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Development of rRNA-Targeted PCR and In Situ Hybridization with Fluorescently Labelled Oligonucleotides for Detection of *Yersinia* Species

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In this report, we present details of two rapid molecular detection techniques based on 16S and 23S rRNA sequence data to identify and differentiate *Yersinia* species from clinical and environmental sources. Near-full-length 16S rRNA gene (rDNA) sequences for three different *Yersinia* species and partial 23S rDNA sequences for three *Y. pestis* and three *Y. pseudotuberculosis* strains were determined. While 16S rDNA sequences of *Y. pestis* and *Y. pseudotuberculosis* were found to be identical, one base difference was identified within a highly variable region of 23S rDNA. The rDNA sequences were used to develop primers and fluorescently tagged oligonucleotide probes suitable for differential detection of *Yersinia* species by PCR and in situ hybridization, respectively. As few as 10^2 *Yersinia* cells per ml could be detected by PCR with a seminested approach. Amplification with a subgenus-specific primer pair followed by a second PCR allowed differentiation of *Y. enterocolitica* biogroup 1B from biogroups 2 to 5 or from other pathogenic *Yersinia* species. Moreover, a set of oligonucleotide probes suitable for rapid (3-h) in situ detection and differentiation of the three pathogenic *Yersinia* species (in particular *Y. pestis* and *Y. pseudotuberculosis*) was developed. The applicability of this technique was demonstrated by detection of *Y. pestis* and *Y. pseudotuberculosis* in spiked throat and stool samples, respectively. These probes were also capable of identifying *Y. enterocolitica* within cryosections of experimentally infected mouse tissue by the use of confocal laser scanning microscopy.

The genus *Yersinia* comprises 11 species, of which *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* possess the potential to be pathogenic in humans and animals. The pathogenicity of these three species is controlled by the common 64- to 75-kb virulence plasmid pYV (or pCD1 for *Y. pestis*) (5). In contrast to the enteropathogenic yersiniae (*Y. enterocolitica* and *Y. pseudotuberculosis*), the plague bacillus (*Y. pestis*) usually harbors two additional virulence plasmids (pCP1 and pMT1).

Y. enterocolitica can be divided into six biogroups (biogroups 1A, 1B, and 2 to 5) and more than 50 serovars (8, 35). *Y. enterocolitica* strains belonging to biogroup 1B are commonly isolated in the United States, whereas strains of other biogroups are ubiquitously distributed. Those isolates formerly called *Y. enterocolitica*-like isolates were reclassified and assigned to eight different species (*Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii*, *Y. mollaretii*, *Y. bercovieri*, *Y. aldovae*, *Y. rhodei*, and *Y. ruckeri*) (1).

Unfortunately, the established cultivation and serological techniques are not sufficient for the diagnosis of all *Yersinia* species (for reviews, see references 8 and 28). There is accumulating evidence that *Y. enterocolitica* may be difficult to recover in chronic infections by using standard cultivation techniques, although indirect immunofluorescence allows detection of the organism within clinical specimens (15, 18). Furthermore, some of the eight nonpathogenic *Yersinia* species share surface antigens with serotypes of *Y. enterocolitica* (8) that are pathogenic in humans, leading to false identification.

Rapid identification of *Y. pestis* is important in the monitoring of enzootic plague and during outbreaks of human plague. Cultivation of *Y. pestis* from clinical specimens requires approximately 2 days; this is followed by biotyping and detection of, e.g., fraction 1 antigen. Several reports have identified unusual *Y. pestis* strains, isolated from patients or rodents, which lack plasmid pCP1 or production of F1 antigen (for a review, see reference 28). Moreover, the pigmentation phenotype characteristic for *Y. pestis* has also been observed with freshly isolated *Y. pseudotuberculosis* strains (10).

Thus, rapid and reliable procedures for the direct detection and differentiation of yersiniae in clinical samples may prove helpful to both clinicians and public health authorities (8, 28).

Therefore, a 16S rRNA-based detection approach was developed, since this molecule has been used extensively to elucidate phylogenetic relationships of bacteria at intra- and intergeneric levels and it is also an excellent target for diagnostic PCR and fluorescent in situ hybridization assays (4, 30). Near-full-length 16S rRNA gene (rDNA) sequences for the three pathogenic *Yersinia* species were determined. A portion of the 23S rRNA gene was also analyzed for *Y. pestis* and *Y. pseudotuberculosis*. These sequence data were used to develop primer sets and fluorescently labelled oligonucleotide probes suitable for group- and subspecies-specific rDNA amplification reactions (PCRs) and for in situ hybridization of pathogenic *Yersinia* species within clinical specimens, respectively.

MATERIALS AND METHODS

Preparation of samples for in situ hybridization and PCR. All bacterial strains used in this study are listed in Table 1. They were grown aerobically in Luria-Bertani (LB) broth at 26°C. Bacterial cells were harvested while in exponential growth phase, centrifuged, washed in 1 M NaCl, resuspended in TE buffer (10 mM Tris, 1 mM EDTA [pH 8]), and diluted to an optical density of 1.0 at 600 nm.

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TABLE 1. Reference organisms, sources, and results of whole-cell hybridizations

Organism and strain[a]	Serotype(s)/ biotype[b]	Accession no.	Hybridization with probe				
			Y.16S-69	Y.ent.16S-184	Y.p.16S-997	Y.pseu.23S-1526	E.car.16S-636
Yersinia enterocolitica							
IP 132[c]	O1, O2, O3/3		+	+	−	−	−
IP 1154[c]	O2, O3/3		+	+	−	−	−
Y 108-c[e,n]	O3*/4	Z75316	+	+	−	−	−
MYO[k]	O3/4		+	+	−	+	−
NF-0[e]	O5/1A		+	+	−	−	−
Y-5,27[f]	O5, O27/2		+	+	−	+	−
0310/90[f]	O6, O30/1A		+	+	−	−	−
MY 79[k]	O9/2		+	+	−	−	−
WA-314[d]	O8/1B	Z75318,[o] Z75324[p]	+	+	−	−	−
8081[c]	O8/1B		+	+	−	−	−
1209-79[d]	O13/1B		+	+	−	−	−
1223-75-1[d]	O20/1B		+	+	−	−	−
Yersinia pestis							
Kuma[g]	—/*antiqua*		+	−	+	−	−
Yokohama[g]	—/*antiqua*		+	−	+	−	−
EV 76[g,n]	—/*orientalis*	Z75317	+	−	+	−	−
A1122[g]	—/*orientalis*		+	−	+	−	−
6/69[h]	—/*orientalis*		+	−	+	−	−
MP6[g]	—/*orientalis*		+	−	+	−	−
M23[g]	—/*orientalis*		+	−	+	−	−
G32[g]	—/*orientalis*		+	−	+	−	−
KIM[g]	—/*mediaevalis*		+	−	+	−	−
Yersinia pseudotuberculosis							
H346.36/88[f]	O1		+	−	+	+	−
2020[j]	O1		+	−	+	+	−
444a[j]	O1		+	−	+	+	−
2019[j]	O1		+	−	+	+	−
H 191/91[k]	O1A		+	−	+	+	−
H 47/91[k]	O1A		+	−	+	+	−
PB1[g]	O1A		+	−	+	+	−
123[i]	O1A		+	−	+	+	−
1468[i]	O1A		+	−	+	+	−
4587[i]	O1A		+	−	+	+	−
H 268/91[k]	O1B		+	−	+	+	−
H 260/91[k]	O1B		+	−	+	+	−
O16[i]	O1B		+	−	+	+	−
O6[i]	O1B		+	−	+	+	−
541[i]	O1B		+	−	+	+	−
487[i]	O1B		+	−	+	+	−
Y-p-T[l]	O3		+	−	+	+	−
YPIII[g,n]	O3	Z21939	+	−	+	+	−
H 267/91[k]	O3		+	−	+	+	−
Yersinia frederiksenii[c]	O3	Z75319,[o] Z75325[p]	−	−	−	+	ND[m]
Yersinia mollaretii[c]	O3	Z75322,[o] Z75328[p]	−	−	−	+	ND
Yersinia intermedia[c]	O3	Z75320,[o] Z75326[p]	+	−	−	−	ND
Yersinia kristensenii[c]	O3	Z75321,[o] Z75327[p]	−	−	−	−	ND
Y. ruckeri[k]		Z75323,[o] Z75329[p]	−	−	−	−	ND
Hafnia alvei DSM 30163			−	+	−	−	ND
Serratia marcescens ATCC 9141			−	−	−	+	ND

Continued on following page

TABLE 1—*Continued*

Organism and strain[a]	Serotype(s)/ biotype[b]	Hybridization with probe				
		Accession no.	Y.16S-69	Y.ent.16S-184	Y.p.16S-997	Y.pseu.23S-1526	E.car.16S-636
Serratia fonticola DSM 4576			−	+	−	−	ND
Rahnella aquatilis DSM 4594			−	−	−	−	ND
Erwinia carotovora ATCC 43762			−	−	+	−	+
Erwinia rhapontici LMG 2462			−	−	−	+	−
Proteus mirabilis[f]			−	−	−	−	ND
Escherichia coli XL-1-Blue			−	−	−	−	ND
Klebsiella oxytoca[f]			−	−	−	−	ND
Citrobacter freundii[f]			−	−	−	−	ND
Salmonella enteritidis[f]			−	−	−	−	ND
Vibrio parahaemolyticus DSM 10027			−	−	−	−	ND
Aeromonas hydrophila DSM 6173			−	−	−	−	ND
Pasteurella multocida ATCC 12945			−	−	−	−	ND

[a] DSM, Deutsche Stammsammlung von Mikroorganismen und Zellkulturen, Brunswick, Germany; ATCC, American Type Culture Collection, Rockville, Md.; LMG, Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium.
[b] —, no serotype detected.
[c] Strain as described in reference 11.
[d] Strain as described in reference 22.
[e] Strain as described in reference 13.
[f] Strain obtained from Institut für Hygiene und Mikrobiologie, Würzburg, Germany.
[g] Strain as described in reference 31.
[h] Strain as described in reference 29.
[i] Strain obtained from I. Semenova, St. Petersburg, Russia.
[j] Strain obtained from J. Lomov, Rostov, Russia.
[k] Strain obtained from Hygiene Institut, Hamburg, Germany.
[l] Strain as described in reference 32.
[m] ND, not determined.
[n] Complete 16S rRNA sequence was determined.
[o] V1 and V2 regions (positions 56 to 197) were sequenced.
[p] V3 region (positions 420 to 650) was sequenced.

One microliter of each cell suspension was used in PCR assays. For in situ hybridization, harvested cells were processed and fixed with paraformaldehyde as previously described (3).

Female BALB/c mice (6 to 8 weeks old), purchased from Charles River Wiga, Sulzfeld, Germany, were inoculated intravenously with 3×10^5 bacterial cells (*Y. enterocolitica* WA-314) and sacrificed 4 days postexposure. The spleen, liver, and lung were aseptically removed from each mouse and cut into small pieces. The tissue pieces were immediately immersed in freshly prepared, cold 3% paraformaldehyde and refrigerated at 4°C for 24 h, to allow complete penetration of the fixative. The fixed tissue was washed in phosphate-buffered saline (PBS) for 2 h, mounted in O.C.T. Tissuetek (Miles Laboratories Inc., Elkart, Ind.), and snap-frozen in liquid nitrogen. Frozen tissue blocks were cut in 5-μm-thick sections with a cryostat and stored at −70°C.

Tissue samples from livers of sterile BALB/c mice were homogenized and spiked with different numbers of *Y. enterocolitica* WA-314. These samples were prepared for PCR analysis by using the QIAGEN (Hilden, Germany) tissue kit as recommended by the manufacturer. Five microliters of each of the resulting preparations was analyzed to evaluate the sensitivity of the different PCR approaches.

Three random stool samples and three random throat swabs submitted to the diagnostic laboratory of the Max von Pettenkofer Institut were collected and prepared for in situ hybridization as follows: 1 g of each stool specimen was resuspended in 9 ml of sterile PBS and processed further as described by Langendijk et al. (25). The throat swabs were placed in 500 μl of sterile PBS in a sterile 1.5-ml Eppendorf tube, and the remaining fluid was expressed from the swab by pressing it against the wall of the tube. The resulting suspension was centrifuged (6,000 × *g*, 10 min) and washed once in sterile PBS. The cell pellet was resuspended in 100 μl of a 1:1 mixture of PBS and 96% ethanol. An aliquot of the fixed specimens was spiked with $\geq 10^6$ *Y. pseudotuberculosis* 487 and *Y. pestis* A1122, respectively. Ten microliters of each sample was analyzed by in situ hybridization.

PCR amplification and sequencing of rDNA. Amplification and sequencing with universal primers were performed as described by Lane (24). Partial-length 16S and 23S rDNAs were amplified with primer pairs 27f-1525r and 1104f-1608r, respectively; one of each primer was biotinylated in each of two reciprocal reactions. Single-stranded DNA was obtained for direct sequencing by using the streptavidin-coated magnetic bead separation technique (17). Single-stranded DNAs were sequenced with multiple internal primers by the *Taq* cycle DyeDeoxy terminator method, combined with an ABI PRISM 373A automatic sequencer (PE Applied Biosystems, Weiterstadt, Germany). Sequences for both rDNA strands were determined. The nucleotide sequence data reported here have been deposited in the EMBL sequence database (Table 1).

PCR with *Yersinia*-specific primers (Table 2 and Fig. 1) was performed with all isolates specified in Table 1, each in a 50-μl reaction mixture containing 20 pmol of each primer, 1 μl of the bacterial cell suspension, 0.20 mM each dATP, dCTP, dGTP, and dTTP (Pharmacia LKB Biotechnology, Freiburg, Germany), 4 mM $MgCl_2$, and 2.5 U of *Taq* DNA polymerase (AmpliTaq; PE Applied Biosystems) in a buffer with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 0.001% (wt/vol) gelatin (PE Applied Biosystems). For each amplification reaction, negative controls containing water, instead of template DNA, were run in parallel. After the initial denaturation (80°C, 5 min), 30 cycles of amplification were carried out in a GeneAmp 2400 thermal cycler (PE Applied Biosystems). Each cycle consisted of 1 min of denaturation at 94°C, 1 min of annealing at 63°C (the exception is given in Table 2), and 2 min of extension at 72°C. Five microliters of each PCR product was analyzed by electrophoresis on 1.5% agarose gels containing 0.5 μg of ethidium bromide per ml.

TABLE 2. PCR primers and hybridization probes

Primer or probe	Sequence (5′-3′)	Target group	Position (nt)[a]	Reference
PCR primers				
Y.16S-86f[b]	GCGGCAGCGGGAAGTAGTTTA	Subgenus	66–86	PS[c]
Y.e.eur.16S-455r	CAATCACAAAGGTTATTAACCTTTATG	*Y. enterocolitica* European serotypes	455–481	PS
Y.e.ame.16S-455r	CAATCCAACAACGTATTAAGTTATTGG	*Y. enterocolitica* American serotypes	455–481	PS
Y.p.16S-455r	CAATGATTGAGCGTATTAAACTCAACC	*Y. pestis* and *Y. pseudotuberculosis*	455–481	PS
B.16S-794r	TACAGCGTGGACTACCAGGGT	Kingdom *Bacteria*	794–814	36
Probes				
Y.16S-69	TAAACTACTTCCCGCTGC	Subgenus	69–85	PS
Y.ent.16S-184	CCCACTTTGGTCCGAAGA	*Y. enterocolitica*[d]	184–202	PS
Y.p.16S-997	CTCTGCCAAATTCTGTGG	*Y. pestis* and *Y. pseudotuberculosis*[d]	997–1013	PS
Y.pseu.23S-1526	CTGCACCGTAGTGCATCG	*Y. pseudotuberculosis*[d]	1526–1549	PS
B.16S-338	GCTGCCTCCCGTAGGAGT	Kingdom *Bacteria*	338–355	3
cB.16S-338	CGACGGAGGGCATCCTCA	Negative control	c338–335	3

[a] Numbering of the target positions corresponds to the *E. coli* numbering described in reference 9.
[b] The annealing temperature for PCR with primer Y.16S-86f was 53°C.
[c] PS, present study.
[d] Exceptions are mentioned in the text.

Development of PCR primers and hybridization probes. An alignment of 29 partial *Yersinia* 16S rRNA sequences was used for the design of PCR primers and hybridization probes. Primer and probe designations, sequences, positions, and references are listed in Table 2.

The oligonucleotides for in situ hybridization were synthesized with a C_6-trifluoroacetyl amino-linker [6-(trifluoroacetylamino)-hexyl-(2-cyanoethyl)-(*N*, *N*-diisopropyl)-phosphoramidit] (MWG, Ebersberg, Germany). Labelling with 5 (and 6)-carboxytetramethylrhodamine, succinimidyl-ester [5(6)-TAMRA,SE] (CT; Molecular Probes, Eugene, Oreg.), 5-(-6-)carboxyfluorescein-*N*-hydroxy-succinimide-ester (FLUOS; Boehringer GmbH, Mannheim, Germany), and Cy5 (Biological Detection Systems, Pittsburgh, Pa.) was performed as described previously (3, 34).

Whole-cell hybridization. In situ hybridization on glass slides was performed as described by Amann et al. (3). For the detection of pathogenic *Yersinia* species, two different probe combinations were hybridized simultaneously to the reference cells and to the clinical samples. Probes Y.16S-69-FLUOS and Y.ent.184-CT were used together for the detection of *Y. enterocolitica*, while probes Y.p.16S-997-FLUOS and Y.pseu.23S-1526-CT were applied simultaneously for the identification and differentiation of *Y. pestis* and *Y. pseudotuberculosis*. The addition of 30% formamide to the hybridization buffer resulted in a specific hybridization of the oligonucleotides to their respective target organisms. To reduce the amount of toxic waste, formamide was not used in the washing buffer in hybridization reactions. According to the formula of Lathe et al. (26), the NaCl concentration was instead decreased in the washing buffer to obtain the same stringency as that of the hybridization buffer.

Probe Y.-16S-69-CT was used to detect *Yersinia* cells within tissue sections. Probe cB.16S-338-CT, complementary to universal probe B.16S-338, was also hybridized to these samples to monitor nonspecific binding of labelled probes to bacterial and human cells. Citifluor (Citifluor Ltd., London, United Kingdom) was used as a mounting medium on hybridized slides, and the slides were examined with a Leica (Heerbrugg, Switzerland) TCS NT scanning confocal microscope equipped with a standard filter set. For probe excitation, an argon-krypton laser was used. Three different fluorochromes (CT, FLUOS, and Cy5) could be detected simultaneously with three different photomultipliers and represented the green (FLUOS), red (CT), and blue (Cy5) channels of the Leica software package. For the tissue sections, optical sectioning (0.5- to 1.0-μm width) was performed to reveal the three-dimensional localization of the probe-conferred fluorescence within the samples. The standard software delivered by the manufacturer was used to further process the digitized images.

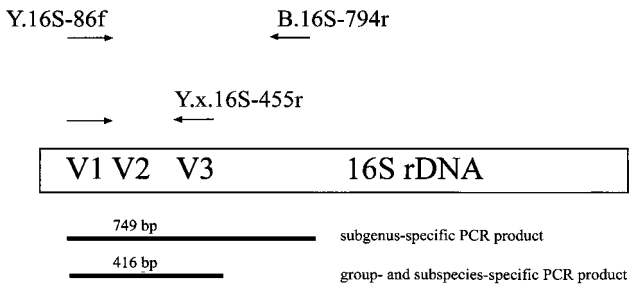

FIG. 1. Positions and specificities of PCR primers and lengths of the amplification products generated with different PCR primers.

RESULTS

Sequence analysis and phylogeny. The PCR primers 27f and 1525r directed the synthesis of a 1,535-bp 16S rDNA fragment. Almost complete double-stranded sequences of these amplicons were determined for the three strains Y-108-c (*Y. enterocolitica* O:3), EV 76 (*Y. pestis* biovar *orientalis*), and YPIII (*Y. pseudotuberculosis* O:3). Only one region of intergeneric variability, corresponding to region V1, and two of intrageneric variability, corresponding to regions V2 and V3, could be detected (27). These regions were also sequenced for seven other *Yersinia* strains. In contrast to the work of Ibrahim et al. (20), the present analysis of 16S rDNA sequences revealed no differences between *Y. pestis* and *Y. pseudotuberculosis*. Therefore, a variable portion of the 23S rDNA of three *Y. pestis* and three *Y. pseudotuberculosis* strains encompassing the region corresponding to *Escherichia coli* positions 1104 to 1608 (9) was sequenced. The sequences of the six strains were identical except for position 1534 (Fig. 2). Direct partial sequencing of the 23S rRNA of *Y. pseudotuberculosis* 487 revealed two different possible bases (C or U) in position 1534.

Sensitivity and specificity of PCR. A subgenus-specific primer set (genus *Yersinia* excluding *Y. frederiksenii*, *Y. mollaretii*, and some strains of *Y. kristensenii*) was developed. Also, for the *Y. pestis-Y. pseudotuberculosis* group and for the two subspecies of *Y. enterocolitica*, specific primers were developed. The specificity of the different amplification reactions was evaluated by PCR by using genomic DNA preparations of closely related bacteria (Table 1) and human DNA. The PCR approach described above generated no amplification products with any of these DNA preparations. Figure 3 shows the results of a PCR assay using the subgenus-specific primer pair Y.16S-86f and B.16S-794r followed by a seminested amplification with primers Y.16S-86f and Y.e.eur.16S-455r. The sensitivity of this seminested PCR approach was compared to previously described alternative PCR systems based on amplification of the *ail* (23) and the *yst* (19) genes. Since no data about sensitivity were given by the authors of these earlier reports, we tested these two PCR approaches in comparison to our seminested PCR. A total of 5×10^3 cells per ml could be detected by amplification of the *ail* and the *yst* genes, whereas the semi-

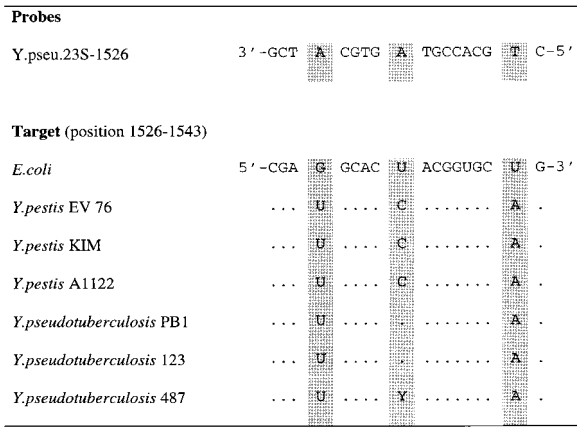

FIG. 2. Alignment of the target region for probe Y.pseu.23S-1526. Numbering of the target positions corresponds to the *E. coli* numbering described in reference 9. Identical nucleotides are represented by dots.

nested-PCR approach presented in this study generated a visible amplicon with 1×10^2 cells per ml.

Sensitivity and specificity of in situ hybridization. Probe Y.16S-69 is targeted to essentially the same rRNA region as primer Y.16S-86f; the theoretical specificity of this probe is therefore the same as that described for primer Y.16S-86f. Primers Y.e.ame.16S-455r and Y.p.16S-455r have also been labelled and tested as hybridization probes. Since these probes failed to hybridize to their respective target organisms, we designed probes Y.ent.16S-184 and Y.p.16S-997 for the detection of *Y. enterocolitica* and the *Y. pestis-Y. pseudotuberculosis* group, respectively.

Probe Y.ent.16S-184 is complementary to all *Y. enterocolitica* sequences but also to some phytopathogenic *Erwinia* species, *Xenorhabdus beddingii*, and *Hafnia alvei* as revealed by a gapped BLAST search (2). The probe target region for Y.p.16S-997 was found in all known *Y. pestis* and *Y. pseudotuberculosis* strains and also in 16S rDNA sequences of two *Erwinia carotovora* strains. For probe Y.pseu.23S-1526–CT, no BLAST matches were identified, although specificity testing showed that all *Y. pseudotuberculosis* strains, some European serotypes of *Y. enterocolitica*, *Y. mollaretii*, *Y. frederiksenii*, and *Serratia fonticola* also hybridized with this probe.

Whereas none of the regions sequenced is suitable for unequivocal identification of the three potentially pathogenic *Yersinia* species, a combination of two different rRNA regions unique for each of these yersiniae could be found. The presence of *Y. enterocolitica* could therefore be unequivocally detected by the combined application of Y.16S-69-FLUOS and Y.ent.16S-184-CT, and *Y. pestis* could be differentiated from *Y. pseudotuberculosis* and other *Yersinia* species by simultaneously hybridizing probes Y.pseu.23S-1526-CT and Y.p.16S-997-FLUOS to the samples. This probe combination was hybridized to three stool samples: no signal was obtained with any of the three samples, although more than 95% of the cells present hybridized with bacterial probe B.16S-338-Cy5. Within the spiked stool samples, *Y. pseudotuberculosis* cells could be easily identified (Fig. 4A). The same probe combination hybridized to specimens prepared from the three throat swabs also unequivocally detected *Y. pestis* within the spiked samples but not within the original samples (Fig. 4B). Probe E.car.16S-636-FLUOS, developed to monitor the presence of *E. carotovora* within the investigated samples, did hybridize to *E. carotovora* but not to any of the *Yersinia* species.

In situ detection of *Yersinia* cells in tissue samples. Spleen and liver cryosections from mice experimentally infected with *Y. enterocolitica* 108-c were hybridized with probe Y.16S-69-CT. All tissue sections showed moderate to strong autofluorescence. Despite such interference, the bacterial cells could be easily detected in all tissues by using confocal laser scanning microscopy, whereas no bacterial cells could be detected in tissue samples hybridized with the negative control probe cB.16S-338-CT. Application of the dual-detector option of the Leica confocal laser scanning microscope (Fig. 4C; left panel) further improved image quality.

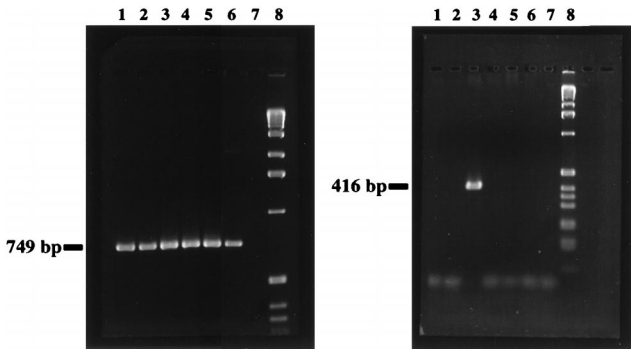

FIG. 3. Result of a seminested-PCR amplification of *Yersinia* rDNA by using primer pair Y.16S-86f–B.16S-794r for the first PCR (left panel) and primer pair Y.16S-86f–Y.e.eur.16S-455r for the second PCR (right panel) analyzed with a 1.5% agarose gel. One microliter of the first PCR mixture served as a template for the second PCR mixture. Lane 1, *Y. pestis* EV 76; 2, *Y. pseudotuberculosis* YPIII; 3, *Y. enterocolitica* Y-108-c; 4, *Y. enterocolitica* WA-314; 5, *Y. intermedia*; 6, *Y. ruckeri*; 7, *H. alvei*; 8, 1-kb ladder (Bethesda Research Laboratories, Eggenstein, Germany).

DISCUSSION

We evaluated the potential of rRNA-targeted PCR and fluorescent in situ hybridization for detection and differentiation of *Yersinia* species with respect to diagnosis of yersiniosis and plague, respectively.

The present analysis of complete and partial 16S rDNA sequences generally confirmed the results of a previous study (20). In contrast to this study was the finding of identical, near-full-length, 16S rDNAs in *Y. pseudotuberculosis* and *Y. pestis*. This is in good concordance with a previous study showing a close relationship between these bacterial species (6) and encouraged us to further investigate a portion of the 23S rRNA which contains the largest variable rRNA region encompassing helices 54 to 59 (16) and a smaller variable region (33) around helix 45. The 23S rRNA sequence data corroborate our 16S rRNA sequences, with the finding of only one base difference between *Y. pestis* and *Y. pseudotuberculosis*. For *Y. pseudotuberculosis* 487, sequencing reactions in both directions indicate that two different bases are present at position 1534. The existence of different rRNA operons within *Y. pseudotuberculosis* 487 is a reasonable explanation for this ambiguity. However, the in situ hybridization results indicate that this sequence ambiguity did not influence the reliable identification of this *Y. pseudotuberculosis* strain by in situ hybridization.

The sequence data obtained from rDNA sequences could be successfully used for the construction of both PCR primers and hybridization probes, allowing a rapid genotype-based detection of *Yersinia* species on different taxonomic levels. Labelled primers Y.e.ame.16S-455r and Y.p.16S-455r did not, however,

hybridize to their target cells in situ. Since these primers perform well in PCR, noncomplementarity of probes and target could be ruled out. The most probable explanation is that RNA-RNA or RNA-protein interactions within 16S rRNA prevent hybridization of the probes. The influence of rRNA higher-order structure on probe-conferred fluorescence after in situ hybridization has long been proposed (4) and has been recently demonstrated (12).

A unique 16S rRNA target region suitable for the detection of *Y. enterocolitica* by in situ hybridization could not be identified. Therefore, an identification approach was developed by employing two different probes with broader specificities. The combined use of probes Y.ent.16S-184 and Y.16S-69 allowed an unequivocal identification of *Y. enterocolitica*. Furthermore, the simultaneous application of probes Y.p.16S-997-FLUOS and Y.pseu.23S-1526-CT proved to be a suitable tool for the differentiation of *Y. pestis* and *Y. pseudotuberculosis*. Nine *Y. pestis* and 19 *Y. pseudotuberculosis* strains were correctly identified by this approach, whereas no false-positive signal was observed with 31 other variably related bacterial species. The only exceptions were some *E. carotovora* strains, which, to our knowledge, have never been isolated from clinical sources. Nonetheless, probe E.car.16S-636 was developed to rule out the presence of this organism within a particular clinical sample. The specificity of this approach was further demonstrated by the successful detection of *Y. pseudotuberculosis* and *Y. pestis* in spiked clinical specimens, since presumably more than 400 bacterial species can be found in stool samples and samples from the oral cavity harbor up to 200 different bacterial species (7).

The potential to detect and identify the closely related yersiniae within 3 h without extensive preparation of nucleic acids from clinical samples is intriguing and may prove useful considering that misidentification of *Y. pestis* as *Y. pseudotuberculosis* and vice versa may occur when identification is based on the detection of capsular antigen F1 or pigmentation (10, 28). Moreover, this investigation showed that *Yersinia* cells infecting different mouse tissues carry enough ribosomes to be detected with fluorochrome-labelled oligonucleotides. The probes readily penetrate tissue samples and bacterial cell walls. This technique is well suited to detect the location of a pathogen within the body, particularly in combination with confocal laser scanning microscopy.

Future studies will evaluate whether in situ hybridization techniques are sensitive enough to detect dormant or metabolically inactive *Yersinia* cells within tissues, where they probably survive intracellularly (18). The importance of this issue stems from the observation that yersiniae associated with chronic infection are often noncultivable (15, 18). However, the sensitivity of in situ hybridization is comparatively low (4). In samples containing fewer than 10^5 cells per ml, more sensitive techniques must be applied. The use of sensitive PCR approaches has been described for the detection of *Yersinia* species (14, 21). Compared to these procedures, the present seminested-PCR assay covers the entire spectrum of pathogenic yersiniae and differentiates *Y. enterocolitica* biotype 1B from other, less pathogenic, *Y. enterocolitica* biotypes with high sensitivity, although rDNA-based PCRs do not discriminate between pathogenic and nonpathogenic *Y. enterocolitica* strains. For this purpose, other published target sequences such as the chromosomal gene for *Y. enterocolitica* heat-stable enterotoxin (*yst* gene) or genes of the virulence plasmid pYV have to be employed in the PCR (19, 21, 23). The *yst*-based PCR, however, showed reduced sensitivity compared to that of our approach, and the latter approaches could lead to false-negative results, since target plasmids can be lost during cultivation.

Both of the molecular methods investigated in this study offer alternatives to more traditional diagnostic methods for detection of yersiniosis. In particular, whole-cell hybridization holds great promise as a rapid, cultivation-independent method for detection of bacterial pathogens within clinical samples.

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FIG. 4. Detection of *Yersinia* species by in situ hybridization. Bars, 10 μm. Binding of at least two differently labelled probes results in distinct mixed colors, as shown in the additive-color illustration (C, right panel). Dual combinations of the red, green, and blue colors result in yellow (green and red), purple (red and blue), and turquoise (green and blue). White is a result of a combination of all three colors. (A and B) The same microscopic fields were viewed by phase-contrast microscopy (left panels) and by epifluorescence microscopy (right panels). Oligonucleotides Y.pseu.23S-1526-CT, Y.p.997-FLUOS, and B.16S-338-Cy5 were simultaneously applied to spiked stool (A) and throat (B) samples. As indicated by the white color, *Y. pseudotuberculosis* hybridized to all three labeled probes, whereas the turquoise color of the *Y. pestis* cells in the throat swab specimen is a result of the simultaneous binding of probes B.16S-338-Cy5 and Y.p.16S-997-FLUOS. (C) In situ detection of *Y. enterocolitica* in spleen sections of an infected mouse (left panel). Tissue sections were hybridized with probe Y.16S-69-CT and detected with the double-exposure option for the green and the red fluorescence of the Leica software package. Single bacterial cells are clearly visible within the spleen sections.

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