogens in animal waste has been based on enumeration of bacterial indicator organisms such as fecal coliforms and fecal streptococci in environmental samples using cultivation techniques (Budnick et al., 1996; Eckner, 1998; Edberg et al., 1990). Thus, most of the historical data which report indicator numbers rather than pathogen numbers are of little use for QMRA. This arose because reliable cultural techniques for pathogens are often expensive or unavailable for environmental samples and the pathogens of interest (e.g. *Cryptosporidium*). Even with well-

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studied bacterial species, there remain the conundrums of growth medium selectivity, cell damage and viability in the absence of culturability (Keep et al., 2006; Kell et al., 1998; Lleo et al., 2005; Oliver, 2005).

Knowledge of the contaminant levels in solid waste should lead to better water management because such matrices are often the primary source of water contaminants and QMRA of water depends on knowing the starting numbers of viable pathogens. Quantification of pathogens in solids is also often more practical than analyzing particulates in downstream diluted water. A promising alternative to culture-based analyses of concentrated wastes appears to be real-time polymerase chain reaction (qPCR). This is a powerful quantitative molecular method which can enumerate a broad spectrum of microorganisms in environmental samples including water and faecal wastes (Shannon et al., 2007; Zhang and Fang, 2006). Detection of less than five genome equivalents per PCR reaction is possible (Klein, 2002), and improvements in sample preparation techniques can now overcome the negative influence of inhibitory substances in environmental samples (Lebuhn et al., 2003; Radstrom et al., 2004). Another critical logistical barrier to quantitative pathogen surveys has been the lack of standardized, well-validated DNA extraction techniques, which are widely applicable to different environmental samples. This barrier appears to be addressed through the development of efficient DNA isolation kits based on bead-beating technology able to extract DNA in a quantitative way from protozoa, fungi, resilient bacterial spores, and mycobacteria as well as the more typical Gram-positive and negative bacteria using essentially the same method (Cook and Britt, 2007; Jiang et al., 2005; Mumy and Findlay, 2004). Bead-beating methods already appear well suited for the extraction of DNA from various problematic matrices including soil and faeces (Cheun et al., 2003; Layton et al., 2006; Lebuhn et al., 2003). Future research needs to validate these methods on the full range of environmental matrices where pathogens are found.

In researching the application of qPCR to environmental matrices, we found there is still limited information on the use of molecular methods for surveys of the range of microorganisms in solid cattle waste-related material, as against species specific studies and work related to environmental waters or wastewater treatment (He and Jiang, 2005; Wery et al., 2006; Wery et al., 2008). Some work to date has described the analysis of microbial indicators in fresh bovine faeces (Lebuhn et al., 2003) and from anaerobic fermenters (Lebuhn et al., 2004, 2005). However, the use and the performance of qPCR as a survey tool for common indicator organisms or pathogens have not yet been reported for different wastes from cattle feedlots.

Cattle manure is a large volume and ubiquitous product of the livestock industry. More than 600,000 tons of this waste material is generated in Australia each year (Tucker, 2008, personal communication). Its high potential as a renewable resource has not been fully realized because there is concern about the waste being a source of water, air and food borne contaminants (Pell, 1997; Rogers and Haynes, 2005; Topp et al., 2008). Manure, particularly aged stockpiled waste, contain high concentrations of inhibitory substances like humic acids but also unknown degradation products, so methods developed for pathogen detection in water or other wastes cannot be used without validation (Lebuhn et al., 2003; Nantavisai et al., 2007; Radstrom et al., 2004). The status of cattle manure as being both a potential risk and a resource points to the need for better management of animal wastes, and, hence better monitoring which this study supports.

The present paper reports on the evaluation of a qPCR method for livestock waste surveys of pathogens based on a comparison of quantitative real-time PCR with cultural methods using the bacterial indicators *Escherichia coli* and *Enterococcus* spp., and the pathogen *Listeria monocytogenes* as models. It compares recovery rates for the principal wastes generated in feedlot operations. qPCR estimates of bacteria have also been compared with estimates obtained using two common cultural methods – most probable number (MPN) and selective plating. This work involves quantification of the numbers of microorganisms occurring naturally in cattle waste material, as well as waste seeded with genetically labeled type culture bacteria.

2. Materials and methods

2.1. Sample collection and sample preparation

Waste samples were collected from five cattle feedlots in eastern Australia between Queensland and Victoria. Three replicate samples were collected from each of the following waste types: fresh faeces, pen manure, freshly harvested pen manure, aged stockpiled or composted manure and waste from carcass composting sites (Table 1). At each sampling point, five sub-samples were collected at a depth of 10 cm below the surface to minimize the influence of solar inactivation on microorganisms within the samples. Samples of fresh faeces were collected from five individual cattle. All five sub-samples of each type were then composited, stored at 4 °C in the dark, and processed within 24 h of collection.

From the final composite samples, 1 g (wet weight) was transferred to 10 mL of Nutrient Broth 2 (Oxoid, Adelaide,

Table 1 – Collected cattle feedlot waste.

Waste stream	Sample age	Moisture content (%)	Comments
Fresh manure	Fresh	80–90	Randomly collected from five cattle
Pen manure	1 week	30–50	Semi-dry faeces mixed with urine
Harvested manure	4 weeks	25–30	Manure from freshly cleaned pens
Aged manure	3–6 months	15–25	Manure from unmanaged stockpiles or compost windrows
Carcass manure	Weeks to months	15–25	Carcasses composted with manure