2007

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Q-307

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Abstract

The goal of this study was to evaluate methanogen diversity in animal hosts to develop the first swine-specific archaeal molecular marker for fecal source tracking in surface waters. Phylogenetic analysis of swine *mcrA* sequences compared to *mcrA* sequences from the feces of five animals (cow, deer, sheep, horse, and chicken) and sewage showed five distinct swine clusters, with three swine-specific clades. From this analysis, six sequences were chosen for molecular marker development and initial testing. Only one *mcrA* sequence (P23-2) showed specificity for swine and was used for environmental testing.

PCR primers for the P23-2 clone *mcrA* sequence were developed and evaluated for swine-specificity. The P23-2 primers amplified products in P23-2 plasmid DNA (100%), pig feces (84%), and swine waste lagoon surface water samples (100%), but did not amplify a product in 47 bacterial and archaeal stock cultures, 477 environmental bacterial isolates, sewage, and water samples from a bovine waste lagoon and polluted creek. Amplification was observed in only 1 sheep out of 260 human and non-swine animal fecal samples. Sequencing of PCR products from pig feces demonstrated 100% similarity to pig *mcrA* sequence from clone P23-2. The minimal amount of DNA required for the detection was 1 pg for P23-2 plasmid, 1 ng for pig feces, 50 ng for swine waste lagoon surface water, 1 ng for sow waste influent, and 10 ng for lagoon sludge samples. Lower detection limits of 10-6 g of wet pig feces in 500 mL of PBS and 10-4 g of lagoon waste in estuarine water were established for the P23-2 marker. This study was the first to utilize methanogens for the development of a swine-specific fecal contamination marker.

Introduction

Swine waste is a significant source of fecal pollution and can cause contamination of soil, groundwater, and surface waters from lagoon overflow and use of lagoon surface water for irrigation. Studies have shown that spills from swine waste lagoons have high pollution potential with increased levels of nitrogen, phosphorous, and *Clostridium perfringens* counts of 40,000 CFU · mL-1. A similar study found that addition of pig manure or fecal slurries to agricultural soils led to persistence of pathogens (*Salmonella, Listeria*, and *Campylobacter*) on the soil surface. Because swine waste can lead to watershed pollution due to runoff from rain events or leaching into groundwater systems, it is important to develop swine-specific fecal markers to identify source of pollution for effective remediation efforts.

Only two potential methods currently exist for identifying swine waste. The STII swine biomarker assay shows specificity, sensitivity, and geographic stability. However, targeting toxin genes for host-specific source tracking may be problematic due to horizontal gene transfer events occurring in eubacterial populations, which may account for the presence of this gene in animals other than swine and humans with diarrhea. A swine specific marker developed using *Bacteroides* spp. also shows potential specificity for swine, although no tests have been conducted to determine the efficacy of this primer for microbial source tracking.

Although many methanogens appear to be specific to the intestinal tract of animals and have the potential for use as host-specific markers of fecal pollution, there are no known archaea markers of swine fecal pollution. Methanogens have been isolated in high numbers from the swine gastrointestinal system in counts of 106-108 methanogens per g wet feces. Only two methanogen species have been isolated and characterized from swine, but molecular studies of swine fecal slurries and waste lagoons have indicated the presence of several unknown methanogens that, if host-specific, may be useful for swine-specific marker development.

Because characterizing host distribution patterns of methanogens is essential to delineate potential host-specific archaeal indicators of fecal pollution, a large-scale examination of methanogen-specific *mcrA* genes in the feces of different host animals was conducted to identify sequences for swine-specific molecular marker development. The *mcrA* gene, encoding the α-subunit of methyl coenzyme-M reductase, was targeted due to the conserved nature of the gene, the specificity of the *mcrA* gene to methanogens, and the use of this gene as an environmental marker for methanogens. This study describes the development of an archaeal swine-specific marker of fecal pollution based on host distribution patterns of methanogens in the feces of six difference animals (pig, deer, cow, sheep, horse, and chicken) and sewage.

Materials and Methods

Cloning and Sequencing *mcrA* genes from animal fecal samples and sewage for swine primer development

DNA was extracted from three fecal samples of six different animals (sheep, deer, cow, pig, chicken, and horse) and sewage using the Power Soil DNA Extraction Kit (MO BIO, Carlsbad, CA). A 470bp region of the *mcrA* gene was amplified using the ML primer pair (Luton et al., 2002) following a modified protocol from Juottonen et al. (2005). Each 50 µL PCR assay contained 20 pmol of each ML primer, 200 µM dNTP, 1.5U Taq Polymerase, 1X ThermoPol Buffer, 0.1% BSA, and 5 µL fecal DNA template (ranging from 50 – 100 ng DNA). Thermal cycler conditions for the reactions were an initial denaturation for 5 min at 95°C, 40 cycles of 95°C for 40 s, 55°C for 1 min and 72°C for 1.5 min, and a final elongation for 1 min at 72°C (Juottonen et al., 2005). Methanogen *mcrA* PCR products from animal samples were cloned into the pGEM-T vector using the pGEM-T Vector System II (Promega, Madison, WI). Clones were screened for positive transformants using the PCR conditions described above (ML primer pair and corresponding PCR conditions). Restriction Fragment Length Polymorphism (RFLP) analysis was conducted on each of 384 clones to elucidate preliminary differences in animal and sewage clones. The *mcrA* insert in each clone was amplified using the ML primers and then digested with *Msp*I and *Taq*I .The restriction fragments were separated on a 3% SynergelTM, and band matching comparisons were performed for each of the RFLP fingerprints using Bionumerics Software v. 3.5 (Applied Maths, Austin, TX). Clones with unique RFLP patterns were identified and at least three clones of each pattern were chosen for sequencing for a total of 206 clones. Plasmids were purified using the ZyppyTM Plasmid Miniprep II Kit (Zymo Research) and sequenced commercially by Macrogen USA using the T7 Promoter Primer. The *mcrA* sequences amplified from fecal DNA of different animals were aligned using ClustalW (DNAStar v. 5.0) with manual inspection of the alignments. Phylogenetic trees were developed for each animal and inter-animal comparisons using the MEGA 3.1 program (Kumar et al., 2004) with 1000 bootstrap pseudoreplicates to confirm branching order. Swine specific sequences were chosen from swine-specific clades for potential primer development and diagnostic testing. Primers were designed for swine clone sequences using DNA Star PrimerSelect v. 5.0 and Integrated DNA Technologies PrimerQuest program. Primers were subjected to BLAST searches and manual comparison with sequences from clones in each clade, and only those primers with little or no overlap with known sequences were synthesized by Integrated DNA Technologies.

Primer Specificity

Samples were tested using two different primer pairs, one for swine-specificity (P23-2) and a universal bacterial primer pair used to determine viability of the DNA template prior to diagnostic testing (Ovreas et al, 1997). PCR analysis of the primers were carried out in 20 µL amplification reactions containing 1X PCR Buffer, 0.1% BSA, 200 µM dNTP, 1 U Taq Polymerase, 0.5 µM each primer, and varying amounts of DNA template. The cycling conditions consisted of an initial 92°C step for 2 min and 30 cycles of: amplification at 92°C for 30 sec, 60°C for 15 sec, and 72°C for 30 sec. A final elongation was performed at 72°C for 6 min. Positive controls contained purified P23-2 plasmid DNA, and negative controls contained an internal amplification control but no other DNA.

An internal amplification control (IAC) was designed for the P23-2 primer pair as a positive control for PCR. The IAC (purchased from IDT, Coralville, IA) was designed by deleting all but 140 bp of the original P23-2 clone *mcrA* sequence to amplify a 120 bp product using the same forward and reverse primers of the P23-2 assay. To determine the appropriate concentration of the P23-2 IAC, serial dilutions of the IAC (100 µM to 10-12 µM) were tested with varying amounts of P23-2 plasmid control ranging from 100 ng to 0.1 pg plasmid DNA in a 20 µL reaction. The P23-2 IAC was also tested with dilutions of pig fecal DNA to determine a suitable IAC concentration (10-9 µM) for the lowest level of detection in feces.

The primers were tested against 15 species of *Methanobrevibacter* and 12 additional methanogen genera (Table 1) to determine specificity. A total of 477 Gram (+) and Gram (-) bacteria isolated on EMB agar and BHI + NaN3 agar from sewage, fluvial, and estuarine water samples were tested for primer specificity. Whole cell PCR was performed using 1 µL cells (approximately 1 x 109 cells · ml-1) in a 20 µL reaction as described above. Fecal samples were collected and processed as described previously (Ufnar et al., 2006). DNA was extracted from fecal samples using the UltraClean Soil DNA Extraction kit and the Powersoil DNA Kit (MO BIO) following the manufacturer instructions. Fecal DNA samples were amplified in 20 µL reactions as described above using 50 ng DNA as template. Animal waste lagoon and sewage samples (500 mL each) were tested to determine the presence or absence of the P23-2 marker in composite samples. Two samples each (500 mL each) from a bovine waste lagoon and an adjacent creek contaminated with lagoon water were collected and each sample was centrifuged at 3,000 X *g* for 15 minutes. DNA was extracted from the resultant pellet using the MO BIO PowerSoil DNA Extraction Kit (MO BIO). Samples (50 mL each) from three different swine waste lagoons (nursery, sow, and finishing farms) were collected, centrifuged at 3,000 X *g* for 15 minutes, and the DNA was extracted from the pellets as described above. Sewer samples (n = 22) were collected from seven different sewers in Gulfport, MS each week for a period of two months. Sewer samples (500 mL) were processed as described previously (Ufnar et al., 2006). Environmental samples (sewage-contaminated water, swine waste lagoon, and bovine waste lagoon and contaminated creek) were amplified in 20 µL reactions following the protocol described above with varying concentrations of environmental DNA. Bovine waste lagoon and adjacent creek DNA samples were added to a 20 µL reaction using serial dilutions of total DNA to determine the presence or absence of the swine-specific methanogen.

Environmental water samples (n = 111) were collected from coastal sampling stations in Harrison County, MS for two, four month periods in 2004 and 2005 and analyzed with the P23-2 primer pair. Ten fluvial water samples were also collected from the Bouie River and tributaries in the Hattiesburg, MS area for PCR analysis using the P23-2 marker. Water samples (500 mL) were processed in a similar manner as the sewage samples by prefiltering and concentrating the bacteria onto a 0.2 µm Supor-200 membrane (Pall Corporation). DNA was extracted from the processed water samples using either the MO BIO PowerSoil DNA kit (MO BIO) or BIO 101 FastDNA Spin Kit for Soil (MP Biomedicals). Environmental water samples were amplified in 20 µL and 50 µL reactions both containing 25 ng and 50 ng of DNA template. Coastal sediment samples (n = 17) were also taken at four Mississippi Department of Environmental Quality (MDEQ) sampling sites. DNA was extracted directly from 0.25 g of surface sediments at 15 and 30 cm increments in the sediment cores using the Ultraclean Soil DNA Extraction Kit (MO BIO). Sediment DNA samples were amplified with 1 µL (50 ng) DNA in a 20 µL reaction.

To verify the identity of the PCR products amplified by the P23-2 primers, amplified DNA from five swine fecal samples collected from two different locations were purified using the Zymoclean DNA Recovery Kit (Zymo Research) and sequenced by Macrogen USA. The sequences were subjected to a BLAST search and aligned using the NCBI bl2seq alignment program (Tatusova and Madden, 1999). The limit of detection for the P23-2 plasmid was determined using serial dilutions of the purified plasmid DNA from 1 to 10-10 ng. One microliter of the plasmid DNA dilution was added to a 20 µL reaction using the conditions described above. In addition, the sensitivity of the P23-2 assay was examined by testing serial dilutions of fresh swine fecal material in PBS. Fecal samples from four pigs (0.25 g wet wt each) were combined and added to 500 mL sterile PBS. The samples were blended in a Waring blender at top speed for 2 min to completely resuspend the feces. Diluted samples were processed and DNA extracted using the procedure described above for fecal samples. DNA was used in amounts of 10 ng, 25 ng, and 50 ng in 50 µL amplification reactions as described above.

The usefulness of the P23-2 PCR assay was determined by testing varying amounts of DNA extracted from swine waste lagoon samples and dilutions of lagoon waste in filter sterilized estuarine water. Dilutions of total DNA from each swine lagoon sample (Nursery surface [NS]; Nursery Anaerobic [NA]; Sow Surface [SS]; Sow Influent [SI]; Finishing Surface [FS]; Finishing Anaerobic [FA]; and Finishing Surface Sludge [FSS]) were assayed in a 20 µL PCR using the protocol above. To determine the level of detection of swine lagoon waste in the environment, swine lagoon surface water (50 mL) was centrifuged at 3000 x *g*, and the pellet (0.25 g) was added to 500 mL of filter sterilized estuarine water. The sample was diluted in ten-fold increments, processed in a similar manner as the environmental water samples, and the DNA extracted using the BIO 101 Spin Kit for Soil. Each dilution was assayed in a 20 µL PCR reaction with varying concentrations of DNA using the P23-2 protocol above.

Acknowledgments

This work was supported by the NOAA OHH Program, Grant No. NA04OAR4600216; USEPA Gulf of Mexico Program, Grant No. MX96401204-0; and USEPA Gulf of Mexico Program, Grant No. MX96429505.

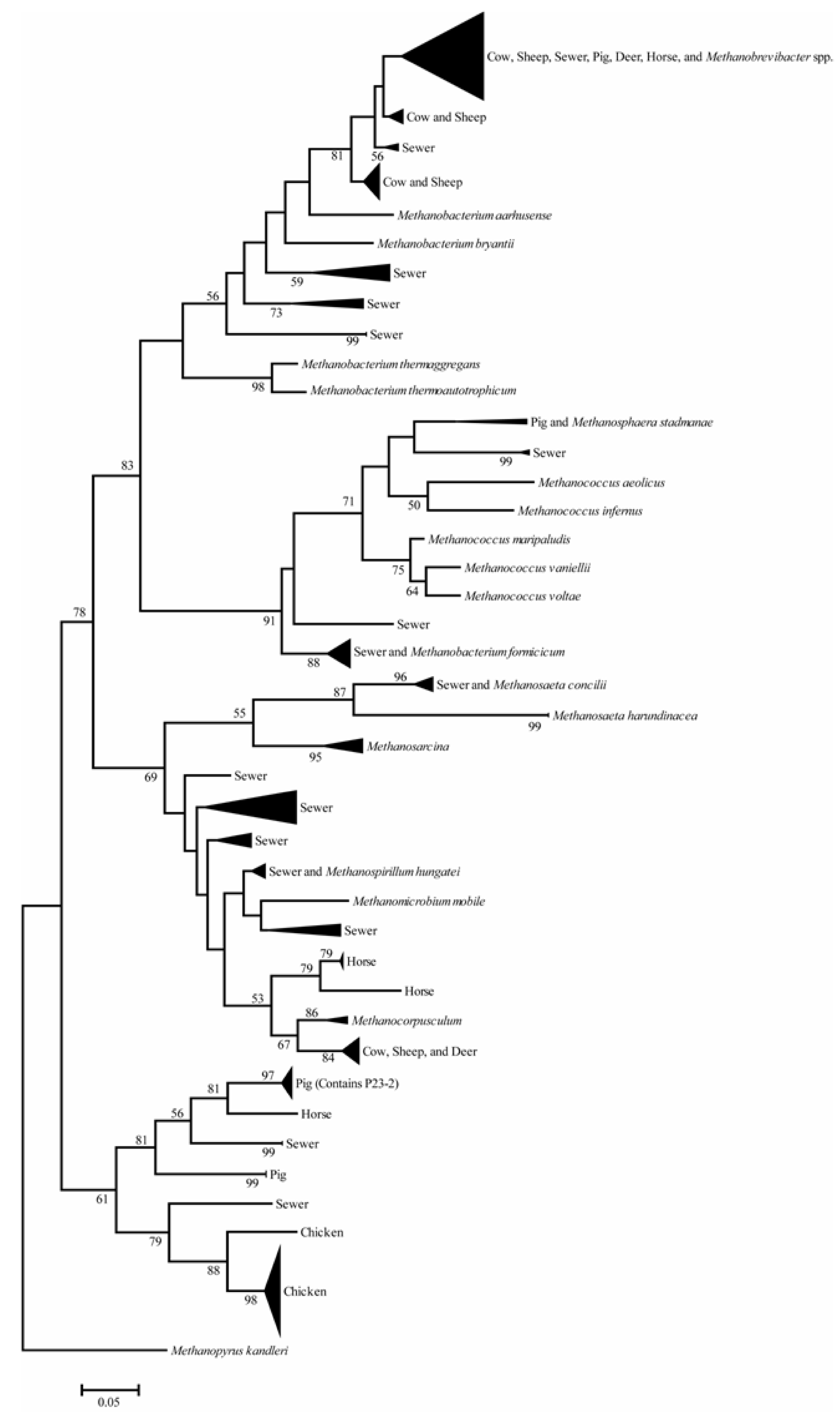

FIG. 1. Phylogenetic relationships among partial *mcrA* DNA sequences (470 bp) of clones recovered from fecal and sewer samples (this study) and previously sequenced *mcrA* genes from methanogen species (italicized). The tree was inferred by neighbor joining method with 1000 bootstrap pseudoreplicates using MEGA 3.1 tree building program and was rooted using the *mcrA* sequence from *Methanopyrus kandleri*. The scalebar represents 5% estimated sequence divergence.

TABLE 1. Fecal samples tested for P23-2 and P23-14 primer specificity

Fecal Samples Tested	# Samples Tested	P23-2 PCR Amplification	P23-14 Amplification
Swine	25	21/25	21/25
Human	50	0/50	0/50
Cow	50	0/50	0/50
Sheep	24	1/24	23/24
Rat	20	0/20	0/20
Horse	20	0/20	0/20
Deer	24	0/24	0/20
Goat	24	0/24	18/24
Chicken	24	0/24	0/24
Dog	24	0/24	0/24

TABLE 2. Environmental samples tested for P23-2 specificity

Expected	Samples Tested	P23-2 Positive Samples
Swine Waste Lagoon:		
Surface Samples	3	3
Sludge Samples	1	1
Sow Influent	1	1
Anaerobic Layer Samples	3	0
Not Expected		
Bovine waste lagoon	2	0
Bovine contaminated creek	2	0
Sewer	22	0
Coastal Water & Creek Samples	111	0
Coastal Sediment	17	0
Environmental Bacteria	477	0
Fluvial Water Samples	10	0

Discussion & Conclusions

The usefulness of a host-specific indicator of fecal contamination is dependent on the specificity of the marker to the host animal. The P23-2 marker developed in this study showed high specificity to swine fecal samples (84%) collected from different farms. More importantly, the P23-2 assay amplified all swine waste lagoon surface samples from three different farms but did not amplify samples from a bovine waste lagoon, creek contaminated with bovine waste, or sewage samples. In addition, sequencing confirmed that the expected products from swine fecal samples showed 100% identity to the P23-2 clone *mcrA* sequence. Inhibitory factors present in DNA extracts from environmental samples including fecal, sewage, animal waste lagoon, and environmental water and sediment samples routinely cause false negative results using PCR. Therefore, an internal amplification control was developed and used in this assay as a positive control to ensure that negative results from environmental samples were not due to PCR inhibition. These data show the usefulness of the P23-2 assay as a method for detecting swine-specific fecal contamination.

Methanogenic archaeal communities can change from the storage tanks to the storage pond in a swine waste system, therefore, samples taken at several locations in three lagoon systems at two different hog farms were tested with the P23-2 markers developed in this study. Swine fecal samples were also collected at two different farms in a different location from the farms sampled for lagoon waste. Amplification using the P23-2 marker in swine feces, surface lagoon water from all lagoons, waste lagoon influent, and lagoon sludge samples showed that the marker is not replaced by another methanogen and is stable in different swine populations. This study showed amplification with the P23-2 marker in all surface lagoon water samples, and is therefore a potential indicator for swine lagoon waste runoff.

Samples collected in swine waste lagoon surface waters showed sensitivity of the P23-2 assay with detection to 50 ng total DNA. PCR sensitivity for the P23-2 plasmid was measured to 10-12 g of DNA, which is comparable to the plasmid detection limit for a previously identified ruminant primer. The P23-2 marker successfully amplified DNA extracted from pig fecal dilutions to 10-6 g fecal material in 500 mL PBS. This level of detection was comparable to the detection of cow feces using the *Bacteroides* ruminant primers described previously. Further, swine lagoon waste was detected to 10-4 g in estuarine water, which suggests that the swine-specific P23-2 marker developed in this study is sensitive and specific and has potential as an indicator of swine lagoon waste in the environment.

The distribution of *mcrA* sequences among the different animal groups suggests both endemic and cosmopolitan methanogen populations with implications for microbial source tracking. This study showed that most methanogen sequences clustered as endemic populations specific to host animals. The few cosmopolitan populations from each animal clustered together and were phylogenetically similar to *Methanobrevibacter* species. This finding is not surprising since *Methanobrevibacter* is the predominant methanogen genus in the animal gastrointestinal tract. *Methanobrevibacter* species include *Methanobrevibacter ruminantium, M. millerae* and *M. ollyae* (ruminant specific); *M. arboriphilus* (plant specific); *M. cuticularis*, *M. curvatus* and *M. filiformis* (termite specific); *M. oralis* (human mouth); *M. gottschalkii* and *M. thaueri* (horse and pig); *M. woesei* (rat and goose); *M. acididurans* and *M. wolinii* (sheep). Although many species of *Methanobrevibacter* are found in more than one animal (i.e. *M. gottschalkii* in horses and pigs and *M. woesei* in different gallinaceous birds), many are believed to inhabit host-specific niches. *Methanobrevibacter ruminantium* occupies a ruminant-specific niche due to a strict growth requirement found only in ruminal fluid, which precludes growth of this organism outside of the rumen. *Methanobrevibacter smithii* occupies a niche specific to the human gastrointestinal system and is considered the predominant methanogen in this system. The similarity of the fecal clones in this study suggests that the only cosmopolitan sequences were related to *Methanobrevibacter* and are most likely separate species of *Methanobrevibacter* inhabiting specific niches in different animal systems.

It has been suggested that differences in host animal gastrointestinal systems create unique environments allowing for host-specific bacterial niches that may be useful for microbial source tracking. This study showed distinct endemic populations of methanogen sequences, suggesting the presence of uncultured methanogens inhabiting host-specific niches in chickens, horses, pigs, sewage, and ruminants. Endemic distributions were observed in pig fecal *mcrA* sequences with several swine-specific clades allowing for microbial source tracking marker development. Several horse-specific and ruminant-specific clusters were also observed representing endemic distribution. Endemic distribution was also represented in the chicken and the sewage environments, with no chicken *mcrA* sequences similar to other animals and only one sewage *mcrA* sequence shared with other animal sequences (similar to *Methanobrevibacter* species). The presence of endemic populations in these different systems suggests evidence for host animal specific methanogen niches in animals with different gastrointestinal systems.

This study showed the potential for using the methanogen-specific *mcrA* gene to identify host-specific methanogens for microbial source tracking, as well as using methanogens as swine-specific markers of fecal pollution. The P23-2 assay developed in this study shows promise as a sensitive, rapid, reliable, and specific method for identifying swine contamination in the environment, although further testing is required to determine the applicability of the assay in different geographical settings.

This study is the first to identify phylogenetic relationships between *mcrA* genes in sewage and the feces of different animals. Understanding the nature and host distribution patterns of methanogens in intestinal systems of different animals will allow for a greater appreciation of host-methanogen interactions, knowledge of uncultured methanogens in different environments, and design of microbial source tracking host-specific markers. Future studies will concentrate on the ecological implications and host distribution patterns of the methanogens in different intestinal environments.