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# Evaluation of a Fluorescence-Labelled Oligonucleotide Probe Targeting 23S rRNA for In Situ Detection of *Salmonella* Serovars in Paraffin-Embedded Tissue Sections and Their Rapid Identification in Bacterial Smears

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**A method for the detection of *Salmonella* based on fluorescence in situ hybridization (FISH) has been developed and applied for the direct detection of *Salmonella* in pure cultures and in formalin-fixed, paraffin-embedded tissue sections. On the basis of the 23S rRNA gene sequences representing all of the *S. enterica* subspecies and *S. bongori*, an 18-mer oligonucleotide probe was selected. The specificity of the probe was tested by in situ hybridization to bacterial cell smears of pure cultures. Forty-nine of 55 tested *Salmonella* serovars belonging to subspecies I, II, IIIb, IV, and VI hybridized with the probe. The probe did not hybridize to serovars from subspecies IIIa (*S. arizonae*) or to *S. bongori*. No cross-reaction to 64 other strains of the family *Enterobacteriaceae* or 18 other bacterial strains outside this family was observed. The probe was tested with sections of formalin-fixed, paraffin-embedded tissue from experimentally infected mice or from animals with a history of clinical salmonellosis. In these tissue sections the probe hybridized specifically to *Salmonella* serovars, allowing for the detection of single bacterial cells. The development of a fluorescence-labelled specific oligonucleotide probe makes the FISH technique a promising tool for the rapid identification of *S. enterica* in bacterial smears, as well as for the detection of *S. enterica* in histological tissue sections.**

Different serovars of *Salmonella* have long been recognized as major causes of infections in humans and animals, causing a variety of clinical manifestations ranging from mild gastroenteritis to severe sepsis (13). Clinical infection is often followed by a subclinical carrier state, in which the convalescing individual may continue to shed salmonellae in feces for a longer period (11).

The genus *Salmonella* can be divided into seven homology groups by DNA-DNA hybridization studies (12, 19); however, the genus is a large group with respect to antigenicity, consisting of more than 2,300 described serovars (25). As DNA homology group V diverges from the others, it has been proposed as a new species, *Salmonella bongori* (28). The remaining subspecies, subspecies I, II, IIIa, IIIb, IV, and VI, belong to the species *Salmonella enterica*. Subspecies I is the largest group, with about 59% of the described *Salmonella* serovars (25), including all the clinically most relevant serovars (18).

The interactions of single bacterial strains in complex ecological systems have been studied by fluorescence in situ hybridization (FISH) techniques. By hybridizing with fluorescence-labelled oligonucleotide probes specifically targeting ribosomes in *Escherichia coli* or *Salmonella typhimurium*, the spatial distribution of bacteria in the intestinal flora of streptomycin-treated mice has been established (20, 27). However, to be able to detect a broad range of clinically relevant *Salmonella* serovars by the FISH technique under natural conditions, a genus-specific RNA-targeted probe is necessary. Such a probe has so far not been available.

The ribosomal genes are universally distributed and contain highly conserved regions as well as variable regions, toward which oligonucleotide probes with various specificities can be developed (26, 32). In growing bacteria the gene is transcribed into a high number of ribosomes, and following fixation these can be used as targets for hybridization with short oligonucleotide probes (16). By end labelling such probes with fluorescent molecules, single bacterial cells can be identified by epifluorescence microscopy (14).

The purpose of this study was to generate a specific oligonucleotide probe targeting the different serovars of *S. enterica* by sequencing and aligning 23S rRNA gene sequences and to evaluate the applicability of the probe for FISH identification of *Salmonella* in bacterial smears and detection in formalin-fixed, paraffin-embedded specimens from animals with clinical *Salmonella* infections.

## MATERIALS AND METHODS

**Bacterial strains and culture methods.** The strains used to test the specificity of the oligonucleotide probe are listed in Tables 2 and 3. *Salmonella* strains were either type strains or clinical isolates identified by serotyping at the Danish Veterinary Laboratory (DVL). Other representatives of the family *Enterobacteriaceae* were type strains or clinical isolates identified by using the API 20E system (bioMérieux, Marcy, France). Gram-positive test strains were all type strains. The bacteria used to test probe specificity and for sequencing were all cultured overnight at 37°C on blood agar (BA; CM331 [Oxoid] supplemented with 5% bovine blood). The strains used to inoculate the experimentally infected mice were grown in nutrient broth (CM1 [Difco]) at 37°C overnight. Samples from lung and liver were plated on BA and Drigalski agar (17), and the plates were incubated overnight at 37°C.

**Fixation of bacteria for smear hybridization.** A single colony from an overnight culture was picked and resuspended in 800 μl of 10% buffered formalin, and the mixture was incubated for 1 h at room temperature. The bacteria were pelleted by centrifugation at 5,000 × *g* for 5 min, washed in 500 μl of 0.1% Nonidet P-40 (Sigma Chemical, St. Louis, Mo.), and resuspended in a 1:1 mixture of a storage buffer (40 mM Tris-HCl [pH 7.5], 0.2% Nonidet P-40) and 96% ethanol. The fixed bacteria were stored at −20°C until use.

**Sequencing of rRNA genes of the bacteria.** *S. typhimurium* DVL 3389-1, *Proteus mirabilis* SN157, and *Citrobacter amalonaticus* UX31 were used for partial sequencing of the 23S rRNA gene. For each isolate a single colony was picked and the bacteria were washed in 1 ml of phosphate-buffered saline (140

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mM NaCl, 22.5 mM phosphate buffer [pH 7.2]) and pelleted at 5,000 × *g* for 5 min. The bacteria were resuspended in 100 μl of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), lysed by boiling for 10 min, and immediately cooled on ice. The lysates were diluted 1:100 in double-distilled $H_2O$ ($ddH_2O$), and 2 μl of this solution was added to 50 μl of a PCR mixture consisting of 50 mM KCl, 2.5 mM $MgCl_2$, 10 pmol of each primer, 100 μM (each) dATP, dCTP, dGTP, and dTTP, and 0.5 U of polymerase (Amplitaq; Perkin-Elmer) in 10 mM Tris-HCl (pH 8.3). Primers with homology to *E. coli* were used for PCR amplification of a segment in the 23S rRNA gene; the forward primer, 5′-TCAGAAGTGCGAATGC-3′, was located at position 1243 (*E. coli* numbering), and the reverse primer, 5′-AACTTACCCGACAAGG-3′, was located at position 1941 (9). Amplification was carried out on a thermocycler, with initial denaturation at 94°C for 2 min and subsequent amplification for 34 cycles, with each cycle consisting of annealing at 45°C for 1 min, extension at 72°C for 2 min, and denaturation at 94°C for 2 min. The 698-base PCR product was purified by using QIAquick spin columns (QIAGEN, Hilden, Germany), according to the manufacturer's instructions, and was verified by gel electrophoresis and ethidium bromide staining.

The nucleotide sequence of the PCR product was determined by cycle sequencing (31) with an Amplitaq FS dye terminator kit and a 373A automatic DNA sequencer (Applied Biosystems/Perkin-Elmer, Foster City, Calif.) by using the same primers used for the PCR amplification. The segments were sequenced twice in both directions.

**Selection of probe sequence.** The sequences obtained were aligned with the published *E. coli* 23S *rrnB* sequence (7), together with sequences from *Salmonella* serovars representing all subspecies (8, 10, 37), *Yersinia enterocolitica* (10), and *Citrobacter freundii* (10), by using the Sequence Navigator software (Applied Biosystems). An 18-mer oligonucleotide probe sequence complementary to the region from positions 1713 to 1730 (*E. coli* numbering) was selected as being specific for *Salmonella* subspecies I, IIIb, and VI. The theoretical specificity was checked by using the CHECK_PROBE program in the RDP Database (24) and in the EMBL database by using the Fasta search tool.

**Oligonucleotide probes.** A *Salmonella* oligonucleotide probe (Sal3; 5′-AATC ACTTCACCTACGTG-3′) the universal bacterial probe (Eub338; 5′-GCTGCC TCCCGTAGGAGT-3′) (3), and a nonsense probe with a sequence complementary to Eub338 (Non338; 5′-CGACGGAGGGCATCCTCA-3′) (35) were used. The probes were all synthesized and labelled with fluorescein (Sal3 and Non338) or the red fluorescent dye Cy3 (Eub338) at the 5′ end (Hobolth DNA syntese, Hillerød, Denmark). The systematic name of the salmonella probe was L-S-Sal-1713-a-A-18 (2); however, for reasons of simplicity it is designated Sal3 in this report.

**Whole-cell hybridization.** The specificity of Sal3 was tested by hybridization with whole bacterial cells. The universal bacterial probe Eub338 served as a positive control. Six-well Teflon-coated slides (NovaKemi, Enskede, Sweden) were coated with poly-L-lysine (Sigma Chemical) according to the manufacturer's instructions. A 1-μl solution of fixed bacteria was spotted in the wells and air dried. Bacteria were dehydrated in 70 and 96% ethanol for 2 min at each concentration. After drying, 8 μl of hybridization solution (0.7 M NaCl, 0.1 M Tris-HCl [pH 8.0], 0.1% sodium dodecyl sulfate, 10 mM EDTA) containing 5 ng of probe per μl was added, and the slide was placed in a moisture chamber and incubated for 3 h at 45°C. Washing was performed by immersing the slide gently in $ddH_2O$, and this was followed by incubation in preheated hybridization buffer at 45°C for 20 min. Finally, the slide was rinsed in $ddH_2O$ and air dried. The slides were mounted in a phosphate-buffered (pH 8.5) medium; the medium contained 20 mg of *n*-propyl gallate (Sigma Chemical) per ml to prevent fading of the fluorescence. The hybridized bacteria were visualized by epifluorescence microscopy at ×400 or ×1,000 magnification on a Leica DMRB microscope equipped with a 100-W mercury lamp and an I3 filter (Leica, Wetzlar, Germany) for excitation at 450 to 490 nm. Nonhybridized bacteria were hardly detectable and were identified by phase-contrast microscopy. Images were captured with a Sony (Tokyo, Japan) DXC-930P 3CCD video camera and a Oculus TCX frame grabber (Coreco Inc.). Pictures were processed as tagged-image file format (TIFF) files on a personal computer running Image-Pro software, version 1.3 (Media Cybernetics, Silver Springs, Md.).

**Murine experimental infections and clinical material.** Monoinfected murine lung and liver tissue samples were prepared by inoculation of bacteria in the lateral tail vein as described by Tegtmeier et al. (33). *S. typhimurium* DVL 3389-1 and *E. coli* ATCC 25299 were used for the inoculations. Bacteria from 10 ml of nutrient broth were harvested and washed in normal saline (0.9% NaCl). The suspensions were adjusted to $10^9$ CFU/ml by resuspending them in normal saline, and the mice received a dose of 0.5 ml. Six-week-old female Balb/c mice were inoculated with either *E. coli* or *S. typhimurium*; controls received only normal saline. After 10 min, the mice were sacrificed by cervical dislocation and their lungs and livers were aseptically removed and placed in a sterile petri dish. To verify the monoinfection with the inoculated strain, the organism was reisolated from each organ by plating on BA and Drigalski agar. A sample from each organ was fixed in 10% buffered formalin for 3 days, embedded in paraffin, cut in 3-μm slides, and mounted on coated microscope slides (Superfrost+; Menzel-Gläser, Braunschweig, Germany).

Samples from pigs and calves with pneumonia or enterocolitis were received at the pathological laboratory at DVL for diagnostic purposes. The samples were delivered unfixed by ordinary mail and were fixed in buffered formalin upon receipt.

**In situ hybridization of tissue specimens.** Tissue sections from the experimentally infected mice or histological sections of clinical material were tested for *Salmonella* by hybridization with Sal3 and Eub338. To test for nonspecific binding the nonsense probe Non338 was used. The slides were dewaxed twice in xylol for 5 min each time and in 99% ethanol for 5 min. With a DAKO-pen (DAKO, Glostrup, Denmark) a circle was drawn around the tissue specimen, and 20 μl of hybridization buffer (0.7 M NaCl, 0.1 M Tris-HCl [pH 8.0], 0.1% sodium dodecyl sulfate, 10 mM EDTA) containing 5 ng of oligonucleotide probe per μl was added. For labelling of all bacteria in the clinical specimen a mixture of fluorescein-labelled Sal3 and Cy3-labelled Eub338 was added to the same hybridization buffer. The slides were placed in a humid chamber and incubated for 16 h at 45°C. Washing, mounting, and detection were performed as described above for whole-cell hybridization. Fluorescent bacteria were detected in the tissue by epifluorescense microscopy with the G/R filter combination (Leica) for the simultaneous excitation of fluorescein and Cy3 at 490 and 575 nm.

**Nucleotide sequence accession numbers.** The sequence data have been deposited in GenBank under accession nos. U88706 (*S. typhimurium* NVL3389-1), U88707 (*C. amalonaticus* UX31), and U88708 (*Proteus mirabilis* SN157).

## RESULTS

**rRNA gene sequencing.** The middle parts of the 23S rRNA genes (*E. coli* positions 1243 to 1841) of *S. typhimurium*, *C. amalonaticus*, and *P. mirabilis* were sequenced and aligned with homologous sequences from other serotypes and species of the family *Enterobacteriaceae*. Two regions with high sequence variability were identified at positions 1470 to 1590 and positions 1710 to 1750, respectively. The homologous sequence from each strain showed a high degree of similarity to the sequence of the *E. coli* 23S *rrnB* gene (7), confirming the close relationship among members of the family *Enterobacteriaceae*. When calculating absolute similarity, including the similarities of hypervariable regions, the *S. typhimurium* sequence was the most closely related to the *C. amalonaticus* sequence, with a similarity of 98%, and to the *E. coli* sequence, with a similarity of 97%, while it was more divergent from the *P. mirabilis* sequence, with a similarity of 90%. When the *S. typhimurium* sequence was aligned with homologous sequences from the other *Salmonella* subspecies, the absolute similarity, including the similarities of hypervariable regions, were 97 to 99.5%, with the highest similarity being to the sequence of subspecies I.

It was not possible to generate a single probe with a sequence that matched those of serovars of *S. enterica* for this part of the 23S gene. Instead, a probe matching the sequences of *Salmonella* subspecies I, IIIb, and VI but with a single mismatch for *Salmonella* subspecies II and IV and two mismatches for *Salmonella* subspecies IIIa was selected. A list of the base compositions within the target area is presented in Table 1. The target sequences were similar within each subspecies with the exception of the sequences of *S. tennessee* and *Salmonella* subspecies II (*S. basel*), which each diverged by one base. The sequences of nonsalmonellae all diverged by at least two mismatches.

**Whole-cell hybridization.** The sensitivity and specificity of the probe were tested by hybridization with fluorescence-labelled probes on smears of formalin-fixed whole cells (Fig. 1A). All strains except *Staphylococcus aureus* were able to hybridize with Eub338. Sal3 was tested against 86 *Salmonella* isolates covering 55 serovars from subspecies I to VI. By using low-stringency hybridization and washing conditions, the probe hybridized and yielded a strong signal for 80 of 86 *Salmonella* isolates tested (Table 2). As predicted from the alignment of the sequence in the target area (Table 1), the probe hybridized to the sequences of the tested serovars of subspecies I, II, IIIb, IV, and VI, although the sequences of serovars of subspecies II and IV had one mismatch. No hybridization was obtained with the four strains of subspecies IIIa, one strain of subspecies V, and one strain of subspecies II (*S. basel*), which all had two to

TABLE 1. Sequence variation in the 23S rRNA genes of different *Enterobacteriaceae* species at *E. coli* positions 1713 to 1730

| Probe or species and serovar (subspecies) | Strain | GenBank accession no. | Sequence in helix 63[a] |
|---|---|---|---|
| Sal3 probe | | | 3′-GTGCATCCACTTCACTAA-5′ |
| *S. enterica* | | | |
| *S. typhimurium* (I) | DVL3389-1, JEO14, JEO294 | U88706, U77920 | 5′-CACGTAGGTGAAGTGATT-3′ |
| *S. typhi* (I) | ATCC 167 | U04734 | .................. |
| *S. dublin* (I) | K771, K228, JEO71 | U77919 | .................. |
| *S. tennessee* (I) | JEO338 | —[b] | ..T............... |
| *Salmonella* subspecies (II) [1,9,12:1,w:e,n,x],[40:d:-] | NSC72, S114655 | U77921 | ..............G.. |
| *S. basel* (II) | JEO297 | — | ..T...........G.. |
| *S. arizona* (IIIa) | S83769, u24 | U77924, U77923 | ............C.G.. |
| *Salmonella* subspecies IIIb [61:i:z],[48:r:z],[60:r:z] | JEO307, JEO823, S109671 | U77922 | .................. |
| *S. houten* (IV) | S84366, S84098 | U77926 | ..............G.. |
| *S. ferlac* (VI) | BR2047 | U77929 | .................. |
| *S. bongori* | | | |
| *S. brookfield* (V) | BR1859 | U77927 | ..T.........C.G.. |
| *E. coli* | | J01695 | T.T..........CCC. |
| *C. freundii* | JEO503 | U77928 | T.T...........G.. |
| *C. amalonaticus* | UX31 | U88707 | T.T..........C... |
| *P. mirabilis* | SN157 | U88708 | ..-......A....CCC.[c] |
| *Y. enterocolitica* | JEO2341 | U77925 | ............C.G.. |

[a] *E. coli* numbering (positions 1713 to 1730) was used.
[b] —, strains were from reference 8.
[c] —, gap in the sequence made by a deletion in the gene.

three mismatches (Table 1). No cross hybridization was observed to any of 64 other representatives of the family *Enterobacteriaceae* tested or to 18 bacterial strains outside of the family *Enterobacteriaceae* which might occur in the intestinal tract of pigs and humans (Table 3).

**In situ hybridization of tissue specimens.** To test the abilities of the probes to hybridize in situ in paraffin-embedded material, murine tissue experimentally infected with either *S. typhimurium* or *E. coli* was prepared. Only tissue from monoinfected organs verified by bacterial culturing was used in the test. In tissue sections from *S. typhimurium*-infected mice, a distinct fluorescent signal from rod-shaped bacteria could be detected when hybridization with Sal3 was performed. Bacteria could be identified as single cells in the liver sinusoids or in the vessels of the lung, as expected from the hematogenous spread of the bacteria (Fig. 1B). The same result was achieved when hybridization with Eub338 was performed. In the tissue from *E. coli*-infected mice, only Eub338 hybridized to bacteria in the tissue, and no signal was obtained when hybridization with Sal3 was performed. To test for nonspecific binding of the probe, tissue sections were incubated with a nonsense probe, probe Non338, which contains the a base sequence complementary to that of Eub338 and which is unable to hybridize to ribosomes. When using this probe no signal was detected at all, confirming the specificities of the probes.

The probes were also tested with formalin-fixed histological sections from 15 pigs and calves with histories of pneumonia or enterocolitis in which *S. typhimurium* or *S. dublin* was found by traditional culture methods (data not shown). For histological sections from all animals the salmonella probe hybridized specifically, and *Salmonella* bacteria could be seen either as clusters of cells in the necrotic debris or as single cells scattered throughout the inflamed tissue. In a few instances single bacteria were detected intracellularly as well. In order to show the spatial localization of salmonella in relation to the indigenous microflora, the fluorescein-labelled Sal3 probe was used together with a Cy3-labelled universal probe in the same slide (Fig. 1C). *Salmonella* bacteria were detected deep in the lamina propria and in the superficial debris, while the indigenous microflora were shown to be localized only superficially in the debris. The number of *Salmonella* bacteria detected in each section varied. In some sections only a few microcolonies were found, while in others numerous bacterial clusters were detected.

For five samples in which *Salmonella* had not been detected by culturing, only Eub338 hybridized to the bacteria. No unspecific binding of the probe was observed when hybridization with the nonsense probe in the clinical material was performed.

## DISCUSSION

In the present study a specific oligonucleotide probe was developed for the in situ detection of *S. enterica* serovars in bacterial smears and in tissue sections by the FISH technique. Selection of the probe sequence was based upon alignment of a sequence segment in the 23S rRNA gene from several salmonella serotypes and other enterobacterial strains. This segment was selected because it contains two regions which have previously been shown to exhibit high degrees of sequence variability in bacteria (9, 34). The stringency of the hybridization conditions was chosen so that it would not discriminate between strains with one mismatch, because this allows for the specific detection of the largest group of *S. enterica* but does

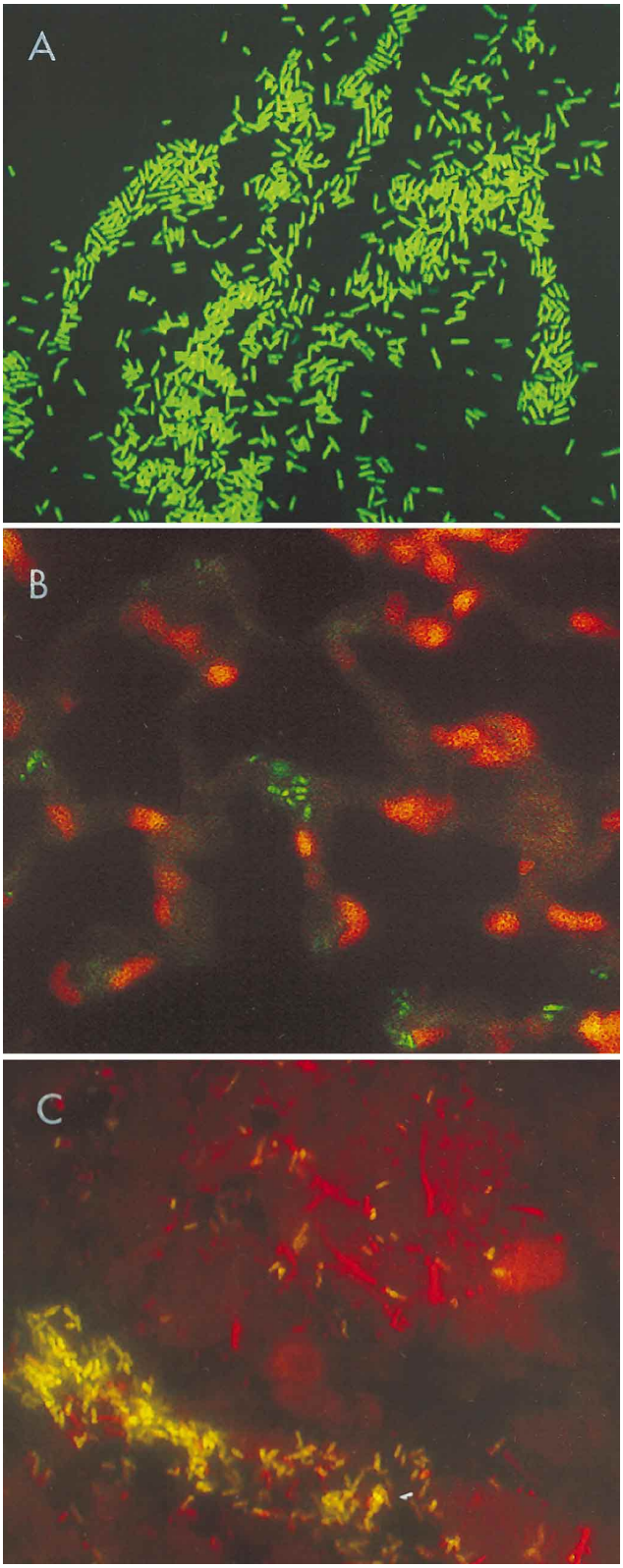


FIG. 1. (A) A smear of *S. typhimurium* hybridized with the fluorescein-labelled salmonella probe (Sal3). (B) Formalin-fixed tissue section of murine lung tissue experimentally infected with *S. typhimurium* and hybridized with Sal3, showing rod-shaped green fluorescent bacteria in the small capillaries of the lung tissue and red autofluorescing erythrocytes. (C) Tissue section from colon of a pig with salmonellosis. By hybridizing with a mixture of Sal3 labelled with fluorescein and the universal bacterial probe (Eub338) labelled with Cy3, the spatial localization of salmonella (yellow) can be determined in proportion to the indigenous microflora (red). The color of salmonella is changed as it hybridizes with both probes. Magnifications, ×450.

not allow for cross hybridization to any of the closely related *Citrobacter* species or *E. coli*. Most important, all tested serovars from subspecies I hybridized with Sal3, because members of this subspecies constitute the most commonly encountered and the most pathogenic serovars (5, 18).

The separation of serovars into seven homology groups or subspecies is based on DNA-DNA hybridization studies (12, 19). The same separation of serovars has been found when comparing 23S rRNA gene sequences (10); the variations were low within genes from serovars of the same subspecies, but they were three times higher when serovars from different subspecies were compared. On the basis of the assumption that the genetic sequence is highly conserved within each subspecies, the specificity of the probe was tested against 55 serovars representing all seven subspecies. This test showed a high degree of conservation in the probe target area, because the probe hybridized only to the subspecies expected from the alignment shown in Table 1. However, one strain from subspecies II failed to hybridize because this strain contained two mismatches instead of one, as for the other subspecies II strains.

Several DNA probes used for the detection of *Salmonella* in food or feces have been published (1, 15, 23). Most probes target chromosomal or plasmid genes, but oligonucleotide probes targeting the 16S and 23S rRNA have also been published (21, 30, 36). Those probes have been applied in detection assays based on denaturated DNA or RNA bound to membranes, eliminating the possibility of the detection and spatial localization of single bacterial cells in tissue samples.

Hybridization to ribosomes in situ is highly dependent on target accessibility (4). Secondary and tertiary structures in the cells, as well as protein binding, might prevent hybridization to the ribosome (4), thus making specific probes meant for other applications useless for the detection of bacteria by the FISH technique. The target sequence of Sal3 is located at helix 63, and in this study we have shown it to be accessible for in situ hybridization of *Salmonella.*

Fixation is another crucial step for in situ hybridization (4, 22). The fixative serves the dual purposes of opening up the bacterial wall for probe penetration and protecting the ribosomes from degradation by endogenous RNase activity. Aldehyde fixatives are most frequently used with gram-negative bacteria (14), whereas alcohol fixation is recommended for gram-positive bacteria (6, 29). In our hands, formalin fixation enabled hybridization of both gram-negative and gram-positive bacteria. The exception was *S. aureus*, for which hybridization with Eub338 was not achieved, probably due to the low level of permeability of the cell wall.

The use of short oligonucleotide probes reduces the time and temperature required for hybridization. By increasing the probe concentration the velocity of the hybridization is further increased; however, this also increases nonspecific staining (35). When using an intermediate probe concentration of 1 to 5 ng/μl, the ribosomes in *E. coli* were saturated after 2 h of incubation with Eub338 (35). When hybridizing to pure cultures, 3 h of incubation was used, with excellent results. However, for reasons of convenience, the tissue samples were hybridized overnight.

To enhance the level of discrimination of the signal from single bacteria in tissue sections, we applied a narrow-band-

TABLE 2. Strains of *Salmonella* tested by in situ hybridization with fluorescence-labelled probes[a]

| Species | Subspecies[b] | Serovar | Strain identification number | No. of strains hybridizing with the following probe: | |
|---|---|---|---|---|---|
| | | | | Sal3 | Eub338 |
| *S. enterica* | I | *S. adabraka* | SN228 | 1 | 1 |
| | | *S. agona* | SN246 | 1 | 1 |
| | | *S. anatum* | SN195, SN244 | 2 | 2 |
| | | *S. berta* | JEO1721 | 1 | 1 |
| | | *S. blockley* | SN229 | 1 | 1 |
| | | *S. bredeney* | SN225, SN224 | 2 | 2 |
| | | *S. choleraesuis* var. *kunzendorf* | SN168 | 1 | 1 |
| | | *S. derby* | SN202, SN203 | 2 | 2 |
| | | *S. dublin* | GRI34285, SN158, SN245 | 3 | 3 |
| | | *S. enteritidis* | JEO1722, SN163 | 2 | 2 |
| | | *Salmonella* 4,12,b.− | SN196, SN197 | 2 | 2 |
| | | *S. hadar* | SN200, SN201 | 2 | 2 |
| | | *S. havana* | SN193 | 1 | 1 |
| | | *S. indiana* | SN243, SN227 | 2 | 2 |
| | | *S. infantis* | SN159, SN204, SN205 | 3 | 3 |
| | | *S. isangi* | SN248 | 1 | 1 |
| | | *S. kottbus* | SN230, SN242 | 2 | 2 |
| | | *S. livingstone* | SN192 | 1 | 1 |
| | | *S. mbandaka* | SN198, SN199 | 2 | 2 |
| | | *S. meleagridis* | SN232 | 1 | 1 |
| | | *S. montevideo* | SN206, SN207 | 2 | 2 |
| | | *S. newport* | S.91389 | 1 | 1 |
| | | *S. ohio* | SN250 | 1 | 1 |
| | | *S. paratyphi* B var *java* | SN259 | 1 | 1 |
| | | *S. pomona* | SN194 | 1 | 1 |
| | | *S. pullorum* | L.41.694 | 1 | 1 |
| | | *S. senftenberg* | SN231, SN249 | 2 | 2 |
| | | *S. tennessee* | JEO338, SN222, SN223 | 3 | 3 |
| | | *S. typhimurium* | NVL3389-1, SN154, SN155, SN158, SN166, SN253, SN254, SN255, SN256, SN257, SN258, SN259, SN260, NVL810 | 14 | 14 |
| | | *S. virchow* | SN226 | 1 | 1 |
| | | *S. worthington* | SN233, SN247 | 2 | 2 |
| | II | | JEO827, JEO822, JEO820, JEO297, NSC72, S114655 | 5 | 6 |
| | IIIa | | S.83769, JEO1691, JEO1690, JEO792 | 0 | 4 |
| | IIIb | | JEO307, JEO821, JEO1831, JEO823 | 4 | 4 |
| | IV | | JEO826, JEO829, JEO817, JEO824, JEO818, JEO825, S.84098, JEO1676 | 8 | 8 |
| | VI | | BR2047 | 1 | 1 |
| *S. bongori* | V | *S. brookfield* | BR1859 | 0 | 1 |

[a] A total of 55 serovars were tested.; 80 of 86 and 86 of 86 strains hybridized with probes Sal3 and Eub338, respectively.
[b] As proposed by Reeves et al. (28).

pass filter combination which allows for simultaneous excitation in two separate bands. This filter allowed for the simultaneous spatial visualization of both salmonella bacteria and the indigenous microflora when hybridization was done with two probes. It also reduced autofluorescence from tissue considerably by turning the color of the tissue red and thereby allowing for the detection of single bacteria (Fig. 1B). When a standard fluorescein isothiocyanate filter was used, only clusters of bacteria could be detected against a background of green autofluorescence.

TABLE 3. Members of the family *Enterobacteriaceae* and selected gram-positive strains tested by whole-cell hybridization with fluorescence-labelled oligonucleotide

| Species | No. of strains | No. of strains hybridizing with the following probe/total no.: | |
|---|---|---|---|
| | | Sal3 | Eub338 |
| *Aeromonas hydrophila* | 1 | 0 | 1 |
| *Citrobacter amalonaticus* | 3 | 0 | 3 |
| *Citrobacter diversus* | 3 | 0 | 3 |
| *Citrobacter freundii* | 16 | 0 | 16 |
| *Citrobacter koseri* | 1 | 0 | 1 |
| *Enterobacter aerogenes* | 1 | 0 | 1 |
| *Enterobacter asburiae* | 1 | 0 | 1 |
| *Enterobacter agglomerans* | 1 | 0 | 1 |
| *Enterobacter cloacae* | 1 | 0 | 1 |
| *Enterobacter sakazakii* | 1 | 0 | 1 |
| *Enterobacter tailorae* | 1 | 0 | 1 |
| *Erwinia herbicola* | 1 | 0 | 1 |
| *Escherichia coli* | 13 | 0 | 13 |
| *Hafnia alvei* | 2 | 0 | 2 |
| *Klebsiella oxytoca* | 1 | 0 | 1 |
| *Klebsiella pneumoniae* | 1 | 0 | 1 |
| *Moganella morganii* | 1 | 0 | 1 |
| *Proteus mirabilis* | 1 | 0 | 1 |
| *Proteus vulgaris* | 1 | 0 | 1 |
| *Providencia heimbachae* | 1 | 0 | 1 |
| *Providencia stuartii* | 1 | 0 | 1 |
| *Pseudomonas aeroginosa* | 1 | 0 | 1 |
| *Pseudomonas alcaligenes* | 1 | 0 | 1 |
| *Serratia marcescens* | 1 | 0 | 1 |
| *Serratia oderiferi* | 1 | 0 | 1 |
| *Shigella sonnei* | 1 | 0 | 1 |
| *Yersinia enterocolitica* | 3 | 0 | 3 |
| *Yersinia frederiksenii* | 1 | 0 | 1 |
| *Yersinia intermedia* | 1 | 0 | 1 |
| *Yersinia kristensenii* | 2 | 0 | 2 |
| *Yersinia pseudotuberculosis* | 1 | 0 | 1 |
| *Yersinia ruckeri* | 1 | 0 | 1 |
| *Vibrio alginolyticus* | 1 | 0 | 1 |
| *Bacillus cereus* | 1 | 0 | 1 |
| *Campylobacter jejuni* | 5 | 0 | 5 |
| *Enterococcus faecalis* | 1 | 0 | 1 |
| *Enterococcus faecium* | 1 | 0 | 1 |
| *Erysipelothrix rhusiopathiae* | 1 | 0 | 1 |
| *Listeria monocytogenes* | 1 | 0 | 1 |
| *Staphylococcus aureus* | 1 | 0 | 0 |
| *Streptococcus equi* | 1 | 0 | 1 |
| *Streptococcus canis* | 1 | 0 | 1 |
| *Streptococcus suis* | 1 | 0 | 1 |
| Total | 82 | 0/82 | 81/82 |

The theoretical sensitivity of detection by the FISH technique is one bacterium in a 3-μm-thick tissue section; however, the number of bacteria in each tissue section from the clinical material varied significantly. By performing hybridization with tissue sections, we were able to detect salmonella bacteria in all the samples which were found to be positive by traditional culture methods. However, when dealing with very low numbers of bacteria, the culture method might still be the most sensitive method because it contains a preenrichment step.

Direct detection of bacteria is highly dependent on the amount and stability of the target, because the intensity of the signal is proportional to the rRNA content (14). Used as a research tool, the FISH technique has previously been applied for studying the spatial distribution of *E. coli* or *S. typhimurium* in the intestines of monoinfected mice (20, 27). This was done under optimal research conditions. We found that this technique is also applicable for the detection of *Salmonella* in clinical samples. The detection of *Salmonella* bacteria in tissue was possible even after the paraffin-embedding process and in the presence of indigenous microflora. Autolysis of the tissue, as a result of the material being shipped unfixed to the laboratory, did not hinder the detection of the bacteria in our study.

In this study we have developed and tested a fluorescence-labelled oligonucleotide probe for the specific detection of *S. enterica* in clinical samples by in situ hybridization. The stability of the ribosome target allowed for the detection of single cells even in clinical material, as well as identification in smears of pure cultures. The results obtained by use of a readily synthesized fluorescence-labelled oligonucleotide probe, combined with a simple hybridization protocol, therefore suggest that the FISH technique can be used for the detection of *S. enterica* serovars.

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