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REAL-TIME PCR DETECTION OF *CAMPYLOBACTER* SPP. IN FREE-RANGING MOUNTAIN GORILLAS (*GORILLA BERINGEI BERINGEI*)

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ABSTRACT: Health monitoring of wildlife populations can greatly benefit from rapid, local, noninvasive molecular assays for pathogen detection. Fecal samples collected from free-living Virunga mountain gorillas (*Gorilla beringei beringei*) between August 2002 and February 2003 were tested for *Campylobacter* spp. DNA using a portable, real-time polymerase chain reaction (PCR) instrument. A high prevalence of *Campylobacter* spp. was detected in both individually identified (22/26=85%) and nest-collected samples (68/114=59.6%), with no statistically significant differences among different gorilla sexes or age classes or between tourist-visited versus research gorilla groups. The PCR instrument was able to discriminate two distinct groups of *Campylobacter* spp. in positive gorilla samples based on the PCR product fluorescent-probe melting profiles. The rare type (6/90 positives, 7%, including three mixed cases) matched DNA sequences of *Campylobacter jejuni* and was significantly associated with abnormally soft stools. The more common type of positive gorilla samples (87/90 positives, 97%) were normally formed and contained a *Campylobacter* sp. with DNA matching no published sequences. We speculate that the high prevalence of *Campylobacter* spp. detected in gorilla fecal samples in this survey mostly reflects previously uncharacterized and nonpathogenic intestinal flora. The real-time PCR assay was more sensitive than bacterial culture with *Campylobacter*-specific media and commercially available, enzyme immunoassay tests for detecting *Campylobacter* spp. in human samples.

Key words: *Campylobacter*, epidemiologic monitoring, gorilla, noninvasive sampling, polymerase chain reaction.

INTRODUCTION

Wild apes are most threatened by habitat loss, poaching, and infectious diseases (Woodford et al., 2002). Along with emerging pathogens, such as Ebola virus, the infectious disease threat comprises a number of infectious agents potentially transmitted from humans with whom the wild apes interact (Wallis and Lee, 1999; LeRoy et al., 2004; Köndgen et al., 2007). Investigation of infectious agents sometimes requires invasively collected tissues (e.g., blood), but diagnostic advances, sample availability, and a preference for limiting wild ape disturbance has increased the use noninvasively collected samples (e.g., feces) for diagnostic assays (Whittier et al., 1999; Goldberg et al., 2008; Jensen et al., 2009). Molecular diagnostics have long been used on noninvasively collected samples from humans to diagnose infection with a variety of pathogens, including many potentially pathogenic to great apes (Lina et al., 1996; Houng et al., 1997). Wider implementation of molecular diagnostic techniques could facilitate free-ranging great ape disease research, especially using noninvasive samples coupled with durable, portable technology capable of providing rapid results in the field (McAvin et al., 2003; Tomlinson et al., 2005).

Mountain gorillas (*Gorilla beringei beringei*) are considered at risk of a number of infectious diseases and could benefit from more rapid diagnostics (MGVP Inc. and WCS, 2007). Actual and potential diagnostic samples, noninvasively collected from mountain gorillas, include feces, urine, and partially eaten food items that may contain respiratory secretions and infectious agents (Watts, 1984; Sleeman et al., 1988). Gastrointestinal parasites have been frequently studied in mountain gorillas because of the ease in collecting

fecal samples and performing microscopic analysis, whereas less is known about bacterial and viral infections (Nizeyi et al., 1999, 2001; Rwego et al., 2008). Molecular detection assays could not only expand the use of noninvasively collected samples, but properly designed real-time polymerase chain reaction (PCR) assays could detect multiple organisms and discriminate between genetic subtypes of target organisms, improving the ability to rapidly evaluate bacterial or viral diseases (Klaschik et al., 2004; Verweij et al., 2004).

Campylobacteriosis is one bacterial disease of potential importance to wild gorilla populations. *Campylobacter* is a gram-negative, microaerophilic bacteria that inhabits the gastrointestinal tract of many animals. The genus comprises pathogenic and nonpathogenic species as well as those that can be opportunistic pathogens. In the developed world, virtually all campylobacteriosis is attributed to one species, *Campylobacter jejuni*, and is considered a foodborne illness commonly associated with animal contamination (Blaser, 1997). In the developing world *Campylobacter* infection has a different epidemiology that includes higher prevalence and incidence of both symptomatic and asymptomatic infections (Coker et al., 2002). Developing world *Campylobacter* epidemiology also is characterized by a younger age-related attack-rate peak, widespread adult immunity to infection, and a higher proportion of *Campylobacter* bacterial infections from species other than *C. jejuni*, particularly in Africa (Oberhelman and Taylor, 2000). Although human-human, and presumably human-gorilla, transmission is believed to be rare, *Campylobacter* spp. could serve as a useful model organism for molecular investigation in wild gorillas (Wassenaar and Newell, 2006). Previous studies indicate that *Campylobacter* spp. occurs with sufficient prevalence (19%) to warrant molecular investigation (Nizeyi et al., 2001).

The objective of this project was to expand molecular technologies to detect potential pathogens in wild mountain gorilla populations using an existing real-time PCR field-detection system with commercially available freeze-dried assay reagents. Specifically we aimed to assess the practicality of performing real-time PCR in the field in Africa and, secondly, to perform a basic epidemiology survey of *Campylobacter* spp. in wild gorillas.

MATERIALS AND METHODS

Gorilla and wildlife sample collection

Fecal samples were collected noninvasively from human-habituated mountain gorillas in the Parc National des Volcans in Rwanda (1°35′S to 1°65′S, 29°35′E to 29°75′E) between August 2002 and February 2003. Approximately 100 g of feces were collected into disposable, sealable, polyethylene bags from identified gorillas immediately after observing defecation ("identified" samples) or from gorilla night nests ("nest" samples). The final epidemiologic data set included 140 gorilla samples ($n=114$ from nests and 26 from identified animals), although additional samples were collected and tested for assay optimization and melting-temperature analyses of PCR products. Duplicate sampling of individual gorillas was avoided by collecting only once from a single nesting site (a group of nests) for each gorilla group. No identified duplicates are included in the final 26 identified samples, but because there could be duplication of these individual gorillas with nests samples, data are treated separately. Additional wildlife samples collected from forest buffalo (*Syncerus caffer*, $n=4$) and an unidentified small carnivore ($n=1$) were incorporated into this study to compare DNA sequences that are PCR-positive for *Campylobacter* spp.

For identified gorilla samples, individual age and sex data were recorded from existing records and assigned to accepted age/sex class by standard definitions. Nest samples that retained their shape (80/114, 70.2%) were assigned to age classes based on measured, maximum lobe width, according to previously published methods (McNeilage et al., 2001). Nest samples were further categorized as silverbacks (adult males >12 yr old), when they exceeded the maximum size for adult females (7.0 cm) and/or had silver/gray hairs present, or as adult females, when nests also contained infant-sized feces (<3.0 cm) that normally occur in their shared nests. For epidemiologic analysis, we used full age classes as well as collapsed classes that combined all adults (silverbacks, adult females, unknown

adults, and blackbacks) and all nonadults (subadult, juvenile, and infant). Gorilla visitation type is well established for all these habituated groups, which at the time of collection were separated into those groups visited by tourists ("tourist" groups, $n=4$) and those visited only by behavioral researchers ("research" groups, $n=3$).

Gorilla sample processing

Most gorilla samples ($n=93$) were processed for total DNA extraction within 12 hr of collection, whereas others ($n=15$) were refrigerated (2–4 C) or frozen (−10 to −20 C) for processing within 7 days. A third group of samples ($n=32$ gorillas and five other wild animals) were used for DNA extraction after storage in guanidine isothiocyanate (GT) buffer (4 M guanidine isothiocyanate, 1 M sodium citrate, 0.7% β-mercaptoethanol, 10 mM ethylenediaminetetraacetic acid [EDTA], pH 7.2, Gibco BRL, Gaithersburg, Maryland, USA), which we have previously shown can be used for long-term storage at ambient temperature (Whittier et al., 1999). Total nucleic acids (DNA and RNA) were extracted using a commercial fecal DNA extraction kit (QIAamp DNA Stool Mini Kit, QIAGEN Inc., Valencia, California, USA). Extracted DNA was either refrigerated before PCR, for analysis within 1 wk, or frozen, if analysis was expected to occur after more than 1 wk of storage.

Human sample *Campylobacter* spp. culture and enzyme immunosorbent assay (EIA) testing

Fresh, human fecal samples were collected voluntarily from more than 120 local conservation personnel as part of the 2003 Mountain Gorilla Veterinary Project (MGVP) employee health program, described elsewhere (MGVP, 2004). Eighteen of these samples were selected for inclusion in this study based on culture results for *Campylobacter* spp. as described below (nine positive, nine negative).

Human samples were cultured for bacterial infections in Rwanda within 2 hr of collection and, subsequently, were frozen until EIA testing and DNA extraction. Sample aliquots were placed in *Campylobacter* thioglycollate enrichment medium (0.16% agar, trimethoprim, vancomycin, polymyxin B, cephalothin, and amphotericin B; Remel Inc., Lenexa, Kansas, USA) and refrigerated at 2–4 C for 24 hr. Enriched media were plated onto *Campylobacter*-selective medium (blood agar with same five antibiotics, Remel Inc.) and incubated in 5% CO_2 at 42 C for 48 hr. The microaerophilic environment was achieved using Pouch-MicroAero gas generators in the AnaeroPouch™ System (Remel/Mitsubishi Gas Chemical America Inc., New York, New York, USA). Positive identification of *Campylobacter* spp. was based on colony morphology (small, gray to yellowish or pinkish gray, and slightly mucoid on selective media), characteristic bacterial morphology and gram-negative staining (small, curved, or seagull-winged gram-negative rods), and biochemical characterization using API® Strips (oxidase positive and catalase positive; bioMérieux Clinical Diagnostics, Marcy l'Etoile, France). Frozen aliquots from nine culture-positive and nine culture-negative human samples were later tested in the United States using a commercial EIA for *Campylobacter* spp. according to the manufacturer's instructions (ProSpecT® *Campylobacter* Microplate Assay, Alexon-Trend, Ramsey, Minnesota, USA). After thawing, total DNA was also extracted for PCR according to the same methods used for the gorilla samples.

Real-time PCR using the R.A.P.I.D.™

DNA extracts were used for analysis in the R.A.P.I.D. instrument (Idaho Technology Inc., Salt Lake City, Utah, USA) according to manufacturer's instructions. This unit is a portable, rapid, forced-air thermocycler with an integrated fluorometer for real-time monitoring of PCR reactions. It is designed for field deployment and can be powered by an auto battery, but security and the manufacturer's agreement for the unit used in this study restricted its use to a laboratory in Ruhengeri, Rwanda. The proprietary freeze-dried *Campylobacter* spp., *Salmonella* spp., *Listeria* spp., and *Escherichia coli* O157 reagents integrate the reaction mix, primers, and hybridization probes. The standard PCR cycle for all assays was denaturation at 94 C for 1 min, followed by 35 amplification cycles of 95 C denaturation (held for 0 sec), and combined annealing and extension at 60 C for 20 sec. Fluorescent hybridization-probe melting-curves ramping from 50 C to 80 C were standard after an initial denaturation at 95 C (for 0 sec). Temperature transition rates were 20 C/sec for all steps, except the melting curve that changed at 2 C/sec. Each PCR reaction contained 10–15% (e.g., 2–3 μl/20 μl) volume of DNA sample, and all cycles included positive and negative kit controls.

The R.A.P.I.D. instrument software (LightCycler® Data Analysis module, version 3.5.28.250, Idaho Technology) derives positive/negative results based on a cutoff value difference between the scores of the flat phase and the growth phase of each fluorescent PCR product curve. Visual inspection of the results

from initial assays revealed intermittent cyclic oscillations of the PCR product curves that often interfered with correct software interpretation and instrument results. In troubleshooting this phenomenon, we ultimately discovered that decreasing the reaction volume eliminated oscillations in the positive controls and improved concordance between replicate assays (data not shown). This led us to deviate from the reagent and instrument instructions by running later reactions at half the normal volume (10 μl) and to score all results visually to detect false-positive and false-negative results. Additionally, questions about stability of the freeze-dried reagents in Rwanda, where we found the laboratory temperature ranged from 13 to 32 C and the humidity from 29 to 94% lead us to re-evaluate and retest initial results after assay optimization in the United States.

PCR product sequencing

To confirm their identity, 15 R.A.P.I.D. PCR reaction products for *Campylobacter* spp. (two controls and 13 samples) were electrophoresed in a 2% agarose gel (Gibco) containing 0.2 μg ethidium bromide (Gibco)/ml in TBE buffer (40 mM Tris, 20 mM acetic acid, 1 mM sodium EDTA; Gibco) at 75 or 100 V for 30–60 min. Target DNA bands were removed and purified with a commercial kit (QIAquick Gel Extraction Kit, QIAGEN) and, because of the proprietary nature of their products, submitted to Idaho Technology for sequencing. Bidirectional sequences for the 15 products were generated by the University of Utah, DNA Sequencing Core Facility (Salt Lake City, Utah, USA).

To further identify *Campylobacter* spp. sequences, original samples of DNA extracts were used as PCR templates for new reactions in a conventional thermocycler (PTC-1160 MJ Research, Cambridge, Massachusetts, USA). Published *Campylobacter* genus level primers C412f (5′-GGATGACACTTTTCGGAGC-3′) and C1288r (5′-CATTGTAGCACGTGTGTC-3′; Linton et al., 1996) were used to generate an ~726–base pair (bp) product. We were unable to optimize the PCR results for all samples, but sequenced products were derived from 25-μl reactions with 1 μl of DNA extract at either full extract concentration, 1:10 dilution, or 1:100 dilution, depending on the sample. In the optimization effort, a number of different PCR premixtures and individual components were used, but the standard reaction varied around 0.4 μM of each primer in commercial PCR reaction mixture for final concentrations of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 20 U *Taq* DNA polymerase. The PCR amplification was 36 cycles of 94 C for 1 min, 55 C for 1 min, and 72 C for 1 min, followed by 72 C for 10 min and included positive and negative controls. The PCR products were electrophoresed in a 2% agarose (Gibco) gel containing 0.2 μg ethidium bromide/ml (Gibco) in TBE buffer (40 mM Tris, 20 mM acetic acid, 1 mM sodium EDTA; Gibco) at 75 or 100 V for 30–60 min. Gels were run with appropriate DNA ladders (Gibco) on benchtop apparatuses (Bio-Rad Laboratories Inc., Hercules, California, USA) with Polaroid® (Bedford, Massachusetts, USA) photograph using ultraviolet transilluminators (Fisher Scientific, Pittsburgh, Pennsylvania, USA; UVP Inc., Upland, California, USA). Products were purified as per the R.A.P.I.D. products described above and were sequenced at the Duke University, DNA Analysis Facility (Durham, North Carolina, USA). Eleven samples (one positive-seeded control, four human, and six gorilla) yielded readable DNA sequences.

Statistical analysis

The unpaired *t*-test was used to compare melting peak temperatures (Steel and Torrie, 1980). Fisher's exact test for association was used for univariate analysis of different variables in 2×2 contingency tables (Steel and Torrie, 1980). All calculations were performed either with GraphPad (QuickCalcs, version 2002–2005, GraphPad Software, Inc., La Jolla, California, USA) or MedCalc (version 11.1.0, 2009, MedCalc Software, Mariakerke, Belgium) online software, and $P<0.05$ was considered statistically significant.

RESULTS

EIA, culture, and PCR comparison of human samples

Table 1 shows that the EIA test disagreed with one of the nine culture-negative samples and three of the nine culture-positive samples. Overall, there was agreement between the culture and EIA in 14 of 18 samples. Results from PCR were positive for most of the culture-negative (seven of nine) and culture-positive (eight of nine) samples.

Melting peak analysis

We found that the melting peaks of positive samples fell into one of two groups: those that peaked at higher

TABLE 1. Comparison of bacterial culture, enzyme immunosorbent assay (EIA), real-time polymerase chain reaction (PCR), and PCR-product melting-peak results for human samples.

Sample	Culture	EIA	PCR	PCR melting peak[a]
Human 01	−	−	+	High[b]
Human 05	−	−	+	Mixed
Human 07	−	−	−	
Human 08	−	−	+	High
Human 11	−	−	+	High
Human 15	−	−	+	High
Human 16	−	−	−	
Human 18	−	+	+	High[b]
Human 33	−	−	+	High
Human 12	+	−	+	High
Human 13	+	+	+	Mixed
Human 14	+	−	+	High
Human 17	+	−	+	High
Human 19	+	+	+	Low[b]
Human 20	+	+	+	High[b]
Human 21	+	+	−	
Human 22	+	+	+	High
Human 24	+	+	+	High

[a] Low = melting peak <62 C; high = melting peak >62 C; mixed = both high and low melting peaks.

[b] PCR products sequenced.

temperatures around 64.5 C, and those that peaked at lower temperatures around 59 C (Fig. 1 and Table 2). All positive controls, the sample seeded with *C. jejuni*, and most of the positive human sample products, melted at the higher temperature, whereas most (358/384, 93.2%) of positive assays for gorillas melted at the lower temperature. Using the obvious separation at 61–62 C, there were significant differences between mean melting-peak temperatures for high (>62 C) and low (<61 C) melting groups of gorilla samples, human samples, and all combined samples. No detectable temperature differences were found between gorilla and human samples at either the high or low melting temperatures (Table 2). A small number of samples ($n=3$ gorillas and $n=2$ humans) were found to have both low- and high-temperature melting peaks, either in single assays or in aggregates of multiple assays. The positive human samples showed no association

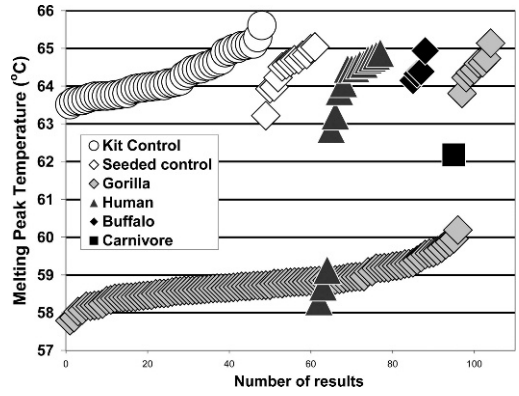


FIGURE 1. Spectrum of melting peak temperatures for R.A.P.I.D. (Idaho Technology Inc., Salt Lake City, Utah, USA) replicates from different types of specimens.

between melting peak temperatures and detection by culture or EIA (Table 1).

Epidemiologic results

We detected no statistically significant differences in prevalence of *Campylobacter* spp. among different sexes, age classes, or tourist and research gorilla groups (Table 3). Specific gorilla family groups ranged in prevalence from 42% to 100% (Table 3), but prevalence was not associated with group size or geographic location within the park (data not shown). Samples from identified gorillas had a significantly higher *Campylobacter* spp. prevalence (85%) than those collected from nests (60%, Fisher's exact test, $P=0.022$).

Considering only the identified samples, which were collected and physically evaluated more immediately after defecation than nest samples, two of the three gorilla samples that were found to have the high melting-peak *Campylobacter* spp. had abnormally soft fecal samples, whereas only one of the 19 with low melting-peak *Campylobacter* spp. had an abnormal stool. This resulted in a statistically significant association between the high melting-peak *Campylobacter* spp. and abnormal stools ($P=0.038$, Fisher's exact test, relative risk = 13 [95% confidence

TABLE 2. Melting peak temperature analysis for R.A.P.I.D. (Idaho Technology Inc., Salt Lake City, Utah, USA) *Campylobacter* spp. polymerase chain reaction products from fecal and control samples showing two distinct products. *P*-values based on two-tailed unpaired *t*-test.

	High melting peak (>62 C)			Low melting peak (<62 C)			
Sample type	No. results	Mean (C)	SD (C)	No. results	Mean (C)	SD (C)	*P*-value
All samples	207	64.36	±0.56	368	59.11	±0.64	<0.0001
Positive controls	113	64.23	±0.55	0			
Seeded samples	28	64.53	±0.58	0			
Gorillas	26	64.64	±0.41	358	59.12	±0.65	<0.0001
Humans	36	64.41	±0.58	10	58.82		<0.0001

interval {CI}=1.6–100, $P=0.016$]; odds ratio = 36 [95% CI=1.6–826, $P=0.025$]).

Sequencing results

The DNA sequences from 15 different initial R.A.P.I.D. PCR products corresponded with a 265-bp sequence of the *Campylobacter* 16s rRNA gene. In this highly conserved region, the R.A.P.I.D. kit control, the *C. jejuni*–seeded PCR product, the buffalo PCR products, and the human and gorilla high melting-peak PCR products all had sequences exactly identical to published sequences for *C. jejuni* (Table 4). The low melting-peak human and gorilla products were, however, unique sequences that most closely resembled (~2%-bp difference) a sequence attributed to *Campylobacter upsaliensis* (LMG8853; Gorkiewicz et al., 2003). The sequence from the low melting-point human product differed from the three gorilla sequences, which were all identical. The PCR product from the unknown carnivore had a relatively intermediate melting peak and a DNA sequence one base pair different from *C. jejuni*.

The DNA sequences from the larger standard PCR products verified the R.A.P.I.D. product sequences for some samples, but not others (Table 5). The seeded control and one human sample (H18), which had been found to have high melting-peak (but unsequenced), R.A.P.I.D., *Campylobacter* spp. product, yielded standard PCR products with sequences 100% identical to *C. jejuni* reference sequences. Likewise the larger, standard PCR sequences from the one human sample (H19) and three gorilla samples (G1, G6, G7) with low melting-peak R.A.P.I.D. products were similar (3–5%-bp differences) to *C. upsaliensis*. A human sample (H01) and three gorilla samples (G2, G4, G5), whose R.A.P.I.D. products were previously sequenced, were found to have different standard PCR products, although two of the gorilla sequences were poor quality with many indeterminate bases. Three of these standard PCR product sequences that did not match their prior R.A.P.I.D. product sequences were either identical (H01) or most similar (G2 and G4) to *Campylobacter gracilis*. An additional human sample (H20) with a previously unsequenced high melting-peak R.A.P.I.D. product was most similar to *Campylobacter hyointestinalis*.

Other assays

Fifty gorilla samples (25 from the largest tourist group and 25 from the largest research group) were tested using R.A.P.I.D. for *Salmonella* spp., *Listeria* spp., and *E. coli* O157 using freeze-dried reagents from Idaho Technology's Pathogen Identification kits. The *Salmonella* spp. assay was successfully pretested using a human sample seeded with pure *Salmonella enterica* from culture. None of the 50 gorilla samples tested positive for any of these agents.

DISCUSSION

The high prevalence of *Campylobacter* spp. detected in both gorilla and human

TABLE 3. Prevalence of *Campylobacter* spp. identified by R.A.P.I.D. (Idaho Technology Inc., Salt Lake City, Utah, USA) polymerase chain reaction (PCR) from mountain gorilla fecal samples, stratified by sample categories.

Sample category	Individually identified samples						Nest collected samples					
	No. sampled	No. negative (%)	No. positive (%)				No. sampled	No. negative (%)	No. positive (%)			
			Total positive	Low[a]	High[b]	Mixed[c]			Total positive	Low[a]	High[b]	Mixed[c]
Age class												
Silverback	7	1 (14)	6 (86)	5 (71)	1 (14)	0	15	5 (33)	10 (67)	10 (67)	0	0
Adult female	9	3 (33)	6 (67)	5 (56)	0	1 (11)	13	4 (31)	9 (69)	7 (54)	1 (8)	1 (8)
Unknown adult[d]							30	13 (43)	17 (57)	17 (57)	0	0
Blackback	3	0	3 (100)	3 (100)	0	0	3	2 (67)	1 (33)	1 (33)	0	0
Subadult/Juvenile	4	0	4 (100)	3 (75)	1 (25)	0	26	9 (35)	17 (65)	16 (62)	0	1 (4)
Infant	3	0	3 (100)	3 (100)	0	0	13	6 (46)	7 (54)	7 (54)	0	0
Unknown subadult[d]							14	7 (50)	7 (50)	7 (50)	0	0
Sex												
Male	12	1 (8)	11 (92)	9 (75)	2 (17)	0	18	7 (39)	11 (61)	11 (61)	0	0
Female	13	3 (23)	10 (77)	9 (69)	0	1 (8)	13	4 (31)	9 (69)	7 (54)	1 (8)	1 (8)
Unknown	1	0	1 (100)	1 (100)	0	0	83	35 (42)	61 (73)	59 (71)	1 (1)	1 (1)
Type of group												
Tourist	5	1 (20)	4 (80)	2 (40)	2 (40)	0	53	16 (30)	37 (70)	37 (70)	0	0
Research	21	3 (10)	18 (90)	17 (85)	0	1 (5)	61	30 (50)	31 (50)	28 (46)	1 (2)	2 (3)
Specific group												
Group 1	16	1 (6)	15 (94)	14 (88)	0	1 (6)	19	11 (58)	8 (42)	6 (32)	0	2 (11)
Group 2	1	1 (100)	0	0	0	0	27	15 (56)	12 (44)	11 (41)	1 (4)	0
Group 3	4	1 (25)	3 (75)	3 (75)	0	0	15	4 (27)	11 (73)	11 (73)	0	0
Group 4	0						6	2 (33)	4 (67)	4 (67)	0	0
Group 5	5	1 (20)	4 (80)	2 (40)	2 (40)	0	9	1 (11)	8 (88)	8 (89)	0	0
Group 6	0						9	0	9 (100)	9 (100)	0	0
Group 7	0						29	13 (45)	16 (55)	16 (55)	0	0
All samples	26	4 (15)	22 (85)	19 (73)	2 (8)	1 (4)	114	46 (40)	68 (60)	65 (57)	1 (2)	2 (2)

[a] Low = PCR product melting peak <62 C.

[b] High = PCR product melting peak >62 C.

[c] Mixed = multiple PCR product melting peaks.

[d] Unknown age classes only used for nest-collected samples.

TABLE 4. Sequence differences at variable positions among *Campylobacter* spp. 265–base pair R.A.P.I.D. (Idaho Technology Inc., Salt Lake City, Utah, USA) polymerase chain reaction PCR products amplified from 15 different samples.[a]

Sequence identification	Melt[c]	Base pair position[b]											
		466	565	568	590	591	592	600	601	602	625	626	635
C. jejuni[b]		T	A	A	A	T	G	C	A	T	G	T	A
C. jejuni seed control	64.05[d]	.	.	.	.	.	.	.	.	.	.	.	.
Positive kit control	63.98	.	.	.	.	.	.	.	.	.	.	.	.
Gorilla 3	64.93	.	.	.	.	.	.	.	.	.	.	.	.
Gorilla 4	64.28[d]	.	.	.	.	.	.	.	.	.	.	.	.
Gorilla 5	64.40[d]	.	.	.	.	.	.	.	.	.	.	.	.
Human 01	64.47	.	.	.	.	.	.	.	.	.	.	.	.
Buffalo 1	64.39	.	.	.	.	.	.	.	.	.	.	.	.
Buffalo 2	64.33	.	.	.	.	.	.	.	.	.	.	.	.
Buffalo 3	64.94	.	.	.	.	.	.	.	.	.	.	.	.
Buffalo 4	64.33	.	.	.	.	.	.	.	.	.	.	.	.
Unknown carnivore	62.18	A	.	.	.	.	.	.	.	.	.	.	.
C. upsaliensis[e]		.	.	.	*G*	*G*	*A*	*T*	*T*	*C*	*A*	.	*G*
Gorilla 1	59.11[d]	A	C	G	G	A	A	T	T	C	.	G	G
Gorilla 2	59.02[d]	A	C	G	G	A	A	T	T	C	.	G	G
Gorilla 6	58.54[d]	A	C	G	G	A	A	T	T	C	.	G	G
Human 19	58.77	A	.	.	G	G	A	n	T	C	n	.	n

[a] *Underlined italics* = reference sequences; a dot (.) = base identical to the *C. jejuni* reference; n = an indeterminate base.

[b] Positions and sequence from *Campylobacter jejuni* strain RM1221 (GenBank accession CP000025 region 37396-38908).

[c] Melting peak temperature (C).

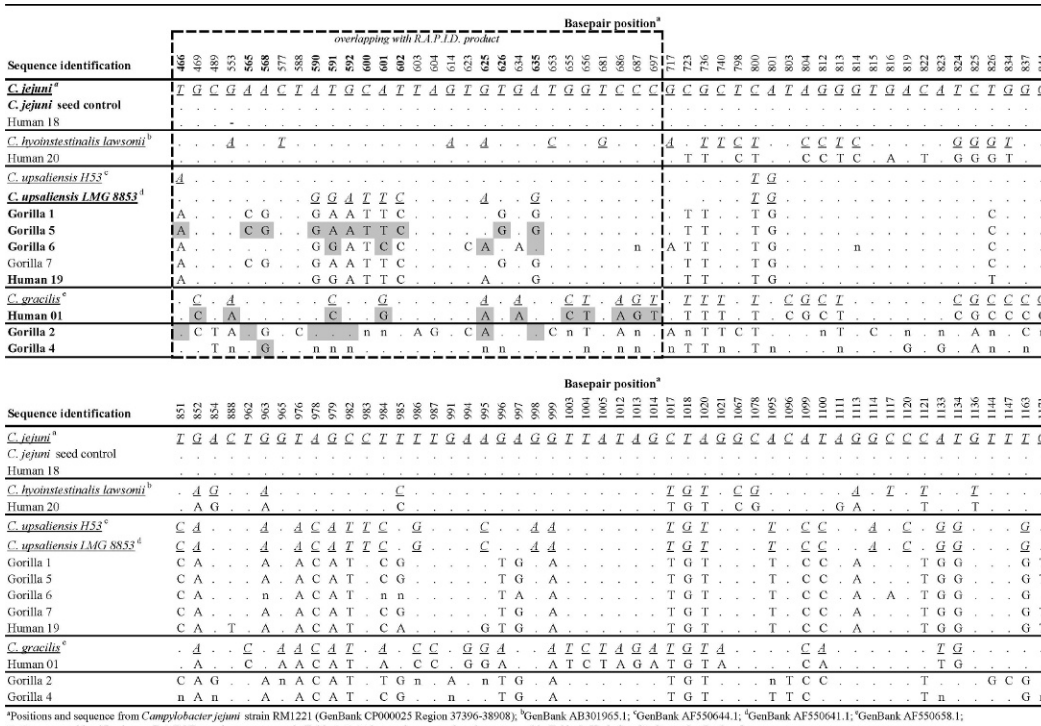
[d] Average of two reactions.

[e] GenBank accession AF550641.1.

samples in this survey was unexpected. Our overall survey result of 65–80% prevalence (depending on collection method) in gorillas is in contrast to *Campylobacter* spp. culture prevalences previously reported for wild mountain gorillas in a nearby park (19% [Kalema, 1995] and 8% [Nizeyi et al., 2001]). The R.A.P.I.D. PCR assay also detected a higher number of positive human samples than either culture or EIA assays were able to detect. Higher sensitivity of molecular, compared with conventional, diagnostics is well established (Munster et al., 2009), but the differences we found could also be a reflection of the specificity of the different methods. The culture protocol and media are fairly selective for *C. jejuni* and *Campylobacter coli* (and selective *against* some other species; Nachamkin et al., 2000), whereas the EIA assay detects a *Campylobacter*-specific antigen shared by *C. jejuni* and *C. coli*, but not by most other species. The PCR assay is less specific, detecting *Campylobacter* at the genus level, and is known to detect at least one species that the EIA will not (*Campylobacter lari*, per manufacturers' inserts).

The high sensitivity and low specificity of a PCR assay like the one used can obscure a clinically useful result. The R.A.P.I.D. instrument, however, is able to quantify starting template DNA, which can correlate to clinical infections (Al-Robaiy et al., 2001), and to distinguish between different PCR products, based on their fluorescent probe melting patterns. This study demonstrated the use of melting-peak analysis in detecting and discriminating what appears be clinically relevant *C. jejuni* (or *C. jejuni*–like) infections that were associated with soft stools in wild gorillas. If we consider solely

TABLE 5. Sequence differences among *Campylobacter* 726–base pair, polymerase chain reaction products amplified from different samples.



[a]Positions and sequence from *Campylobacter jejuni* strain RM1221 (GenBank CP000025 Region 37396-38908); [b]GenBank AB301965.1; [c]GenBank AF550644.1; [d]GenBank AF550641.1; [e]GenBank AF550658.1;
Bold sequence identifications = R.A.P.I.D. products sequences in Table 4; **Bold** base pairs = same locations reported in Table 5; *Underlined italics* = GenBank reference sequences;
"." = base identical to *C. jejuni* reference; n = indeterminate base; shaded = discrepancy with sequence from same sample by R.A.P.I.D.

the high melting peak and the mixed products compatible with a diagnosis of *C. jejuni*, the gorillas sampled in this study had an overall prevalence of only 3% for nest samples and 11% for identified samples, which are similar to the prevalences reported in previously published studies.

Sample quality, as reflected by collection method, affected the results in a predictable manner. Nest samples collected up to many hours after defecation had a lower proportion of positives than samples from identified gorillas collected immediately after defecation. One gorilla group (Group 1) was overrepresented in the identified samples, which potentially confounded results and inflated identified-sample prevalence. However, that same group had the lowest *Campylobacter* spp. prevalence in the more evenly sampled nest results and, therefore, was unlikely to falsely increase the identified-sample prevalence. The likely explanation for the lower nest-sample prevalence is that *Campylobacter* spp., a group of enteric, microaerophilic, and thermophilic bacteria, usually do not survive very long outside of the gastrointestinal tract, are susceptible to desiccation and light, and fail to grow below 30 C (Hazelberger et al., 1998). This fragility and potential overgrowth by other organisms could limit detection of diagnostic nucleic acids in samples left longer in the environment, such as the nest-collected samples.

In addition to the possibility of environmental and sample-handling factors resulting in undetected *Campylobacter* spp. infections, retention of PCR inhibitors that prevent product formation is also a common cause of false-negative results for

fecal DNA extracts (Wilson, 1997). Although removal of PCR inhibitors was incorporated in the standard DNA extraction methods, this survey found evidence of sporadic PCR inhibition. Even with the optimized assays, we found a high proportion (>20%, data not shown) of discordance between replicate pairs of assays during single-instrument runs. We also found one human sample (H21) that was culture positive and EIA positive but PCR negative. It is, therefore, possible that more gorillas shed *Campylobacter* spp. than even the 80% prevalence we detected in fresh, identified samples.

We speculate that this high prevalence mostly represents normal gorilla intestinal flora and a not yet fully described *Campylobacter* species. Most of the *Campylobacter* spp. we detected in wild gorillas was the low melting-peak type that generated multiple, consistent DNA sequences that were most similar, but not identical, to *C. upsaliensis*. One of the drawbacks of this survey was the collection and handling of gorilla fecal samples for only DNA detection with PCR. Although future research is planned, the lack of preserved samples with potentially viable bacteria prevented isolation and further description of the *Campylobacter* spp. we detected.

The initial R.A.P.I.D. results revealed few apparent coinfections (three of 90 positives) with multiple *Campylobacter* spp. in the same gorilla sample. That finding suggests that the common (low melting peak) gorilla *Campylobacter* sp. may not only be nonpathogenic but also might even be somewhat protective to gorillas by competitively excluding the seemingly more pathogenic, high melting-peak *C. jejuni* or *C. jejuni*–like species, similar to what has been shown with *Campylobacter* spp. in chickens (El-Shibiny et al., 2007). The DNA sequencing of 726-bp, standard PCR product, however, revealed a more complicated distribution of *Campylobacter* spp. infections in both gorillas and humans. These sequences included two human isolates not originally detected (*C. gracilis* and *C. hyointestinalis*–like) as well as five samples that showed either a different *Campylobacter* sp. than initially detected or evidence of a mixture of multiple DNA sequences. Aside from the DNA of the *C. jejuni*, which we used as a PCR control, no other *Campylobacter* organisms or DNA were used in our laboratory, making lab contamination with these other species unlikely. We aim to clarify the nature and spectrum of these organisms with future studies isolating live bacteria in addition to detecting DNA.

Employing the R.A.P.I.D. instrument had some additional drawbacks. In addition to the problematic PCR growth-curve oscillations briefly outlined in the methods, we detected a seemingly related, lower-than-optimal PCR sensitivity using the standard R.A.P.I.D. protocol of 20-µl reactions, and also experienced an apparent pre-expiration degradation of R.A.P.I.D. reagents in the field. The reagent issue was likely due to widely fluctuating climactic conditions in the field laboratory and was remedied with quicker use of additional reagent lots. The cycle oscillations and suboptimal detection rates were remedied by using smaller reaction volumes, which we can only speculate may have eliminated an unknown thermodynamic effect. Lastly, at the time of our investigation, the military classification of the R.A.P.I.D. instrument inconveniently prevented our ability to fully test the unit in the forest in which it is designed and capable of operating.

Overall, however, we achieved our objectives of showing that the R.A.P.I.D. instrument was practical to use in a remote field laboratory and enabled us to complete a survey for *Campylobacter* spp. in wild gorillas. Without this or similar technology, the apparently novel low melting-peak *Campylobacter* sp. we found may have gone undetected or been lumped together with the another type or types. This distinction could be particularly important if the typical patterns of variable *Campylobacter* spp. pathogenicity exist in wild

gorillas as this study has suggested. The ability shown here to rapidly detect and easily distinguish between similar types of organisms, in the field, using noninvasively collected samples, could greatly expand diagnostic capabilities for many wildlife professionals.

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