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Quantitative PCR Method for Sensitive Detection of Ruminant Fecal Pollution in Freshwater and Evaluation of This Method in Alpine Karstic Regions

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A quantitative TaqMan minor-groove binder real-time PCR assay was developed for the sensitive detection of a ruminant-specific genetic marker in fecal members of the phylum *Bacteroidetes*. The qualitative and quantitative detection limits determined were 6 and 20 marker copies per PCR, respectively. Tested ruminant feces contained an average of 4.1×10^9 marker equivalents per g, allowing the detection of 1.7 ng of feces per filter in fecal suspensions. The marker was detected in water samples from a karstic catchment area at levels matching a gradient from negligible to considerable ruminant fecal influence (from not detectable to 10^5 marker equivalents per liter).

Groundwater resources from alpine and mountainous karst aquifers play an important role in public water supplies throughout the world (15). Occasionally, karst springs show vulnerability to fecal contamination from point sources like leaking septic systems and nonpoint sources like wildlife and grazing livestock (29). The identification and apportioning of the sources of fecal contamination could make management and mitigation of this problem much easier and more cost-effective (28). Microbial source tracking (MST) serves this purpose and makes it possible to pinpoint pollution sources. Anaerobic bacteria of the phylum *Bacteroidetes* are highly abundant in feces (32), have been proposed as fecal indicator organisms (2, 3, 13), and exhibit host adaptation on the genetic level (10, 16). More recently, these bacteria became the target of MST efforts (20, 21). Bernhard and Field (4) identified source-specific genetic markers and developed methods for their qualitative detection, which were subsequently applied in practice (5, 6, 8) and improved (27). The aim of this study was to establish a method for the sensitive quantification of ruminant fecal pollution in spring water and groundwater from alpine karstic regions important for public water supplies.

Sampling and DNA extraction of feces and water. More than 300 fecal samples were collected in a large alpine catchment area in the Northern Calcareous Alps (area, 100 km^2; latitude, 47°35′N to 47°43′N; longitude, 15° to 15°20′E; for a description of the study area, see reference 12) and to some extent from the larger area of eastern Austria. For pooled fecal samples, 10 single samples were combined and homogenized. Samples were collected in sterile fecal sampling tubes and stored at −20°C. DNA was extracted from 100 mg of each fecal sample using the Ultra Clean Soil DNA kit (MoBio Laboratories, Carlsbad, CA) in combination with bead beating (FastPrep FP120, Bio-101, Vista, CA; speed setting, 6 for 30 seconds). DNA was stored at −20°C. Water samples were collected in clean and autoclaved Nalgene (Nalge Europe, Hereford, United Kingdom) sampling bottles, stored in dark cooling boxes at 4°C during transport, and processed within 6 h after collection. A given volume of spring water was filtered through Isopore 0.2-μm polycarbonate membrane filters (Millipore, Bedford, MA). Three independent filtrations were done for each sample. Immediately after filtration, the filters were frozen and stored at −80°C until nucleic acid extraction. Nucleic acid extraction was performed as described by Griffiths et al. (17); samples were dissolved in 50 μl of sterile distilled water and stored at −80°C. All analyzed DNA extracts of fecal and aquatic origin contained amplifiable bacterial DNA as checked by applying a universal bacterial PCR assay (34).

Assay development. The following published 16S rRNA gene sequences were aligned with the Vector NTI software (InforMax, Oxford, United Kingdom): AF233400, AF233402, AF233403, and AF233404 (4) and AF294903, AF294904, AF294905, AF294906, AF294908, and AF294909 (5). Primers BacR_f and BacR_r (Table 1) were designed from the derived consensus sequence using Primer Express software (Applied Biosystems, Foster City, CA). The designed primers were used to amplify a 118-bp fragment from 10^{-2} dilutions of DNA extracts from ruminant fecal samples to obtain additional sequence information for quantitative PCR (qPCR) probe design. PCR was performed on an iCycler (Bio-Rad, Hercules, CA) using the following program: 95°C for 3 min; 30 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 45 s; and 72°C for 3 min. Reaction mixtures (25 μl) contained 2.5 μl of sample DNA dilution, 200 nM BacR_f, 200 nM BacR_r, 10 μg bovine serum albumin (Boehringer Mannheim, Mannheim, Germany), and 12.5 μl of iQ Supermix (Bio-Rad). All PCR products were checked by agarose gel electrophoresis for correct size. PCR was performed from DNAs of two single and one pooled fecal sample each for cattle, deer, and chamois from representative locations in the study area. The PCR amplicons

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TABLE 1. Primers and probe for the BacR qPCR assay developed in this study

Name	Function	Sequence (5′–3′)	Length (bp)	Annealing temp (°C)
BacR_f	Forward primer	GCGTATCCAACCTTCCCG	18	58
BacR_r	Reverse primer	CATCCCCATCCGTTACCG	18	58
BacR_p	TaqMan MGB probe	FAM-CTTCCGAAAGGGAGATT-NFQ-MGB[a]	17	68

[a] FAM, 6-carboxyfluorescein; NFQ, nonfluorescent quencher; MGB, minor groove binder.

were then cloned into a pGEM-T vector (Promega, Madison, WI) and transformed into *Escherichia coli* JM 109. After purification of the plasmid DNA with the Quantum Prep Plasmid Miniprep Kit (Bio-Rad), the cloned inserts were sequenced by MWG-Biotech (Ebersberg, Germany). The sequences retrieved by cloning showed very high similarity, even between different ruminant sources (sequence identities above 74%). The TaqMan minor-groove binder probe BacR_p (Applied Biosystems) (Table 1) was designed using Primer Express. TaqMan minor-groove binder probes offer the additional advantage of being shorter and more specific than regular 5′ nuclease probes (1, 22).

qPCR was monitored on an iCycler iQ Real-Time Detection system. The optimized reaction mixture composition was 2.5 μl of sample DNA dilution, 100 nM BacR_f, 500 nM BacR_r, 100 nM BacR_p, 10 μg bovine serum albumin (Boehringer Mannheim), 12.5 μl of iQ Supermix (Bio-Rad), 2 mM additional $MgCl_2$ (Bio-Rad), and water to a final volume of 25 μl. The PCR program was as follows: 95°C for 3 min and 50 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 45 s. All reactions were performed in triplicate and in at least two 10-fold dilution steps. One of the specific clones (GenBank accession number DQ364808) was used for the generation of a plasmid standard-dilution series for qPCR detection. The concentration of the plasmid standard solution was measured photometrically. The standard was 10-fold serially diluted in a 5-ng/μl poly(dI-dC) solution as nonspecific DNA background (Roche Diagnostics, Mannheim, Germany). A total of six 10-fold serial dilutions of plasmid standard (5 to 5×10^5 gene copies) were run in triplicate on every well plate, as well as a no-template control and a no-amplification control (containing standard and 0.01% sodium dodecyl sulfate).

Evaluation setup. For the determination of the limit of detection in applied use, raw water from an alpine karst spring was filtered through a 0.22-μm Steritop membrane filter (Millipore). Three pooled fecal samples each (each consisting of 10 single samples) from cattle, deer, and chamois were analyzed in this experiment. One gram of wet feces from each sample was suspended in 50 ml of filtered spring water, and 100-fold dilution steps were prepared down to a concentration of 2×10^{-10} g wet feces per ml using the same water. One milliliter from each dilution step was filtered through 0.2-μm polycarbonate membrane filters (Millipore), the DNA was extracted, and qPCR was performed. Additionally, 100 and 500 ml of the spring water used for the suspensions were filtered and analyzed as negative controls.

To assess the occurrence of marker in the study area, samples were taken from the following water sources: spring KPAS (a well-protected spring site), spring LKAS2 (a relatively vulnerable karstic spring site; mean water residence time, 0.8 to 1.5 years; quick discharge response after precipitation; described in detail in reference 12), a river (a small river, influenced by several villages), a watering pond (situated in a fenced game-feeding compound), and a watering brook (running through a remote game-feeding compound). Sampling dates were as follows: KPAS, November 2005; LKAS2, June 2005; LKAS2 flood, August 2005 during a flood event; and river, watering pond, and watering brook, December 2005.

Characteristics of the BacR qPCR assay. Absolute quantification of the copy number of the ruminant-specific marker was achieved by analyzing a plasmid standard-dilution series with a known copy number in every measurement run. The marker copy number of any unknown sample could be determined from the respective standard regression curves (PCR efficiencies, >98% for all runs; $R^2 > 0.99$). The qualitative detection limit was in the range of a few copies of the marker per reaction volume. Marker quantification was possible in the range from 20 to 10^7 marker copies per reaction volume. The presence of PCR inhibitors was assessed by analyzing DNA from water and fecal samples in 10-fold dilution steps. No inhibitory effects could be observed in any water samples, allowing the use of undiluted DNA extracts. For fecal samples, we tested 10 representative fecal-DNA extracts in four serial 10-fold dilutions. Inhibition was apparent in the undiluted DNA extract. In order to rule out any inhibition, we used 100-fold-diluted fecal-DNA extracts in the test of specificity.

Source specificity. The specificity of the assay was tested on DNA extracted from fecal samples from ruminant and nonruminant animals, as well as humans (Table 2). Among the group of ruminants, single samples were tested to measure the frequency of presence of the marker in ruminant fecal samples. For all other groups, the absence of the marker was demonstrated by analyzing pooled samples derived from 10 single samples whenever available. The prevalence of the marker among ruminants was 100%. Amplification was absent in all nonruminant sources ($n = 146$; 101 samples from nonruminant animals, 30 samples from human individuals, 13 samples from cesspits of alpine huts, and 2 samples of municipal wastewater from a small village in the study area) (Table 2).

Limit of detection in applied use. It was not possible to relate the copy numbers of our marker directly to a cell count of *Bacteroidetes* cells carrying this marker because the strain has not been isolated yet. We chose to express the results as marker equivalents detected in the PCR assay and assessed the number of those equivalents present in pooled ruminant feces suspended in water as the environmental matrix of interest. After filtration and DNA extraction, the marker equivalent concentration was determined, signifying the actual copy number of marker still detectable after the losses incurred in the preceding steps. Pooled samples were chosen in this experiment to reflect the average marker concentrations present in ruminant feces. Detection was possible in all nine pooled sam-

TABLE 2. Specificity of the BacR qPCR assay tested on ruminant and nonruminant animal fecal samples and human pooled fecal samples from cesspits and wastewater

Source	Scientific name	Sample type[a]	No. of samples[b]	No. BacR positive	No. BacR negative
Cattle	*Bos taurus*	Single	14	14	0
Deer	*Cervus elaphus*	Single	12	12	0
Chamois	*Rupicapra rupicapra*	Single	12	12	0
Roe deer	*Capreolus capreolus*	Single	6	6	0
Sheep	*Ovis aries*	Single	6	6	0
Goat	*Capra hircus*	Single	5	5	0
Capricorn	*Capra ibex*	Single	2	2	0
Cesspits and wastewater	*Homo sapiens*	Wastewater	15	0	15
Human	*Homo sapiens*	Pooled	3 (30)	0	3
Horse	*Equus caballus*	Pooled	3 (30)	0	3
Pig	*Sus scrofa domestica*	Pooled	3 (30)	0	3
Fox	*Vulpes vulpes*	Single	2	0	2
Cat	*Felis catus*	Single	3	0	3
Dog	*Canis familiaris*	Single	3	0	3
Chicken	*Gallus gallus*	Pooled	1 (10)	0	1
Turkey	*Meleagris gallopavo*	Pooled	1 (10)	0	1
Swan	*Cygnus cygnus*	Single	1	0	1
Duck	*Anas platyrhynchos*	Pooled	1 (10)	0	1
Black grouse	*Tetrao tetrix*	Single	2	0	2

[a] Single, single fecal sample; pooled, sample pooled from 10 single fecal samples of the respective source or wastewater sample.
[b] Numbers in parentheses are the original sample numbers before pooling of samples; about 50% of the samples used in this experiment were collected in an alpine karstic catchment area; the rest originated from all over eastern Austria.

ples down to a filtered volume containing 2×10^{-8} g wet feces, while higher-dilution steps contained no detectable marker. The marker was present in ruminant feces at an average concentration of 4.1×10^9 marker equivalents per g wet feces. The values showed little variation, which is a consequence of the pooling process rather than being attributable to population dynamics (Table 3). From these results, we extrapolated the lowest detectable concentrations of ruminant feces. We tried to give a realistic estimate of the detectable marker equivalent concentration by assuming a Poisson distribution in the samples and defining a detection frequency of 95% (according to reference 23, the following formula applies: $S = X_m \pm \sqrt{X_m}$, where S is the 95% confidence interval and X_m is the average). Taking this into account, the reliable detection of the marker can only be expected in PCRs containing an average of six or more copies. The average detection limit of ruminant fecal material was 1.7×10^{-9} g per analyzed filter (Table 3).

TABLE 3. Marker equivalent concentrations in suspended feces and detection limits in suspensions

Source	No. of samples[a]	No. of marker equivalents in suspended feces (10^9 copies/g feces)		Detectable concn[b] (10^{-9} g feces/filter)	
		Avg	Range	Avg	Range
Cattle	3 (30)	3.7	3.0–4.3	1.7	1.4–2.0
Deer	3 (30)	4.5	3.8–5.7	1.4	1.1–1.6
Chamois	3 (30)	4.2	1.6–6.7	2.0	0.9–3.9

[a] Numbers in parentheses indicate the original sample numbers before pooling of samples.
[b] Detection limits were calculated as the minimum amount of feces detectable in a filtration volume, assuming a 95% detection probability based on a Poisson distribution, i.e., six marker equivalents per PCR reaction volume. They are the reciprocal value of the number of marker equivalents per g feces multiplied by 6 for the minimum average marker equivalent number reliably detectable.

TABLE 4. Levels of ruminant marker equivalent concentrations measured in samples taken throughout the study area[a]

Sampling site	Filtration vol (liters)	No. of marker equivalents per liter	
		Avg	Range
Spring LKAS2, flood	1.5	3.2×10^5	1.2×10^5–5.3×10^5
Watering brook	1.0	1.2×10^4	0.6×10^4–1.7×10^4
Watering pond	2.0	5.9×10^2	4.4×10^2–8.1×10^2
River	0.3	3.7×10^2	1.5×10^2–6.5×10^2
Spring LKAS2	4.5	Detected[b]	Detected[b]
Spring KPAS	4.5	ND[c]	ND

[a] Three filtrations per sample.
[b] Detectable close to the detection limit in the investigated volume but not reliably quantifiable.
[c] ND, not detectable.

qPCR has been demonstrated to be useful in the quantification of microorganisms in the environment (9, 18, 24, 25, 31, 33). The general applicability of probe-based qPCR in spring water was demonstrated by our group (30). Bernhard and Field (5) determined detection limits in the range from 2.8×10^{-5} to 2.8×10^{-7} g feces per liter using a qualitative PCR assay for their ruminant *Bacteroides* markers (approximately 100 times less sensitive than the BacR qPCR). In another publication, the detection limit of a qPCR assay for the detection of *Rhodococcus coprophilus* was 1 CFU per reaction (26). Since this bacterium is present at levels between 5.5×10^3 and 3.6×10^6 CFU per gram of herbivore feces, this method exhibits a sensitivity even lower than that of the above-mentioned conventional PCR assay for detection of *Bacteroides* marker (5). The high sensitivity of the BacR assay is also demonstrated by comparing it with cultivation-based fecal indicators. As an example, the concentrations of cultivable *E. coli* organisms in feces from cattle, deer, and chamois range from 4.0×10^6 to 7.7×10^7 CFU per g, with an average of 2×10^7 CFU per g (11) compared to the average of 4.1×10^9 marker equivalents per g measured in this study. This results in a 100-times-higher sensitivity with the BacR qPCR assay. On the other hand, the PCR method allows no assessment of the viability of fecal bacteria. Future investigation will have to establish correlations and differences between viability-based assays and direct-detection methods in the investigated environment.

Occurrence of the marker throughout the study area. To investigate the quantitative occurrence of the ruminant marker in the study area, water samples were taken from selected aquatic habitats covering a presumptive fecal-pollution gradient ranging from well-protected springs to heavily influenced surface waters. The marker could be found at concentrations ranging from not detectable in 4.5 liters (KPAS) to 10^6 marker equivalents per liter (LKAS2 flood) (Table 4). Strong differences in occurrence were obvious and in accordance with the expected different levels of ruminant fecal influence. The LKAS2 site showed low marker levels in early summer, when fecal indicators were not detectable, while the levels during a strong summer flood event were very high, corresponding to high fecal-indicator counts in the same sample (1.6×10^3 *E. coli* organisms per liter) (unpublished data). To our knowledge, no method with comparable performance for the specific

detection of ruminant fecal contamination has been available up to now. In addition, the method is relatively fast and simple. In contrast, most established MST methods are either extremely laborious, like library-based typing methods (28), or not sensitive enough for spring water sources (26).

Possible limitations of the assay. For future applications, it will be necessary to estimate the persistence of the marker in the investigated environment as related to the hydrological situation. For the alpine karstic system, we performed preliminary experiments testing the stability of the marker in highly diluted fecal suspensions in spring water at ambient temperatures (4°C) and found no strong reduction of detectable marker levels during an incubation period of 2 months (data not shown). This result is in accordance with findings for human-specific *Bacteroidetes* markers (27). However, in aquatic systems with higher temperature and trophic status, an increased decay of the detectable marker can be expected. In addition, it will be necessary to investigate the occurrence of the marker in soils and sediments potentially influencing the water body of interest. It can be assumed that the source-specific organisms detected by this assay are highly adapted to the intestinal tracts of warm-blooded ruminant animals and are thus unlikely to proliferate in a soil habitat. Nevertheless, soil might be an intermittent storage reservoir for these bacteria.

Potential applications. The high sensitivity and specificity of the assay apparently meet the set requirement for the detection of fecal contamination in karstic spring water. After additional evaluation, the assay might allow the specific allocation of fecal pollution in alpine water sources, enabling target-oriented measures in the catchment area and thus facilitating watershed management (7, 19). Furthermore, it could also provide additional information for quantitative microbial risk assessment studies as part of water safety plans recommended by the WHO (35), allowing the relative estimation of ruminant fecal input compared to other sources. The current study was restricted to the areas of eastern Austria. The speculation of a cosmopolitan occurrence of the BacR marker is supported by studies from the United States, where fecal rRNA gene clones that exhibited sequences identical to those of the primer and probe binding sites of this assay were retrieved (5, 14). Evaluation in other regions will determine the method's general usefulness for scientists doing MST studies, as well as water suppliers trying to improve source water quality.

Nucleotide sequence accession numbers. Sequences are available at GenBank under accession numbers DQ364808 to DQ364822.

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