Potential of *Enterococcus faecalis* as a Human Fecal Indicator for Microbial Source Tracking

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ABSTRACT

Regulatory agencies are interested in a fecal indicator bacterium with a host range limited to humans because human fecal contamination represents the greatest hazard to humans, yet is a relatively easy nonpoint source to remedy. Watersheds with human fecal contamination could be given first priority for cleanup. A fecal indicator bacterium with a host range limited to humans and a few other warm-blooded animal species would also simplify microbial source tracking because only a few animal species would be required for any host origin database. The literature suggests that the fecal indicator bacterium *Enterococcus faecalis* has a limited host range. On this basis, we selected this bacterium for study. Of 583 fecal streptococcal isolates obtained on Enterococcosel agar from Canada goose, cattle, deer, dog, human, chicken, and swine, 392 were considered presumptive enterococci and were subsequently speciated with the API 20 Strep system. Of these isolates, 22 were *Ent. durans* (5.6%), 61 were *Ent. faecalis* (15.6%), 98 were *Ent. faecium* (25.0%), 86 were *Ent. gallinarum* (21.9%), and 125 were unidentified (31.9%). The host range of the *Ent. faecalis* isolates was limited to dogs, humans, and chickens. Media were developed to isolate and identify *Ent. faecalis* quickly from fecal samples and this scheme eliminated *Ent. faecalis* isolates from dogs. When the remaining *Ent. faecalis* isolates were ribotyped, it was possible to differentiate clearly among the isolates from human and chicken. It may be that combining the potentially limited host range of *Ent. faecalis* with ribotyping is useful for prioritizing watersheds with fecal contamination.

Species of fecal indicator bacteria with a host range limited to humans are of the most interest to water managers, not only because human feces are commonly presumed to be the reservoir of many human pathogens (Hurst, 1997), but also because the likely sources of these bacteria (e.g., malfunctioning septic systems) are easier to remedy than other sources (e.g., wildlife defecating in the water). Watersheds with human fecal contamination could be given first priority for cleanup.

Of the fecal indicator bacteria, most regulatory agencies are interested in *Escherichia coli* and *Enterococcus faecalis* (e.g., USEPA, 2001). The host range of *E. coli* is not limited to humans and is found in the intestines of many warm-blooded animals (Ørskov, 1984). The host range of *Ent. faecalis* is contradictory. Almost all researchers agree that *Ent. faecalis* is found in humans (Geldreich and Kenner, 1969; Oragui and Mara, 1981; Pourcher et al., 1991; Ruoff, 1990; Turtura and Lorenzelli, 1994; Wheater et al., 1979), but this agreement does not extend to other warm-blooded animals. On the one hand, *Ent. faecalis* is also found in cattle (Rutkowski and Sjogren, 1987; Wheater et al., 1979), dogs (Devriese et al., 1987; Geldreich and Kenner, 1969; Wheater et al., 1979), horses (Devriese et al., 1987), chickens (Devriese et al., 1987; Geldreich and Kenner, 1969; Pourcher et al., 1991; Rutkowski and Sjogren, 1987; Turtura and Lorenzelli, 1994), rabbits (Devriese et al., 1987; Wheater et al., 1979), rodents (Geldreich and Kenner, 1969; Wheater et al., 1979), sheep (Devriese et al., 1987; Geldreich and Kenner, 1969; Rutkowski and Sjogren, 1987; Wheater et al., 1979), swine (Devriese et al., 1987; Geldreich and Kenner, 1969; Oragui and Mara, 1981; Wheater et al., 1979), and wild birds (seagull, Pourcher et al., 1991; duck and seagull, Wheater et al., 1979). On the other hand, Pourcher et al. (1991) found *Ent. faecalis* to be present in chickens and seagulls, but absent or in low numbers in cattle, horses, rabbits, sheep, and swine.

Therefore, the evidence suggests *Ent. faecalis* is found in humans, dogs, and chickens, and may or may not be limited to other warm-blooded animals. If the host range is indeed limited to humans and a few other animals, then it may be possible to differentiate among these limited number of host species using phenotypic or genotypic methods associated with *microbial source tracking* or *bacterial source tracking*. This area of research is based on the assumption that specific markers or strains of bacteria are associated with specific animal species (e.g., Amor et al., 2000). While most research on phenotypic methods has concentrated on multiple antibiotic resistance (Hagedorn et al., 1999; Harwood et al., 2000; Parveen et al., 1997; Wiggins, 1996; Wiggins et al., 1999), most research on genotypic methods has concentrated on ribotyping (e.g., Carson et al., 2001; Parveen et al., 1999), pulsed field gel electrophoresis (e.g., Kariuki et al., 1999), and various polymerase chain reaction (PCR) methods (e.g., Buchan et al., 2001; Dombek et al., 2000; Farnleitner et al., 2000). Whether or not a microbial source tracking method is phenotypically or genotypically based, many of these methods require a host origin database to identify environmental isolates. The advantage of a limited host range for *Ent. faecalis* is that only a few warm-blooded animal species would be required for the database, and this would result in a considerable savings in time and cost and would permit waters contaminated with human feces to be given priority quickly.

This study had three objectives. First, we determined if the host range of *Ent. faecalis* was limited to humans, dogs, and chickens as suggested by Pourcher et al. (1991). To test this, we isolated fecal streptococci from the feces of humans and six nonhuman animals, Canada goose (*Branta canadensis*), cattle (*Bos domestica*), deer (*Odocoileus virginianus*), dog (*Canis familiaris*), chicken

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Abbreviations: ATCC, American Type Culture Collection; BHI, Brain Heart Infusion.

(*Gallus gallus domesticus*), and swine (*Sus scrofa*), and speciated these isolates with the API 20 Strep system (bioMérieux, Hazelwood, MO). This is the system that Pourcher et al. (1991) used. Second, because the API 20 Strep system was too expensive and time consuming to use in the identification of thousands of isolates necessary to confirm the potential limited host range of *Ent. faecalis*, we developed a scheme to isolate and identify *Ent. faecalis* from fecal samples quickly. Third, where *Ent. faecalis* was found in other animals, we determined if it was possible to use one genotypic method, ribotyping, to distinguish among *Ent. faecalis* isolates from humans and these other animals.

MATERIALS AND METHODS

Selection and Identification of *Enterococcus faecalis* Isolates from Feces

A minimum of two humans or nonhuman animals was individually sampled. All individuals of each species had no association with each other (e.g., Canada goose samples were from different flocks). In the case of deer, samples were obtained from separate penned deer at the University of Georgia Whitehall Deer Research Facility (Athens, GA) and wild deer outside the facility fences. It was possible to sample the wild deer easily because they were naturally attracted to the penned deer. To reduce geographic variability, all individuals were sampled in the vicinity of Athens, GA. Sampling of humans complied with all federal and institutional guidelines on the use of human subjects. With the exception of deer, freshly deposited feces from Canada goose, cattle, dog, humans, chicken, and swine were sampled with a CultureSwab Plus containing Amies gel without charcoal (Baltimore Biological Laboratories, Baltimore, MD). All swabs were processed within 12 h. Because the deer feces were pelletized and difficult to sample with a swab, three pellets from each scat pile were aseptically retrieved with ethanol flame-sterilized forceps and were placed in a Whirl-pak bag (NASCO, Modesto, CA). The pellets were suspended in 20 mL of 0.1% sterile peptone and were mixed by hand from outside the bag until the suspension was uniform. The suspension was sampled with disposable, sterile 10-μL loops.

All swabs and loops were streaked onto triplicate, 5-cm plates of Enterococcosel agar (Difco Laboratories, Sparks, MD) and incubated in Ziploc bags (Dow Brands, Indianapolis, IN) at 37°C. After 48 h, 10 randomly selected black (indicating esculin hydrolysis) isolates from each of the three Enterococcosel plates were streaked onto KF (Kennel Faecal) Streptococcus agar medium (Difco) and incubated at 37°C for 48 h. Therefore, a maximum of 30 isolates was obtained from each individual. Colonies with a diameter of >1 mm (probably other bacterial species) were not sampled. One randomly selected dark red to black colony (indicating transformation of triphenyltetrazolium chloride to an acid azo dye) from each plate was streaked onto Brain Heart Infusion (BHI) agar medium (Difco) amended with 6.5% NaCl and incubated at 37°C for 48 h. This percentage of NaCl ensured that *S. bovis* and *S. equinus* were eliminated (Mundt, 1986).

Each isolate was examined microscopically under phase contrast at 750× for coccal cell morphology. To ensure each isolate was catalase negative, a catalase test with 8.82 *M* H_2O_2 was performed (MacFaddin, 1976) with *E. coli* American Type Culture Collection (ATCC, Manassas, VA) no. 11775 (catalase positive) as the control. Isolates with a coccal cell morphology that were catalase negative were restreaked for purity onto BHI agar with 6.5% NaCl and incubated at 37°C for 48 h. A loopful of cells was removed from each plate and inoculated into the API 20 Strep system according to the manufacturer's specifications. When speciated, all *Ent. faecalis* isolates had to have a species identification of ≥90% (minimum of good identification). All other identified isolates had to have species identification of ≥80% (minimum of acceptable identification).

Identification of Isolates Based on Selected Defined Biochemical Characteristics

Fecal isolates that were catalase-negative, esculin-positive cocci and grew on BHI agar with 6.5% NaCl were considered as presumptive *Enterococcus* spp. Because *Ent. faecalis* was the species of primary interest, biochemical tests as described in *Standard Methods for the Examination of Water and Wastewater* (Clesceri et al., 1998) and *Bergey's Manual of Systematic Bacteriology* (Mundt, 1986) were used to differentiate this species from the other enterococcal species. Five biochemical characteristics were selected: arginine hydrolysis, and carbon utilization of pyruvate, arabinose, L-sorbose, and raffinose (Table 1). The media for arginine hydrolysis and utilization of pyruvate, arabinose, L-sorbose, and raffinose were as described by Gross et al. (1975). When media by Gross et al. (1975) were tested against ATCC type strains *Ent. faecalis* no. 19433, *Ent. faecium* no. 19434, and *Ent. gallinarum* no. 49573, false positives were observed (data not shown). For this reason, new basal media for carbon source utilization and arginine hydrolysis were developed. In the case of carbon source utilization, the original medium contained (g L^{-1}): carbon source (10.0), tryptone (10.0), yeast extract (5.0), NaCl (5.0), bromothymol blue (0.04), and agar (2.5). In the case of arginine hydrolysis, the medium contained (g L^{-1}): L-arginine monohydrochloride (5.0), Bacto peptone (5.0), yeast extract (3.0), glucose (1.0), brom cresol purple (0.02), and agar (2.5). The carbon utilization medium was changed to (g L^{-1}): carbon source (10.0), NaCl (5.0), NH_4NO_3 (1.0), KH_2PO_4 (1.0), $MgSO_4 \cdot H_2O$ (0.2), bromothymol blue (0.04), and agar (2.5).

Table 1. Selected biochemical and physical characteristics of *Enterococcus* spp. as defined in *Standard Methods for the Examination of Water and Wastewater*, 20th ed. (Clesceri et al., 1998) and *Bergey's Manual of Systematic Bacteriology* (Mundt, 1986).

Characteristic	*Ent. faecalis*	*Ent. faecium*	*Ent. avium*	*Ent. gallinarum*
Esculin hydrolysis†	+	+	+	+
Growth in medium with 6.5% NaCl	+	+	+	+
Coccal cell morphology	+	+	+	+
Presence of catalase	−	−	−	−
Arginine hydrolysis	+	+	−‡	−
Pyruvate utilization	+	−	−‡	−
Arabinose utilization	−	+	+	−
L-sorbose utilization	−	−	+	−
Raffinose utilization	−	−	−	+

† As determined on Enterococcosel agar (Baltimore Biological Laboratories, Baltimore, MD).
‡ Reactions are variable.

Varying amounts of yeast extract (0.1, 0.2, 0.5, 1.0, and 5.0 g L^{-1}) were tested. Therefore, the original carbon utilization medium was changed by reducing or eliminating carbon sources other than the carbon source being tested. The arginine hydrolysis medium was changed to (g L^{-1}): L-arginine monohydrochloride (5.0), NH_4NO_3 (1.0), KH_2PO_4 (1.0), $MgSO_4{\cdot}H_2O$ (0.2), glucose (1.0), yeast extract (0.2 g), bromcresol purple (0.02), and agar (2.5). Similar to the carbon utilization medium, extraneous carbon sources besides arginine and glucose were reduced or eliminated. The pH values were adjusted to 7.4 for carbon utilization and 6.8 for arginine hydrolysis media. The revised media, without the agar and indicator dye, were sterilized by passage through a 0.22-μm membrane filter (Corning, Corning, NY). The agar and indicator dye were made at a 10× concentration and autoclaved separately. The agar–dye mixture was combined with the filter-sterilized medium and dispensed.

The entire identification scheme was adapted for use in sterile 96-well microtiter plates (Falcon, Bedford, MA). To test this scheme, a random sample of 74 of the 125 API 20 Strep "unidentified" isolates, as well as all 61 *Ent. faecalis* isolates, were selected. A single colony of each isolate was removed from a BHI agar plate with a sterile plastic stab, and the colony was resuspended in 150 μL of saline–phosphate buffer (NaCl, 8.5 g L^{-1}, K_2HPO_4, 0.65 g L^{-1}; KH_2PO_4, 0.35 g L^{-1}; pH 7.0) contained in one assigned well of the microtiter plate. Additional wells were reserved for *Ent. avium* ATCC no. 14025, *Ent. faecalis* ATCC no. 19433, *Ent. faecium* ATCC no. 19434, *Ent. gallinarum* ATCC no. 49573, and an uninoculated control. Separate microtiter plates were created of BHI broth (Difco) with 6.5% NaCl, arginine hydrolysis medium with and without arginine, and pyruvate, arabinose, raffinose, and L-sorbose carbon utilization media, for a total of seven microtiter plates. Each well contained 150 μL of the appropriate medium. Each well was inoculated by transferring approximately 10 μL of inoculum with a sterile 96-place replicator (Sigma Chemical Co., St. Louis, MO), and, with the exception of BHI agar with 6.5% NaCl, each well was covered with 75 μL of sterile mineral oil. The remaining portion of unused saline–phosphate suspension was used to observe cell morphology and to determine catalase production. Microtiter plates were incubated for 18 h at 37°C, with the exception of arginine hydrolysis medium, which was incubated for 48 h. Microtiter plates were observed from underneath for the presence of a ring of growth. Wells containing arginine medium without arginine were observed for the presence of a yellow color (acid production from glucose utilization); if positive, then the corresponding wells with arginine were observed for the presence of a purple color (arginine hydrolysis positive). This duplicate system was necessary because the uninoculated medium is purple. Wells containing pyruvate, arabinose, L-sorbose, and raffinose were scored as positive if the entire well appeared greenish or yellow. Isolates that were catalase-negative cocci that grew in BHI broth with 6.5% NaCl, were arginine hydrolysis positive, and utilized pyruvate but not the other carbon sources were considered *Ent. faecalis*.

DNA Extraction and Quantification

DNA was extracted from 31 *Ent. faecalis* isolates obtained from humans and chickens. A single colony of each isolate was inoculated into 10 mL of Luria–Bertani broth (pH 7.5; Sambrook et al., 1989) contained in a 16- by 150-mm test tube and secured flat on a rotating shaker at 75 rpm at 37°C. After 18 h, a 2.0-mL sample was removed and the DNA extracted with a commercial kit (DNeasy; Qiagen, Valencia, CA). The manufacturer's protocol was modified by adding lysozyme (40 mg L^{-1}; Sigma) to the lysis buffer, lowering the lysis temperature from 70 to 55°C, lengthening the lysis time from 30 to 90 min, and decreasing the elution buffer from 200 to 100 μL (to concentrate the DNA). A portion of the DNA was mixed with Hoechst Dye no. 33258 (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's directions, and the genomic DNA was quantified with a fluorometer (DynaQuant DQ200, Amersham Pharmacia Biotech). DNA from *E. coli* strain B (Sigma) was used as the standard.

Ribotyping of *Enterococcus faecalis* Isolates

Ribotyping was done as described by Parveen et al. (1999) with some modifications. Briefly, two 2-μg samples of DNA from each isolate and the *Ent. faecalis* ATCC no. 19433 control were separately digested overnight, one with the restriction enzyme *Eco*RI and the other with the restriction enzyme *Pvu*II (both from Roche Molecular Biochemicals, Indianapolis, IN). The digested DNA was stained and loaded into a 1% agarose (high strength analytical grade) gel. The gel was electrophoresed at 58 volts for 3 h with a horizontal gel system. Digoxigenin-labeled (DIG-labeled) Marker III (Roche) was the molecular weight marker and occupied every fifth lane of the gel. Control lanes contained no DNA and DNA from *Ent. faecalis* ATCC no. 19433. DNA was transferred by Southern blotting to a nylon membrane with a vacuum blotting system (VacuGene XL, Amersham Pharmacia Biotech) and the DNA on the membrane was cross-linked with UV light. Following a 2-h prehybridization at 42°C, the membrane was hybridized at the same temperature for 20 h to DIG-labeled cDNA from *E. coli* total ribosomal RNA. Membranes were prepared for chemiluminescence by a series of washing steps before a chemiluminescent substrate for alkaline phosphatase was added. Membranes were placed in a FluorChem 8000 imager (Alpha Innotech, San Leandro, CA) and images saved as TIFF files. The TIFF files were imported into GelCompar II (Applied Maths, Kortrijk, Belgium) for analysis. DNA fragments < 1375 base pairs were ignored because they were often indistinct. Of the 31 *Ent. faecalis* isolates, 28 produced clear banding patterns; the DNA of the remaining three isolates was consistently degraded and these isolates were discarded. Lanes were normalized within the gel with molecular weight marker and variations among the gels were assessed with the *Ent. faecalis* ATCC no. 19433 strain. Optimization (maximum percentage shift allowed between two different patterns for the patterns to still be considered a match) and tolerance (maximum percentage shift allowed between two bands on different patterns for the bands to still be considered a match) were each set at 1.00%. The normalized banding patterns for both enzymes were stacked with *Eco*RI on the top and *Pvu*II on the bottom to create one combined ribotype pattern for each isolate. Similarity indices were determined with Dice's coincidence index (Dice, 1945) and the distance among clusters calculated with the unweighted pair-group method using arithmetic averages (UPGMA; Lyon, 1999). The banding pattern of the control the *Ent. faecalis* ATCC no. 19433 strain varied from gel to gel and a similarity index of 90.0% was required for all the banding patterns to be considered the same ribotype. Based on variability of the intergel *Ent. faecalis* control, banding patterns of all other isolates had to have a similarity index of $\geq$90.0% to be considered the same ribotype.

RESULTS

A total of 583 isolates was obtained on Enterococcosel agar from humans and six other warm-blooded animal species: Canada goose, cow, deer (both penned and wild), dog, chicken, and swine (Table 2). Fecal samples from two of the four humans did not have any isolates

Table 2. Number of isolates obtained on Enterococcosel agar (Baltimore Biological Laboratories, Baltimore, MD) from the feces of seven warm-blooded animal species. Isolates were subsequently streaked on KF Streptococcus agar and Brain Heart Infusion (BHI) agar (both from Difco Laboratories, Sparks, MD), the latter amended with NaCl (65 total g L^{-1}). Each isolate was observed microscopically under phase contrast at 750× for coccal cell morphology and absence of catalase production.

		Number of isolates				
Source	Number	Enterococcosel	KF	BHI agar + NaCl	Morphology	$Catalase^{-}$
Canada goose	1	29	29	2	2	2
	2	14	14	2	2	2
	3	13	13	2	2	2
Cattle	1	30	30	29	29	28
	2	30	30	17	17	13
	3	30	30	30	30	28
Deer (penned)	1	30	30	30	30	19
	2	30	30	24	24	21
	3	30	30	27	27	11
Deer (wild)	1	30	30	29	29	29
	2	30	30	29	29	27
	3	30	30	27	27	24
Dog	1	30	30	30	30	30
	2	30	30	28	28	26
Human	1	20	20	20	20	20
	2	27	27	27	27	27
Chicken	1	30	30	13	13	12
	2	30	30	18	18	14
	3	30	30	12	12	9
Swine	1	30	30	27	27	25
	2	30	30	28	28	23
Total	NA	583	583	451	451	392

on Enterococcosel agar. Restreaking isolates from Enterococcosel agar onto KF Streptococcus agar did not result in the loss of any of the isolates, but subsequently restreaking from KF Streptococcus agar to BHI agar with 6.5% NaCl, a test to segregate enterococci from streptococci, decreased the 583 isolates to 451 isolates, depending on the animal species. All human isolates and most isolates from cattle (84.4%), deer (92.2%), dog (96.7%), and swine (91.7%) grew on BHI agar with 6.5% NaCl. In contrast, only 6 of 66 (9.1%) of the isolates from Canada goose and 43 of 90 (47.8%) from chicken grew on BHI agar with 6.5% NaCl. Thus, all isolates from humans, most isolates from cattle, deer, dog, and swine, and a few isolates from Canada goose and chicken fit the general description for enterococci. All the isolates that grew on BHI agar with 6.5% NaCl were cocci when observed under phase contrast microscopy. Of the 451 remaining isolates, 392 (86.9%) were catalase negative, an additional test to identify enterococcal species. Therefore, the percentage of false positives on BHI agar with 6.5% NaCl was 13.1%.

Each of the 392 isolates from Table 2 was confirmed with the API 20 Strep system (Table 3). Of the 392 isolates, 22 were *Ent. durans* (5.6%), 61 were *Ent. fae-*

Table 3. Speciation of enterococcal isolates from the feces of cattle, penned and wild deer, dog, human, chicken, and swine as determined with the API 20 Strep system (bioMérieux, Hazelwood, MO). All *Enterococcus faecalis* isolates had a species identification of ≥90% (minimum of good identification), whereas all other identified isolates all had a species identification of ≥80% (minimum of acceptable identification).

			Enterococcus					
Source	Number	Number of isolates	*avium*	*durans*	*faecalis*	*faecium*	*gallinarum*	Unidentified
Canada goose	1	2	0	2	0	0	0	0
	2	2	0	0	0	2	0	0
	3	2	0	0	0	2	0	0
Cattle	1	28	0	1	0	5	17	5
	2	13	0	0	0	10	3	0
	3	28	0	0	0	11	10	7
Deer (penned)	1	19	0	4	0	2	1	12
	2	21	0	2	0	0	5	14
	3	11	0	0	0	1	7	3
Deer (wild)	1	29	0	2	0	9	14	4
	2	27	0	3	0	7	15	2
	3	24	0	0	0	8	7	9
Dog	1	30	0	0	30	0	0	0
	2	26	0	0	0	22	2	2
Human	1	20	0	0	19	0	0	1
	2	27	0	0	0	17	2	8
Chicken	1	12	0	0	10	0	0	2
	2	14	0	7	2	2	3	0
	3	9	0	1	0	0	0	8
Swine	1	25	0	0	0	0	0	25†
	2	23	0	0	0	0	0	23†
Total	NA	392	0	22	61	98	86	125

† Of the 48 isolates from swine, 28 (58%) were identified as *Aerococcus viridans*.

calis (15.6%), 98 were *Ent. faecium* (25.0%), 86 were *Ent. gallinarum* (21.9%), and 125 were unidentified (31.9%). *Enterococcus avium* was not found in any human or nonhuman animal. *Enterococcus durans* was found in Canada goose, cattle, deer, and chicken, but not in dog, human, or swine. Most importantly, *Ent. faecalis* was only found in dog, humans, and chickens. In contrast, with the exception of Canada goose for *Ent. gallinarum* and swine for both *Ent. faecium* and *Ent. gallinarum*, *Ent. faecium* and *Ent. gallinarum* were found in every human and nonhuman animal. In the case of swine, 28 of 48 isolates (58.3%) were identified as *Aerococcus viridans* and the remaining 20 isolates were unidentified. Therefore, deer and cattle accounted for 79 of 86 (91.9%) of the *Ent. gallinarum* isolates,

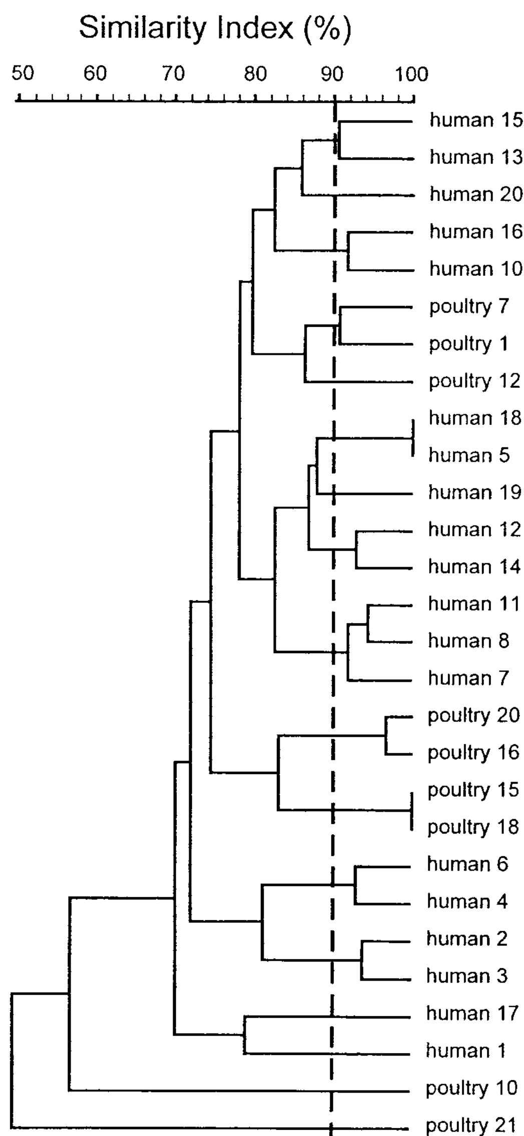

Fig. 1. Dendrogram of the ribotype patterns of 28 *Enterococcus faecalis* isolates from human and chicken in Athens, GA. The dendrogram was derived from the combined ribotype pattern of each isolate (*Eco*RI on the top and *Pvu*II on the bottom). The similarity index is given on the top scale. Based on the variability of the intergel control, *Ent. faecalis* American Type Culture Collection (ATCC) no. 19433, the cutoff was 90% (dashed vertical line).

whereas dogs, humans, and chickens accounted for 61 of 61 (100%) of the *Ent. faecalis* isolates. The highest number of isolates were unidentified (125 of 392 isolates; 31.9%); the most prevalent *Enterococcus* species was *Ent. faecium*, with 98 of 392 isolates (25.0%).

The best basal medium for carbon utilization contained (g L^{-1}): NH_4NO_3 (1.0), KH_2PO_4 (1.0), $MgSO_4 \cdot H_2O$ (0.2), yeast extract (0.2), NaCl (5.0), carbon source (10.0), bromothymol blue (0.04), and agar (2.5). This same medium, amended with glucose (1.0 g L^{-1}) and arginine (5.0 g L^{-1}) as carbon sources, was also satisfactory for arginine hydrolysis. Among the four ATCC type strains, false positives consistently appeared in the carbon utilization tests whenever the yeast extract was $\geq$0.5 g L^{-1}; when the yeast extract was lowered to 0.1 or 0.2 g L^{-1}, all ATCC strains were true to type.

When the 61 *Ent. faecalis* isolates identified by the API 20 Strep system were inoculated into our arginine hydrolysis medium and the new basal media containing pyruvate, arabinose, L-sorbose, and raffinose, all 31 isolates from humans and chickens were confirmed as *Ent. faecalis* (arginine hydrolysis positive, arabinose negative, pyruvate positive, and raffinose negative). However, the 30 remaining isolates from dog were identified as arginine hydrolysis negative by our tests. Because of this discrepancy, the 30 *Ent. faecalis* isolates from dog were not ribotyped. Of the 74 isolates selected from the 125 isolates that the API 20 Strep system listed as "unidentified," 72 (97.3%) were confirmed as unidentified (i.e., not corresponding to profiles for *Ent. avium*, *Ent. faecalis*, *Ent. faecium*, or *Ent. gallinarum*). The two remaining isolates were both identified as *Ent. faecalis* by our identification scheme.

Gels typically showed 9 to 11 bands for *Eco*RI and 11 to 13 bands for *Pvu*II; when the banding patterns were combined (with *Eco*RI on the top and *Pvu*II on the bottom), this was considered sufficient for good discrimination among ribotypes. When the gels were analyzed at the 90% similarity index, the 28 *Ent. faecalis* isolates, 19 from humans and 9 from chickens, formed 17 ribotypes (Fig. 1). The isolates separated into 11 ribotypes for humans and 6 for chickens. None of the ribotypes was shared between humans and chickens. Therefore, ribotyping was able to differentiate clearly among the isolates from human and chicken.

DISCUSSION

Enterococcus faecalis had a limited host range, and was found in humans, dogs, and chickens, but not in Canada goose, cattle, deer (wild and penned), or swine. This is the first report of the absence of *Ent. faecalis* in deer. Our results (392 isolates) agree closely with those of Pourcher et al. (1991; 308 isolates), who found *Ent. faecalis* to be present in humans and chickens, and absent or in low numbers in cows, horses, pigs, rabbits, and sheep. They did not test dog fecal samples. Other researchers (Devriese et al., 1987; Geldreich and Kenner, 1969; Oragui and Mara, 1981; Rutkowski and Sjogren, 1987; Turtura and Lorenzelli, 1994; Wheater et al., 1979) did not observe this limited host range for

Ent. faecalis. The discrepancy is probably caused by different isolation and identification media, as there is no putative single isolation medium for enterococci, including *Ent. faecalis*, and identification schemes vary considerably. Pourcher et al. (1991) identified their *Ent. faecalis* isolates with the API 20 Strep system, whereas other researchers typically used complex media. Our carbon utilization studies with the basal medium of Gross et al. (1975) show a large number of false positives caused by the excessive amounts of yeast extract. When the yeast extract level was lowered to 0.2 g L^{-1}, these false positives were eliminated. These results are consistent with those of Wagner et al. (1995), who observed that carbon utilization patterns for *Bradyrhizobium* spp. were dependent on the amount of yeast extract in the medium. When the level of yeast extract was reduced, false positives were eliminated. Our best basal medium contained 0.2 g yeast extract L^{-1}, a 25-fold reduction over the 5.0 g of yeast extract L^{-1} recommended by Gross et al. (1975).

A total of 392 fecal isolates that were identified as esculin-positive, catalase-negative, NaCl-tolerant cocci were subsequently speciated by the API 20 Strep system. Because the API 20 Strep system is time consuming and expensive to use, we developed an alternative identification scheme to identify *Ent. faecalis* isolates quickly. This required the development of a new basal medium. When the 61 *Ent. faecalis* and 74 "unidentified" isolates (as identified by the API 20 Strep system) were tested against our identification scheme, 30 of 61 *Ent. faecalis* isolates and 72 of 74 "unidentified" isolates were correctly identified. However, all 30 dog isolates that were identified as *Ent. faecalis* by the API 20 Strep were labeled as "unidentified" by our identification scheme. Under our scheme, all the dog isolates were arginine hydrolysis negative, whereas the API 20 Strep system recorded all these isolates as arginine hydrolysis positive. *Enterococcus faecalis* is identified in *Standard Methods for the Examination of Water and Wastewater* (Clesceri et al., 1998) as arginine hydrolysis positive. Because the API 20 Strep medium for arginine hydrolysis is proprietary, it is not possible to compare the two media. Therefore, the reason for this difference is unclear. Nevertheless, this was a fortuitous result, because it eliminated the dog isolates from ribotyping. In addition, two isolates listed as "unidentified" by the API 20 Strep system were identified as *Ent. faecalis* by our new scheme. This difference was caused by the pyruvate utilization test. Both isolates were pyruvate positive in our tests, but this is not a carbon source tested in the API 20 Strep system. Currently, we are identifying *Ent. faecalis* isolates by removing isolates directly from Enterococcosel plates, suspending each isolate in 150 μL of saline–phosphate buffer, and inoculating each isolate with a replicator into separate microtiter plates containing BHI broth (Difco) with 6.5% NaCl, arginine hydrolysis medium with and without arginine, or pyruvate, arabinose, raffinose, and L-sorbose carbon utilization media. This system appears to work well (Hartel, unpublished data, 2001).

It may be that combining the potentially limited host range of *Ent. faecalis* with a genotypic method, ribotyping, is useful for microbial source tracking of fecal contamination because it reduces the amount of host origin sampling required. A potentially limited host range also means that obtaining isolates from hard-to-sample wild animals is unnecessary. At a 90% similarity index, there was a good separation observed between chicken and human ribotypes. Quednau et al. (1999) observed similar results when they analyzed chromosomal DNA and were able to separate *Ent. faecium* isolates among humans, chickens, and swine. Although ribotyping is highly reproducible (Farber, 1996), it is also time consuming and costly. By limiting the number of enterococcal isolates to be tested, this reduces both the time and cost to identify potential human fecal contamination. Such screening would be useful to water managers.

If fecal contamination occurs in an urban environment where the presence of chickens is minimal, then it may be possible to use the limited host range of *Ent. faecalis* isolates to indicate human fecal contamination. Under these circumstances, ribotyping may be unnecessary. Rutkowski and Sjogren (1987) developed a single medium to identify specific ecological or physiological groups of streptococci and used the percentages of these groups to differentiate between human and nonhuman sources of fecal contamination. Although we did not attempt to link other enterococcal species or ratios of various enterococcal species to determine sources of fecal contamination, this area deserves more attention. These ratios may also include species not examined closely here (e.g., *Aerococcus viridans* in swine). This approach is consistent with the "toolbox" approach advocated by some researchers (C. Hagedorn, personal communication, 2001; see http://www.bsi.vt.edu/biol_4684/BST/BSTmeth.html [verified 28 Mar. 2002]) for doing microbial source tracking. Under these circumstances, ribotyping would simply serve to confirm phenotypic tests.

There were three noteworthy observations in the host ranges of the speciated enterococci. First, *Enterococcus avium* was not observed among the 392 speciated isolates. This is probably because this species does not grow or grows slowly in medium containing 6.5% NaCl (Mundt, 1986). Second, 91.9% of the *Ent. gallinarum* isolates were found in deer and cattle. This enterococcal species is normally found in the intestines of domestic fowl (Mundt, 1986). This difference is probably because of cattle and deer grazing in pastures with land-spread broiler litter. Georgia is ranked number one in the USA for broiler litter production (Georgia Agricultural Statistics Service, 1999), and broiler litter is commonly land-spread in Georgia as part of good agronomic practice. Third, two human fecal samples did not contain enterococci. This absence may be because what constitutes normal colonic bacteria may vary when individuals are evaluated (Willard et al., 2000). Nevertheless, Pourcher et al. (1991) were able to isolate *Ent. faecalis* from humans and human sewage.

It is still possible that *Ent. faecalis* does not have a limited host range because, even when our results are combined with those of Pourcher et al. (1991), the total

number of animal species tested, as well as the number of individuals tested within a species, is small. More animal species need to be tested more extensively to confirm the limited host range for *Ent. faecalis* observed here. In addition, the effect of temporal and geographic variability also needs to be considered. Devriese et al. (1987) noted that numbers of *Ent. faecalis* increased with age in the intestines of chicken. Similarly, Rivas et al. (1997) established that *Streptococcus agalactiae* ribotypes in several New York dairy herds were geographically dependent.

CONCLUSIONS

If a primary interest of water managers is to identify human fecal contamination, then one way to do this may be to focus on a fecal indicator bacterium with a host range limited to humans. This would also reduce the number of isolates needed for a host origin database that some microbial source tracking methods require. We suggest that *Ent. faecalis* is a good candidate bacterium because it appears to have a host range limited to humans and a few other animals. Determining this potentially limited host range was difficult because published media for speciating *Ent. faecalis* gave too many false positives. Therefore, two new media were developed to eliminate this problem. Combining the potentially limited host range of *Ent. faecalis* with ribotyping for microbial source tracking was advantageous because ribotyping was able to clearly differentiate among *Ent. faecalis* isolates from human and chicken. It may be that other bacteria with a limited host range are also useful for tracking other host animal species. We are currently field-testing our method with environmental samples.

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