PCR Assay Specific for Chicken Feces

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Bacteroidales **are fecal anaerobic bacteria that are common in the digestive systems and feces of warm-blooded animals. Some strains of** ***Bacteroidales*** **have been reported to be host-specific. In this study,** ***Bacteroidales*** **strains from chicken feces were examined for their potential use as indicators of chicken fecal contamination.** ***Bacteroidales*** **16S rRNA gene sequences from chicken feces were amplified, cloned and sequenced. Phylogenetic analysis was performed using these sequences and published** ***Bacteroidales*** **16S rRNA gene sequences from human and bovine feces. Primers were designed based on putative chicken feces-specific 16S rRNA gene sequences and the primer pairs were tested for specificity in PCR assays. One set of primers, chBact F1 and chBact R16, specifically amplified DNA from chicken feces in a PCR assay, but did not amplify wild turkey, cat, bovine, or deer fecal DNAs. In addition, DNA from feces contaminated straw-based chicken litter produced a product in the PCR assay. However, DNA from feces contaminated wood shavings-based chicken litter was not amplified. The PCR assay described here may prove a useful tool for the detection of chicken feces and for source tracking in watersheds with fecal contamination.**

INTRODUCTION

A current trend in animal production is the confinement of large numbers (thousands to tens of thousands) of animals in relatively small spaces. The resulting large quantities of fecal waste must be managed to minimize environmental and public health risks. Standard tests (e.g., quantitative tests for bacteria such as coliforms, *Escherichia coli* or *Enterococcus*, etc.) can detect fecal contamination, but they do not reveal the source(s) of the contamination (Savichtcheva and Okabe 2006; Yan and Sadowsky 2007). Understanding the origin of fecal pollution is critical for determining public health risks and for determining the actions required for remediation. Microbial source tracking utilizes fecal microorganisms to trace the source(s) of fecal pollution and includes microbiological, genotypic, phenotypic, and chemical methods (Field and Samadpour 2007; Stoeckel and Harwood 2007; Wuertz and Field 2007).

Bacteroidales, fecal anaerobic bacteria, are abundant in the digestive tracts and feces of warm-blooded animals. The presence of *Bacteroidales* species in water is considered indicative of fecal contamination (Allsop and Stickler 1985; Fiksdal and others 1985). Previous studies have shown that *Bacteroidales* present in the feces of different species, or group of species with similar digestive systems (e.g., ruminants), are genetically distinct. PCR-based assays have been developed that discriminate between human, ruminant (cattle, elk, etc.), pig, and horse fecal contamination based on these genetic differences (Bernhard and Field 2000b; Dick and others 2005; Mieszkin and others 2010). Several attempts have been made to develop assays that discriminate chicken fecal contamination from other animal feces with varying levels of success (Wheeler and others 2002; Johnson and others 2004; Hassan and others 2007; Lu and others 2007). The most recent study describes an assay specific for chicken fecal contamination based

on a *Brevibacterium* DNA sequence marker (Weidhaas and others 2010). In this study, *Bacteroidales* strains from chicken feces were examined for their potential use as specific indicators of chicken fecal contamination.

MATERIALS AND METHODS

Extraction of DNA from feces

Fecal samples were collected using sterile spatulas and transferred into sterile 50 ml centrifuge tubes. Samples were transported to the lab on ice and stored at –80˚C. Chicken feces and three samples of feces contaminated chicken litter (one straw, the other two wood shavings) were collected from the University of Arkansas poultry research farm in Fayetteville, AR. Wild turkey and deer feces were collected at Sequoyah National Wildlife Refuge near Vian, Oklahoma. Cat and bovine feces were collected in Tahlequah, Oklahoma.

Fecal samples were pooled for DNA extraction: equal weights of individual feces were combined for each DNA sample. Each DNA sample contained feces from a minimum of two individuals. DNA was extracted from feces using a Powersoil DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA) or by phenol extraction (Ausubel and others 1999). Phenol extraction was used for all avian fecal samples including chicken litter. Feces were resuspended in sterile water and the aqueous layers were extracted sequentially with equal volumes of equilibrated phenol (pH 7.9), phenol:chloroform (1:1) and chloroform. DNA samples containing less than 2.0 ng/ μl of DNA were precipitated using sodium acetate and ethanol. If initial DNA amplification tests were unsuccessful a PowerClean DNA Clean Up Kit (MO BIO Laboratories Inc., Carlsbad, CA) was used to further purify the fecal DNA.

PCR amplification of chicken fecal DNA and phylogenetic analysis of 16S rRNA sequences

Bacteroidales 16S rRNA gene primers, Bac32F and Bac708R, were used to amplify chicken fecal DNA using the protocol described by Bernhard and Field (Bernhard and Field 2000a; Bernhard and Field 2000b). PCR products were cloned using a Zero Blunt® TOPO® PCR Cloning Kit with One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen Corporation, Carlsbad, CA). Plasmid DNA was prepared using a QIAprep Spin Miniprep Kit (Qiagen Inc. - USA, Valencia, CA) and plasmids were sequenced at the University of Arkansas DNA Resource Center (Fayetteville, AR). Sequences were examined using Bellerophon (Huber and others 2004) for putative chimeras. Exact duplicate sequences were removed from the set prior to alignment. Nucleotide sequences of the 16S rRNA genes from this study have been deposited in GenBank under accession numbers HQ896738-HQ896767.

A phylogenetic tree based on *Bacteroidales* 16S rRNA gene sequences from chicken, human, and cattle feces was inferred using the neighbor-joining method and Kimura-2 parameter model (MacVector 11.1, MacVector, Inc., Cary, NC). Bootstrap values were obtained from a consensus of 1,000 neighbor-joining trees. *Bacteroidales* 16S rRNA gene sequences from non-avian sources were obtained from GenBank. Accession numbers for *Bacteroidales* 16S rRNA gene sequences from human hosts were: AB200217-AB200225, AB200228-AB200229, and AB215082-AB215084. Accession numbers for *Bacteroidales* 16S rRNA gene sequences from bovine hosts were: AJ006457, AJ011682-AJ011683, and AJ009933.

Primer design and testing

The computer program MacVector was used to design primer pairs for PCR assays. Putative chicken feces-specific 16S rRNA gene sequences were identified based on the phylogenetic tree described above. Selected sequences were then aligned and consensus sequences constructed. Primer pairs for PCR assays were designed based on the consensus sequences. Forward primers were derived from variable regions within the

16S rRNA genes while reverse primers were derived from conserved regions. Primer pairs were tested for host specificity in PCR reactions using DNA samples from chicken litter and, chicken, wild turkey, deer, cattle, and cat feces. All DNA samples used in this study successfully produced PCR products in assays with *Bacteroidales* primers Bac32F and Bac708R as described above. Reaction mixes consisted of 10 ng of DNA (chicken fecal and litter samples) or 20 ng of DNA (all other fecal samples), 0.4 μM each primer, and 12.5μl GoTaq® Green Master Mix (Promega Corporation, Madison, WI) in a final volume of 25μl. One primer pair, chBact F1 (CCGATGGTTTCAAGGGATTGC) and chBact R16 (TCTAAGCATTTCACCGCTACACC), was specific for chicken feces producing a PCR product of 532 bp. The chBact F1 and chBact R16 primer pair was designed using the consensus sequence based on chicken *Bacteroidales* 16S rRNA gene clones 2, 8, 28, 37, and 39. (See phylogenetic tree in Figure 1 for putative chicken-specific clusters of *Bacteroidales* 16S rRNA gene sequences.) Amplification with the chBact F1 and chBact R16 primers was performed under the following conditions: 95˚C for 2 min, 27 cycles of 95˚C (30 sec), 63.1˚C (30 sec), and 72˚C (40 sec) with a final extension at 72˚C for 6 min.

RESULTS AND DISCUSSION

Thirty partial *Bacteroidales* 16S rRNA gene sequences from chicken feces were aligned with corresponding partial *Bacteroidales* 16S rRNA sequences from human and bovine feces. Phylogenetic analysis revealed several clusters of *Bacteroidales* 16S rRNA sequences from chicken feces that were well separated from human and bovine strains (Figure 1). Oligonucleotide primers were designed using consensus sequences produced from putative chicken feces-specific *Bacteroidales* sequences. Several primer pairs were tested in PCR reactions containing DNA from animal feces, but only one primer pair was specific for chicken feces. PCR analysis of fecal DNAs with the chBact F1 and chBact R16 primer pair showed that chicken fecal DNA was amplified, but wild turkey, cat, deer and bovine fecal DNAs did not produce PCR products (Figure 2). DNA samples from chicken litter were also tested in PCR assays using the chBact F1 and chBact R16 primer pair. DNA from straw-based chicken litter was amplified while DNA from wood shavings-based chicken litter was not amplified (Figure 2). Although our preliminary results are promising, additional testing of the assay using other animal feces and additional samples of poultry litter and chicken feces are needed to confirm the validity of the assay.

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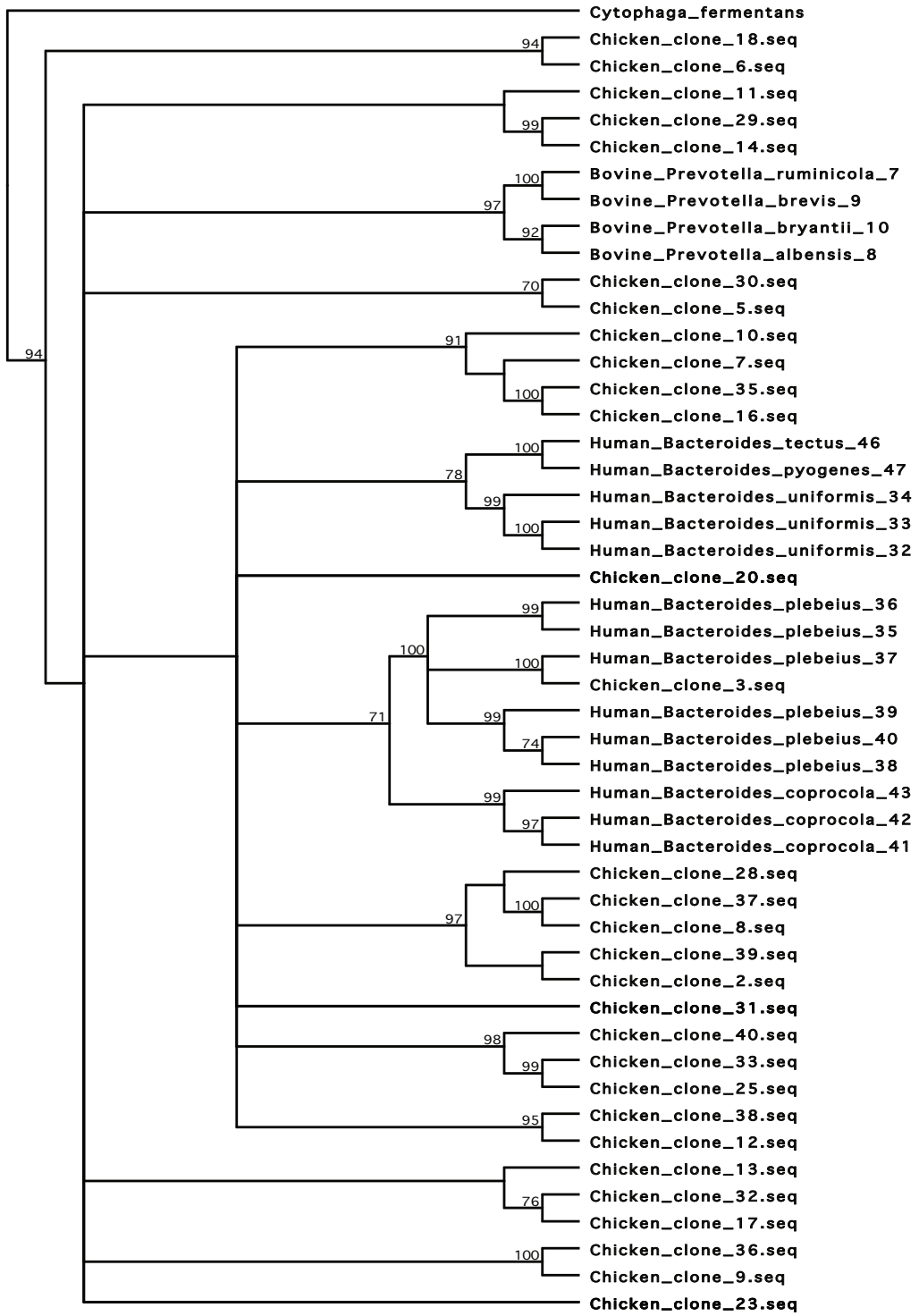

Figure 1. Phylogenetic tree of 16S rRNA gene sequences showing the relationships between *Bacteroidales* from chicken feces (this study) and *Bacteroidales* from human and bovine feces (GenBank). The tree was inferred using the neighbor joining method with Kimura 2-parameter correction. Numbers at nodes represent bootstrap values and values <70% are not shown. The corresponding partial 16S rRNA gene sequence from *Cytophaga fermentans* (M58766) was used to root the tree.

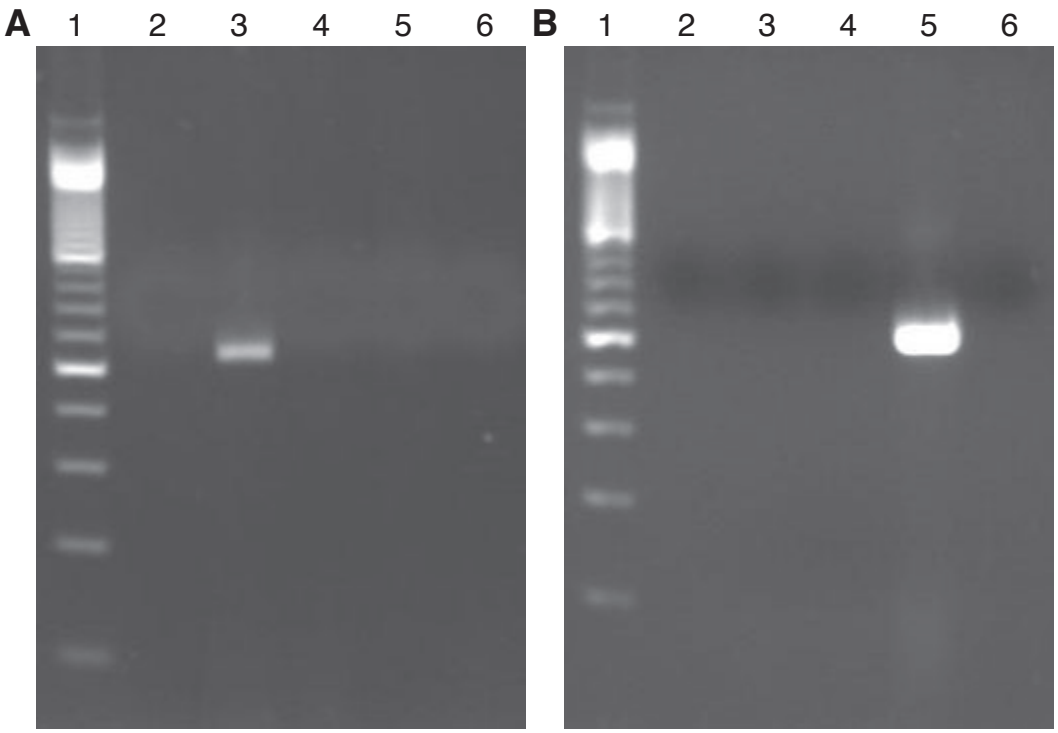

Figure 2. Results from PCR assays using the chBact F1 and chBact R16 primer pair and various DNA samples. A) lane 1, molecular weight standard; lane 2, negative control; lane 3, 10ng DNA from straw-based chicken litter; lane 4, 20 ng DNA from wild turkey feces; lane 5, 20 ng DNA from cat feces; lane 6, 20 ng DNA from deer feces. B) lane 1, molecular weight standard; lane 2, negative control; lane 3, 10 ng DNA from wood-shavings based chicken litter; lane 4, 10 ng DNA from wood-shavings based chicken litter (different sample); lane 5, 10 ng DNA from chicken feces; lane 6, 20 ng DNA from bovine feces.

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