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# A microbial diagnostic microarray technique for the sensitive detection and identification of pathogenic bacteria in a background of nonpathogens

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**Abstract**

A major challenge in microbial diagnostics is the parallel detection and identification of low-bundance pathogens within a complex microbial community. In addition, a high specificity providing robust, reliable identification at least at the species level is required. A microbial diagnostic microarray approach, using single nucleotide extension labeling with *gyrB* as the marker gene, was developed. We present a novel concept applying competitive oligonucleotide probes to improve the specificity of the assay. Our approach enabled the sensitive and specific detection of a broad range of pathogenic bacteria. The approach was tested with a set of 35 oligonucleotide probes targeting *Escherichia coli*, *Shigella* spp., *Salmonella* spp., *Aeromonas hydrophila*, *Vibrio cholerae*, *Mycobacterium avium*, *Mycobacterium tuberculosis*, *Helicobacter pylori*, *Proteus mirabilis*, *Yersinia enterocolitica*, and *Campylobacter jejuni*. The introduction of competitive oligonucleotides in the labeling reaction successfully suppressed cross-reaction by closely related sequences, significantly improving the performance of the assay. Environmental applicability was tested with environmental and veterinary samples harboring complex microbial communities. Detection sensitivity in the range of 0.1% has been demonstrated, far below the 5% detection limit of traditional microbial diagnostic microarrays.


*Keywords:* Microbial diagnostic microarray; *gyrB*; Sequence-specific end labeling of oligonucleotides; Pathogen detection; MDM

Microarrays are genomic tools originally developed to monitor gene expression, also applied for the detection of specific mutations in DNA sequences, and lately employed in the parallel detection and identification of microorganisms in environmental or clinical samples. Although the use of microbial diagnostic microarrays (MDMs)[1] has increased lately [1–12], environmental-scale application still presents significant challenges with regard to specificity, sensitivity, and quantification [13].

MDM sensitivity can be defined in different ways: the amount of nucleic acid needed for successful detection, the ratio of microbial nucleic acid to background (i.e., host nucleic acid), and the ratio of target to nontarget microbial nucleic acid. The latter type of sensitivity threshold, directly related to the relative abundance of the target microbe(s) within the microbial community, is the one most frequently limiting the applicability of MDMs. The current reported sensitivity threshold of MDMs lies in the range of 1–5% relative abundance [2,11,14]. MDMs for microbial community analysis need to be capable of detecting microbes from a broad phylogenetic range. In turn, their application can

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[1] *Abbreviations used:* MDMS, microbial diagnostic microassays; SAP, shrimp alkaline phosphatase; SSC, standard saline citrate; SSELO, sequence-specific end labeling of oligonucleotides; WMM, weighted mismatch.

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tolerate a relatively low sensitivity with regard to relative abundance. On the other hand, high specificity and sensitivity are primary requirements for MDMs applied for pathogen detection. Such MDMs, used in clinical, veterinary, food, and public health microbiology, typically rely on species- or genus-specific PCR amplifications to increase sensitivity and specificity of detection [5,15,16]. As a drawback, they are then limited to a narrow range of pathogens. To cover a range of different pathogens, this approach requires multiple or multiplex PCRs to be performed, ultimately limiting the number of pathogens that can be targeted. Epidemiological and public health surveys in addition to the screening of veterinary, food, or water samples for pathogens would greatly benefit from a different method combining broad coverage with high specificity and increased sensitivity. The specificity of MDMs is defined predominantly by the degree of conservation of the marker gene and the length of the oligonucleotide probe [25]. The *gyrB* gene, encoding the subunit B of the bacterial gyrase, meets all the requirements for a phylogenetically useful protein-coding gene: it can be found in most, bacterial species and does not appear to be frequently horizontally transmitted. Furthermore, the rate of *gyrB* evolution not only is faster than that of the ribosomal genes but also appears fast relative to other protein-coding housekeeping genes (http://seasquirt.mbio.co.jp) [17]. Short oligonucleotide probes enable the discrimination of single nucleotide differences. In combination with a high-resolution phylogenetic marker, an optimal performance can be achieved with regard to MDM specificity.

Here we report on the development of an MDM approach for the sensitive and specific detection of a broad range of pathogenic bacteria from samples harboring complex microbial communities.

## Materials and methods

### *Probe design and microarray fabrication*

The *gyrB* sequence database was established by downloading sequences from the NCBI database (http://www.ncbi.nlm.nih.gov) and by sequencing the *gyrB* genes of those strains used for microarray validation. Alignment and neighbor-joining phylogenetic tree of the gyrB sequences were constructed using the ARB software package [18], which was subsequently also used for the probe design. The most important factor considered during probe design was the placement of the diagnostic mismatch(es) as close to the 3′ end as possible. This feature is very important for the sequence-specific end labeling of oligonucleotides (SSELO). Furthermore, all probes were designed with a 3′ terminal cytosine residue that was added via terminal labeling of the oligonucleotides (upon the presence of a corresponding template). Other factors considered during probe design were similar melting temperature (targeted 60 °C), and length between 17 and 28 nucleotides. Outputs of the Probe Match function were imported into CalcOligo 2.03 [19]. CalcOligo was used to create an Excel table indicating predicted melting temperatures (based on the nearest neighbor model and SantaLucia parameters [20]), length and GC content of the probes, and number of weighted mismatches between each probe–target pair. Nearest neighbor $T_m$ values were calculated with concentration settings of 250 nmol for oligonucleotide and 50 mmol for $Na^+$. Factors for weighting mismatches in CalcOligo were as follows: positions, 5′ 1st 0.3; 5′ 2nd 0.6; 5′ 3rd 0.8; 3′ 1st 4.0; 3′ 2nd 2.0; 3′ 3rd 1.2; all other positions 1.0; basepairs, dArC 1.2; dTrC 1.2; dGrU 0.7; dTrG 0.4; all other mismatched basepairs 1.0 (where "d" refers to the probe on the array and "r" to the target sequence). Probe–target pairs with weighted mismatch values of up to 0.5 were expected to yield positive hybridization under the conditions applied. A complete list of the oligonucleotides used in this study can be found in Table 1. Control oligonucleotide (Msi_294) targeting *pmoA* gene of the *Methylosinus trichosporium* OB3b was taken from the diagnostic microarray for methanotrophs [2].

Oligonucleotides for immobilization were custom synthesized (VBC Genomics, Vienna, Austria) with a 5′ primary amino group, followed by a C6 spacer and five thymidine residues preceding the probe sequence. A 384-well flat bottomed plate (Ritter GmbH, Schwabmünchen, Germany) was prepared with 30 µl of 50 µM oligonucleotide solution in ArrayIt spotting buffer (TeleChem Inc., Sunnyvale, CA, USA). Microarrays were spotted with an OmniGrid spotter (1 TeleChem SMP3 pin) at 55% relative humidity and 21 °C onto aldehyde silylated slides (CEL Associates Inc., Pearland, TX, USA). Arrays were spotted in triplicate to allow a statistical correction of the errors. Slide processing was carried out as described before [19]. Processed slides were stored desiccated at room temperature in dark.

### *DNA templates*

Strains used for microarray validation are listed in Table 2. Genomic DNA samples from pure cultures were purified using the DNeasy extraction kit (Qiagen, Hilden, Germany), following manufacturer's instructions. Archived veterinary samples used for the testing of the method's performance were kindly provided by Clyde Hutchinson (Institute of Zoology, London, UK). These DNA samples were prepared from animal specimens known to be contaminated with one or more bacterial pathogens using the DNeasy extraction kit (Qiagen). Genomic DNA from soil samples was extracted using UltraClean Soil DNA Kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions.

### *DNA amplification*

An approximately 1200-bp-long fragment of the *gyrB* gene was amplified using universal primers UP1 (5′-GAA GTC ATC ATG ACC GTT CTG CAY GCN GGN GGN AA RTT YGA-3′) and UP2r (3′-TACTGNCTRCGNCTRCA

Table 1
Oligonucleotide probe set

| Name[a] | Intended specificity | RC oligonucleotide sequence (5′ – 3′) | L | $T_m$ | GC% |
|---|---|---|---|---|---|
| Mav_1653 | *Mycobacterium* group III[b] | CGACATCTTCGAGACCACCGAGTA | 24 | 61.9 | 56.0 |
| Mav_1657 | *Mycobacterium* group III[b] | ATCTTCGAGACCACCGAGTACGA | 23 | 61.3 | 54.2 |
| Mav_1659 | *Mycobacterium* group III[b] | CTTCGAGACCACCGAGTACGACTT | 24 | 61.8 | 56.0 |
| Mtb_2112 | *Mycobacterium* group I[c] | GAACAAGTACGCCAAGGACCG | 21 | 61.8 | 59.1 |
| Hpy_1516 | *Helicobacter pylori* | GATGAAAGCGTGATGGAAGT | 20 | 55.0 | 47.6 |
| Hpy_1647 | *Helicobacter pylori* | TTTCTATGAAGACGGCTTGAAACAATT | 27 | 58.1 | 35.7 |
| Hpy_1960 | *Helicobacter pylori* | GTGAGCTTGAAAATGAGCGAGC | 22 | 60.3 | 52.2 |
| Hpy_2242 | *Helicobacter pylori* | GAGAGTGAAATCTTTTTAGTGGAGGG | 26 | 59.3 | 44.4 |
| Prt_1882 | *Proteus* spp. | AACTATATGGATAAAGAAGG | 20 | 48.3 | 33.3 |
| Prt_2152 | *Proteus* spp. | GCGCGTAAAGCACGTGAGATGA | 22 | 62.1 | 56.5 |
| Sal_1451 | *Salmonella* spp. | AGGCACCCCTGGCCGT | 16 | 63.5 | 76.5 |
| Sal_1457 | *Salmonella* spp. | CCCTGGCCGTCACTGG | 16 | 61.6 | 76.5 |
| Sal_1950 | *Salmonella* spp. | AGGTCTGATTGCGGTGGTTTC | 21 | 61.1 | 54.5 |
| Eco_1402 | *Escherichia* spp. and *Shigella* spp. | CAGCGCGAGGGTAAAATTCAC | 21 | 60.0 | 54.5 |
| Eco_1404 | *Escherichia* spp. and *Shigella* spp. | GCGCGAGGGTAAAATTCACCGT | 22 | 62.0 | 56.5 |
| Eco_1472 | *Escherichia* spp. and *Shigella* spp. | GCGAGACTGAAAAAACCGG | 19 | 58.1 | 55.0 |
| Eco_1521 | *Escherichia* spp. and *Shigella* spp. | AACCTTCACCAATGTGACCGAGTT | 24 | 61.0 | 48.0 |
| Aer_1195 | *Aeromonas* spp. | TTCTGGCCGAGCCCGAC | 17 | 63.1 | 72.2 |
| Aer_1374 | *Aeromonas* spp. | GAACCAGAACAAGACCCCGATC | 22 | 60.9 | 56.5 |
| Aer_1585 | *Aeromonas* spp. | GAGGATTACAGCAAGAAGGCCAAGT | 25 | 61.2 | 50.0 |
| Aer_1984 | *Aeromonas* spp. | GCCAAGCAGGGTCGCAA | 17 | 60.3 | 66.7 |
| Cam_1556 | *Campylobacter* spp. | TTTTGGCTAAAAGATTTCGTGAACTTG | 27 | 59.1 | 35.7 |
| Cam_2027 | *Campylobacter* spp. | CTTATGTGCGTCCTATAGTTTCAAAAG | 27 | 58.1 | 39.3 |
| Cam_2221 | *Campylobacter* spp. | GCTGATTGTCAAAGTAAAGATC | 22 | 53.4 | 39.1 |
| Cje_2000 | *Campylobacter jejuni* | GACAAACCAAAGGAAAACTTGGTTCAA | 27 | 59.5 | 39.3 |
| Cje_2016 | *Campylobacter jejuni* | ACTTGGTTCAACTTATGTGCGTC | 23 | 59.4 | 45.8 |
| Yer_1740 | *Yersinia* spp. | AGATGATATCGGTGTGGAAGTGG | 23 | 60.3 | 50.0 |
| Yen_1512 | *Yersinia enterocolitica* | GAGCTTCCAGACGTTCACCAATAATA | 26 | 59.2 | 44.4 |
| Yen_1425 | *Yersinia enterocolitica* | GCAGACTTACAAGATGGGTGTGC | 23 | 61.6 | 54.2 |
| Yer_1407 | *Yersinia* spp. | TGAAGGTAAAGTTCACGAGCAGACTTA | 27 | 60.3 | 42.9 |
| Yer_1704 | *Yersinia* spp. | GATCCACCCGAAAGTGTTCTATTTCT | 26 | 59.1 | 44.4 |
| Vch_1776 | *Vibrio cholerae* | GGTTGAGTCAGCCATGAATGAGAAG | 25 | 61.6 | 50.0 |
| Vch_1795 | *Vibrio cholerae* | GAGAAGCTGGCGGATTTCCTAG | 22 | 61.1 | 56.5 |
| Vch_1839 | *Vibrio cholerae* | AAACGTTTGTTCGAAGATTATTGATG | 26 | 57.0 | 33.3 |
| Vib_1627 | *Vibrio* spp. | AAAGAAGGCTTCTCGAAGAAAG | 22 | 57.1 | 43.5 |

Sequences listed are those of the RC oligonucleotide probes that were used in the labeling reaction. All sequences are listed without the 3′ terminal C residue. Indicated $T_m$ and G + C% values were calculated using CalcOligo 2.03.

[a] Numbers at the end of the probe names refer to their relative positions on the *E. coli gyrB* gene.

[b] *M. avium*, *M. intracellulare*, *M. maloense*.

[c] *M. tuberculosis*, *M. bovis*, *M. gastri*.

NCTRCCGAGCGTGTAGGCATGGGACGA-5′). Alternative primers UP1G (5′-GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGG-3′) and U P2Ar (3′-TACYGNCTRCGNCTRCANCTRCCGAGCGTGTAGGCATGGGACGA-5′) were used for those strains where UP1-UP2r amplification failed (these cases were in agreement with sequence information, if available) [17]. PCRs were performed in 100-μl aliquots, consisting of 1× PCR buffer, 2 mM $MgCl_2$, 4 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 50 μM for each of the four dNTPs, 150 nM for each primer, with 50–100 ng DNA as template. For soil and veterinary DNA samples and for DNA mixtures amplification was performed with all four primers in one "multiplex" reaction, using the FailSafe PCR PreMix E (Epicentre, Madison, WI, USA). Amplification conditions were 95 °C for 5 min, followed by 35 cycles of 1 min at 95 °C, 1 min at 58 °C, 2 min at 72 °C, followed by a final elongation step of 10 min at 72 °C (http://seasquirt.mbio.co.jp/icb/protocols/protocols.php). PCR products were subsequently purified using a commercial PCR purification kit (Qiagen) according to manufacturer's instructions and eluted in 30 μl $dH_2O$. DNA concentration was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and adjusted to a final concentration of 50 ng/μl.

### *Cloning and sequencing*

Purified PCR products were cloned using the Topo ta Cloning Kit for Sequencing (Invitrogen) following manufacturer's instructions. Chemically competent cells (25 μl; provided with the cloning kit) were transformed with 6 μl of the ligation reaction. LB plates containing 100 μg/ml ampicillin were inoculated with 100 μl of the transformation reaction and incubated overnight at 37 °C. Template for the colony PCR was prepared by resuspending single colonies in 20 μl $dH_2O$ and denaturing the cell suspension for 10 min at 95 °C in a

Table 2
Bacterial species used for microarray validation

| Name | Designation | *gyrB* Accession Nos. |
|---|---|---|
| *Aeromonas hydrophila* | SSM 4131 | DQ386876 |
| *Campylobacter jejuni* | NCTC 12145 | DQ445256 |
| *Enterobacter cloacae* | SSM 2554 | DQ386885 |
| *Escherichia coli* | SSM 1771 | DQ386875 |
| ETEC (*E. coli* enterotoxic) | SSM 4135 | DQ386871 |
| EHEC slt-I (*E. coli* enteroh emorragic) | SSM 4134 | DQ386872 |
| EHEC slt-II (*E. coli* enteroh emorragic) | SSM 4140 | DQ386873 |
| EIEC (*E. coli* enteroinvasive) | SSM 4137 | DQ386888 |
| EPEC (*E. coli* enteropathogenic) | SSM 4136 | DQ386874 |
| *Helicobacter pylori* | SSM 4138 | DQ386880 |
| *Mycobacterium avium* | SSM 4139 | DQ386879 |
| *Mycobacterium tuberculosis* | H37Rv | DQ445257 |
| *Proteus mirabilis* | SSM 2105 | DQ386881 |
| *Pseudomonas putida* | SSM 2506 | DQ386884 |
| *Salmonella* spp. | SSM 1592 | DQ386877 |
| *Serratia marcescens* | SSM 2560 | DQ386886 |
| *Shigella* spp. | SSM 2023 | DQ386882 |
| *Staphylococcus aureus* | SSM CI-1 | DQ386887 |
| *Vibrio cholerae* | SSM 2508 | DQ386878 |
| *Yersinia enterocolitica* | SSM 4130 | DQ386883 |

*C. jejuni* and *M. tuberculosis* strains were kindly provided by M. Hess, C. Neubauer and W. Prodinger. All other strains were environmental or clinical isolates from the culture collection of the Department of Biological Sciences, University of Sassari.

thermocycler with subsequent cooling to 4 °C; 1 μl of this suspension was used as a template for 50-μl PCRs. M13 PCRs were performed as described above but using M13 forward (5′ GTAAAACGACGGCCAG 3′) and M13 reverse primers (3′ GTCCTTTGTCGATACTG 5′) and 50 °C annealing temperature. PCR products were subsequently purified using PCR purification kit (Qiagen) according to manufacturer's instructions and eluted in 30 μl $dH_2O$. DNA concentration was measured using a NanoDrop spectrophotometer (NanoDrop Technologies). Sequencing reactions were performed using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing reaction was done in 10-μl reaction aliquots consisting of 4 μl purified PCR product, 2 μl sequencing mix and 400 nM primer (M13 forward or M13 reverse). Sequencing conditions were 95 °C for 2 min, followed by 25 cycles of: 30 s at 95 °C, 15 s at 50 °C, 4 min at 60 °C with no final elongation step. Sequencing reactions were purified using Sephadex G-50 (Amersham Biosciences, Piscataway, NJ, USA) columns and run on the automated ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences were analyzed using Sequencher v 4.5 (Gene Codes Corp. Ann Arbor, MI, USA) and subjected to a preliminary nucleotide–nucleotide BLAST analysis within the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST) prior to importing to the *gyrB* database in ARB where proper phylogenetic analysis took place. Accession numbers of the *gyrB* sequences of the strains used in this study are listed in Table 2.

### *Labeling control*

An internal positive control, a *pmoA* PCR product from *M. trichosporium* OB3b, was included in each labeling and hybridization experiment and subsequently applied for normalization of the results. Genomic DNA extraction and *pmoA* PCR amplification were performed as described before [2]. PCR product was purified using PCR purification kit (Qiagen) according to manufacturer's instructions. DNA concentration was measured using a NanoDrop spectrophotometer (NanoDrop Technologies) and adjusted to a final concentration of 50 ng/μl.

### *Vibrio cholerae-specific gyrB PCR amplification and sequencing*

*V. cholerae*-specific *gyrB* primers, Vch718for (5′ GTGG CAAGATCCATTCGCAAACT 3′) and Vch1776rev (5′G CTTCTCATTCATGGCTGACTCAACC 3′) were developed using the ARB software package. PCR was performed in 100-μl volume using the FailSafe PCR PreMix E (Epicentre), 4 U *Taq* DNA polymerase (Invitrogen), and 150 nM of each primer. Amplification conditions were 95 °C for 5 min, followed by 35 cycles of 1 min at 95 °C, 1 min at 65 °C, 2 min at 72 °C, followed by a final elongation step of 10 min at 72 °C. After PCR amplification, PCR products were purified, cloned, and sequenced as described above.

### *DNA labeling*

For the sequence-specific end labeling of the oligonucleotides, a modified protocol of Rudi and co-workers [21] was applied. After purification, PCR products were treated with shrimp alkaline phosphatase (SAP) (Roche Diagnostics GmbH, Penzbeg, Germany) to dephosphorylate remaining nucleotides. Samples consisting of 20 μl purified PCR product (50 ng/μl), 2 μl Thermo Sequenase reaction buffer (Amersham Biosciences), and 4 μl SAP (1 U/μl) were incubated at 37 °C for 30 min, followed by 10 min at 95 °C to inactivate enzyme activity. The SAP-treated *gyrB* PCR products were then used for the cyclic labeling reaction. The control *pmoA* PCR product was further diluted to a final concentration of 5 ng/μl.

For the labeling a set of reverse complement oligonucleotides lacking the 3′ terminal cytosine residue was custom synthesized (VBC Genomics). Lyophilized oligonucleotides were dissolved to a final concentration of 100 pmol/μl and stored at −20 °C. For the labeling reaction, an oligonucleotide mix ("RC mix") containing each reverse-complement oligonucleotide at a final concentration of 1 pmol/μl was prepared. Competitive oligonucleotides, when used, were added at the same concentration to the RC mix.

The cyclic labeling was performed in 10-μl aliquots consisting of 1× Thermo Sequenase reaction buffer, 10 ng SAP-treated control *pmoA* PCR product, 1 pmol of each reverse-complement oligonucleotide, 10 pmol of Tamra-

ddCTP (PerkinElmer Life and Analytical Sciences, Boston, MA, USA), 10 pmol of each ddATP, ddTTP, ddGTP (Roche Diagnostics GmbH), 3 U *Taq* DNA polymerase (Invitrogen) and 100 ng of SAP-treated *gyrB* PCR product. Reaction conditions were 25 cycles of 30 s at 95 °C followed by 75 s at 60 °C, carried out in a thermocycler. After cyclic labeling, samples were used directly for hybridization without further purification.

For experiments investigating the possibility of using up to 1000 different probes, RC oligonucleotides were added at a final concentration of 0.1 pmol/μl. Labeling experiments were also performed using all four nucleotides in the form of Tamra-labeled ddNTPs (PerkinElmer Life and Analytical Sciences) at the final concentration of 10 pmol and omitting all silencing (unlabeled) ddNTPs.

### *Sensitivity tests*

To establish the detection sensitivity of the microarray analysis, experiments were performed with environmental DNA samples spiked with 1 and 0.1% of the DNA of targeted pathogens.

### *Microarray hybridization*

Hybridization was carried out as described before [19]. Labeled targets (10 μl) were mixed with 200 μl hybridization buffer (prewarmed to 65 °C). Final concentration of the hybridization buffer was: 6× SSC, 1× Denhardt's reagent (Sigma, St. Louis, MO, USA), 0.1% SDS. Hybridization was performed overnight at 55 °C. After hybridization, slides were washed in a 2× SSC, 0.1% SDS wash solution for 5 min, followed by two wash cycles for 5 min in 0.2× SSC and a final wash for 5 min in 0.1× SSC, all at room temperature. Slides were dried with an oil-free air gun and scanned immediately.

### *Scanning and data analysis*

Microarrays were scanned at three lines to average and at 10-μm resolution using a GenePix 4000 A laser scanner (Axon Instruments, Foster City, CA, USA). PMT gain was adjusted to scan the spots below the saturation level. Scanned images were saved as multilayer tiff images and analyzed with the GenePix Pro 6.0 software (Axon Instruments). Microsoft Excel was used for statistical analysis and presentation of the results. Microarray hybridization results were normalized to the signal obtained from the internal control oligonucleotide (Msi_294) and expressed as percentage, with 100% equaling the signal of the control probe. Probes were considered to be positive during validation if their normalized signal was at least 10% (of the control signal, Msi_294). Microarray images are presented in the rainbow color mode of GenePix software, displaying signal inensities by color, ranging from red (strongest) through yellow and green to blue (no signal).

## Results and discussion

### *Methodology adaptation*

Sequence-specific end labeling of oligonucleotides [21–23], originally developed for membrane-based macroarrays, offers a significant improvement in the detection limit of MDMs by focusing labeling onto the regions actually used in hybridization to the microarray. The principle of the method is shown in Fig. 1. Capture oligonucleotides are immobilized on the microarray. Reverse complements of the capture oligonucleotides (RC oligonucleotides) are end labeled in a linear amplification reaction upon the availability of the corresponding target sequence. The entire mixture is then hybridized to the microarray to sort out the sequences that have been labeled. The amount of labeled nucleic acids, especially of those without a real capture probe on the microarray, is in this way drastically decreased compared to other labeling approaches where the entire PCR product is labeled. As a consequence, the level of nonspecific, background hybridization, the major factor limiting detection sensitivity, is also decreased, resulting in a significant improvement in the sensitivity of detection. SSELO was adapted to glass microarrays and for application on relatively complex systems.

The specificity of the assay was shown to be determined primarily by the stringency of the annealing step during labeling rather than that of the subsequent hybridization. The optimum annealing temperature under the conditions applied was shown to be 60 °C, below and above which false positive and negative results started to emerge. When targets were labeled under optimized conditions, increasing the hybridization temperature from the standard (55 °C) to 60 °C did not further improve specificity. The few false positive signals also were not affected. The only effect observed was an overall (28–81%) decrease in the intensity of all the signals.

One of the mechanisms conferring specificity to the labeling step is the application of labeled ddCTP in combination with the other three unlabeled ddNTPs (silencing ddNTPs). In this way the chance that an unexpected hybridization of the RC oligonucleotide to a PCR product yields a labeled oligonucleotide is decreased by 75% (assuming a random chance of the next nucleotide in the 3′ direction on the PCR product being G, serving a template for the addition of the labeled ddCTP). However, this system seriously limits the options for probe design to sequences showing a cytosine following the RC oligonucleotide. Even though our probe set was designed with regard to this rule, we checked whether relaxing it would compromise specificity. An environmental sample was labeled under standard conditions and in a modified reaction where all four ddNTPs were labeled. Labeling performed using all four Tamra-ddNTPs in combination (and no silencing ddNTP) showed false positive results in only 1 of 35 probes (Sal_1950) (Fig. 2). These results indicate that, if the number of potential probes is lim-

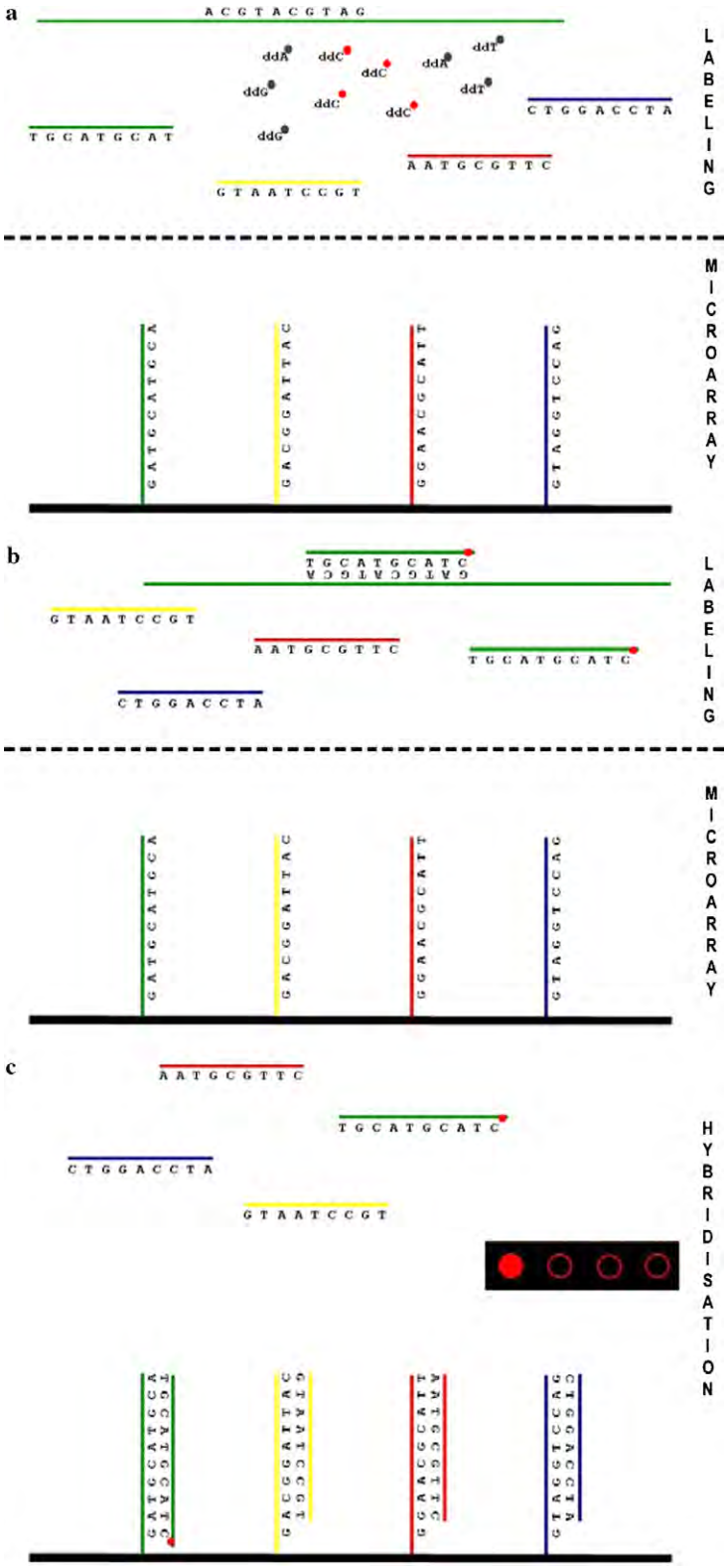


Fig. 1. SSELO principle. (a) Mixture of *gyrB* PCR product(s), RC oligonucleotides, labeled ddCTPs, and unlabeled (silencing) ddNTPs. Probes (capture oligonucleotides) on the microarray are the exact reverse-complement sequences of the oligonucleotides for labeling. Silencing ddNTPs (ddATP, ddGTP, ddTTP) are added to the reaction to minimize the risk of nonspecific elongation by the labeled ddCTP. (b) Sequence-specific end labeling of the RC oligonucleotide for which a corresponding PCR product was present. Elongation by a single, fluorescently labeled ddCTP. (c) Hybridization of the RC oligonucleotides against the microarray containing the capture oligonucleotides as probes. Signal is developed only where the corresponding RC oligonucleotide has been labeled in the previous labeling step.

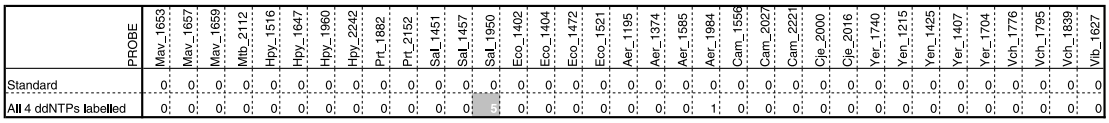


Fig. 2. Effect of using all four ddNTPs labeled versus only ddCTP, with the other three ddNTPs unlabeled (standard). Hybridization results after normalization to positive control probe are shown (see Materials and methods for details). An environmental (soil) sample was labeled under standard conditions and in a modified reaction where all four ddNTPs used were labeled. A weak false positive result occurred in only 1 of 35 probes (Sal_1950).

Table 3
Effect of decreasing the concentration of the RC oligos in the labeling reaction

| Experiment/probes | Mav_1653 | Mav_1657 | Mav_1659 | Vch_1776 | Vch_1795 | Vch_1839 | Vib_1627 |
|---|---|---|---|---|---|---|---|
| *M. avium*, 1 pmol RC | $19 \times 10^3$ | $16 \times 10^3$ | $18 \times 10^3$ | — | — | — | — |
| *M. avium*, 0.1 pmol RC | $25 \times 10^3$ | $27 \times 10^3$ | $29 \times 10^3$ | — | — | — | — |
| *V. cholerae*, 1 pmol RC | — | — | — | $138 \times 10^3$ | $67 \times 10^3$ | $127 \times 10^3$ | $61 \times 10^3$ |
| *V. cholerae*, 0.1 pmol RC | — | — | — | $161 \times 10^3$ | $103 \times 10^3$ | $107 \times 10^3$ | $59 \times 10^3$ |

*M. avium* and *V. cholerae* pure cultures were used to generate target for microarray hybridization using 1 pmol (standard amount) and 0.1 pmol RC oligos per labeling reaction. Absolute signal intensities, displayed as arbitrary units, are displayed. Arrays were scanned at 750 V PMT. Only data for relevant probes are shown.

ited, it may be a feasible compromise to sacrifice the extra specificity conferred by the silencing ddNTPs and in turn allow for probes with any nucleotides at the 3′ end.

A crucial factor determining the potential performance of the method is the maximal number of probes that can be employed in parallel. The standard protocol applies 1 μl of a mixture of RC oligonucleotides with 1 pmol/μl final concentration for each oligonucleotide. Oligonucleotide stock solutions are usually 100 pmol/μl in concentration; increased concentrations are often prone to instability caused, e.g., by precipitation, uneven mixing, etc. Therefore, at the amount of RC oligonucleotides applied, the maximal number of probes is limited to the range of 100–200 (our current array and hence the labeling procedure employs 35 oligonucleotide probes in one assay). Decreasing the amount of RC oligonucleotides from 1 to 0.1 pmol did not, however, cause any significant drop in the signal intensities of the corresponding spots, indicating that the method could theoretically be adapted to 1000–2000 different oligonucleotides (Table 3). However, the increasing number of potential interactions between the oligonucleotides might be a limitation.

### *Probe set validation*

The probe set was validated with pure cultures of reference strains. Of the 35 probes validated only 2 (i.e., Prt_1882 and Cje_2016) exhibited false negative hybridization results. Several probes gave rise to false positive signals with nontarget strains exhibiting significant sequence similarity to the respective probes (calculated weighted mismatches from between 1.0 and 2.7). However, a competitive oligonucleotides system that successfully resolved this problem was developed (see below). An overview of the validation data is presented in Fig. 3.

### *Competitive oligonucleotides*

To suppress false positive signals a series of competitive oligonucleotides (CO oligonucleotides) was designed and tested. Competitive oligonucleotides were designed as a variation of an RC oligonucleotide showing false positive signals with nontarget strains. The probe sequence was altered in a way to design a new probe that was a prefect match toward the strain exhibiting false positive signals. These CO oligonucleotides should therefore have a higher specificity toward the sequence giving rise to the false positive signal than the corresponding RC oligonucleotides. To ensure the silencing feature, CO oligonucleotides were synthesized with a 3′ phosphate modification (VBC Genomics) disabling their labeling. Of the seven competitive oligonucleotides developed (Table 4), six silenced the false positive signal that they were targeted against. Fig. 4 shows hybridization profiles of *Enterobacter cloacae* obtained with and without CO oligonucleotides. One of the competitive oligonucleotides (CO_1) failed to silence the false positive signal below the detection threshold, although an over 4× decrease in the signal was achieved. It has to be noted that this was the strongest false positive signal obtained. Furthermore, this was the signal resulting from the highest similarity between the RC oligonucleotide and the sequence giving rise to the false positive signal (weighted mismatch value 0.7), therefore having the lowest difference in the binding specificities for the CO and RC oligonucleotides. The application of the CO oligonucleotides had no statistically significant effect on the specific (i.e., expected) signals obtained with the targeted strains. In conclusion, the above results indicated that the application of competitive oligonucleotides during the labeling step can significantly increase specificity without compromising sensitivity.

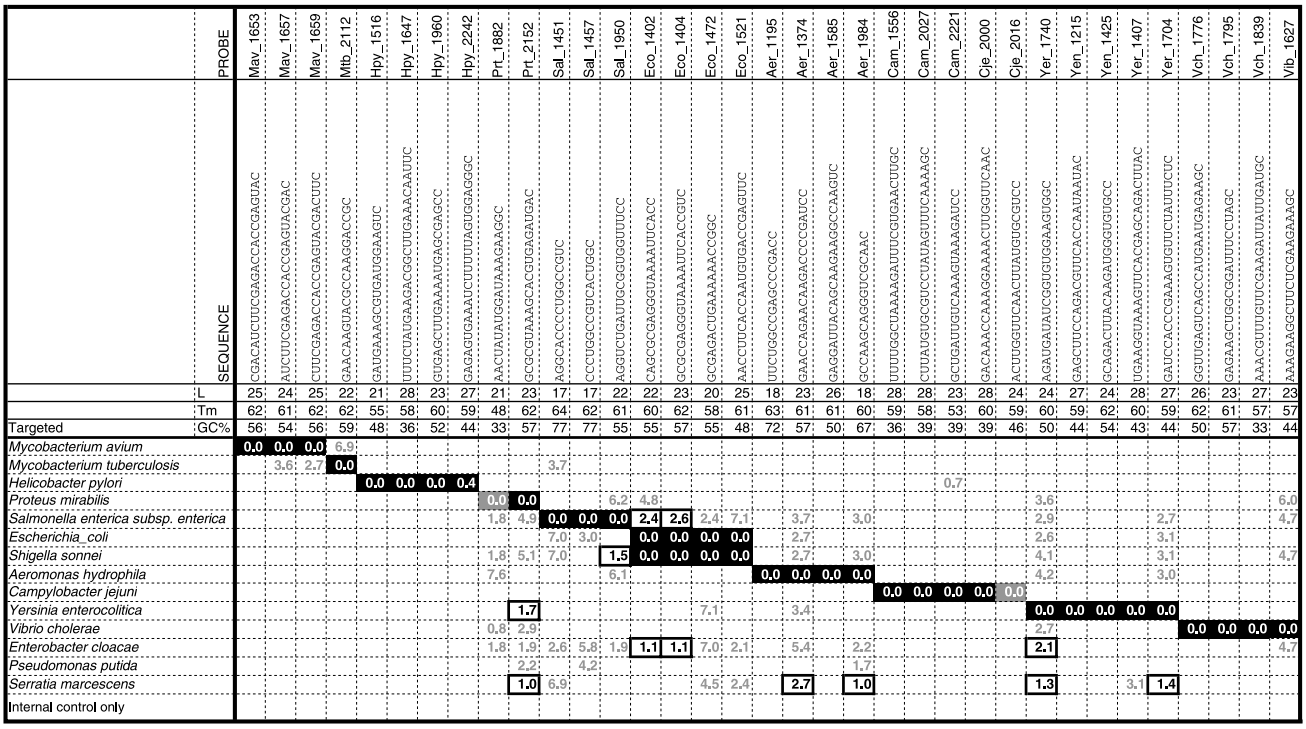


Fig. 3. Probe set validation. For each species, genomic DNA was purified from pure cultures and *gyrB* was amplified by PCR, labeled and hybridized on a separate microarray each. Summarized results (predicted and experimentally established probe specificities) are shown. Black fill indicates expected positive results, gray fill indicates negative results where hybridization was predicted, and thick black framing indicates positive results not predicted. Numbers indicate the number of weighted mismatches as described in the relevant section under Materials and methods.

Table 4
Set of competitive oligonucleotides (CO)

| CO | Against RC | Against strain(s) | CO sequence (5′–3′) | wMM | Signal without CO | Signal with CO |
|---|---|---|---|---|---|---|
| CO_1 | Prt_2152 | *Serratia marcescens* | GCGCGTAAAGC**G**CGTGAGATGA | 0.7 | 76.0 | 13.9 |
| CO_2 | Eco_1402 | *Enterobacter cloacae* | CAGCGCGA**A**GG**C**AAAATTCACC | 1.1 | 19.6 | 0.3 |
| | Eco_1404 | | | 1.1 | 45.2 | 0.7 |
| CO_3 | Eco_1402 | *Salmonella* spp. | CAGCG**A**GA**T**GG**C**AAAATTCACC | 2.4 | 4.9 | 0.4 |
| | Eco_1404 | | | 2.6 | 12.1 | 0.0 |
| CO_4 | Aer_1374 | *Serratia marcescens* | GAAC**A**AGAACAA**A**ACCCCGATCC | 2.7 | 7.4 | 0.0 |
| CO_5 | Aer_1984 | *Serratia marcescens* | GCCAAGCAGGG**G**CGCAAC | 1.0 | 11.4 | 0.7 |
| CO_6 | Yer_1740 | *Serratia marcescens* | AGATGATATCGG**C**GTGGAAGTGGC | 1.3 | 25.0 | 0.2 |
| CO_7 | Yer_1740 | *Enterobacter cloacae* | AGATG**G**TATCGG**C**GTGGAAGTGGC | 2.1 | 3.5 | 0.0 |

Positions of mismatches with the original probe (RC oligonucleotide; see sequence in Table 1) are indicated by boldfaced and underlined characters. Weighted mismatch (wMM) values for the RC oligonucleotide vs the sequence yielding false positive signal are indicated.

### *Probe set development*

As every spotted probe encounters its perfect reverse-complement match (either fluorescently labeled or not) during hybridization, it is not this step in the SSELO approach that primarily determines probe specificity. This was also confirmed by obtaining virtually the same hybridization profiles at 55 and 60 °C hybridization temperatures (as detailed above). The specificity-conferring step of the assay is the cyclic labeling, the extension of the RC oligonucleotides by a single fluorescently labeled dideoxy nucleotide. Therefore the most crucial region is the 3′ end of the oligonucleotide probe, followed by the central region. This is reflected in our parameters for calculating weighted mismatches, significantly differing from the parameters that we use for the approach where the hybridization on the array is the step determining specificity [21].

Therefore, essential requirements during probe design are to tune the probe set in such a way that their behavior is compatible with the conditions of the cyclic labeling reaction and that probes have sufficient discriminating power. We defined three essential guidelines for the probe design: (i) position of the diagnostic mismatch(es) as close to the 3′ end as possible, (ii) similar melting temperature ($60 \pm 2$ °C, if possible), and (iii) similar probe length (17–28 nucleotides). Probes potentially forming hairpin structures or 3′ self-dimers that could lend themselves to self-priming were avoided. Probe–target pairs with weighted mismatch (wMM) values of up to 0.5 were expected to yield positive

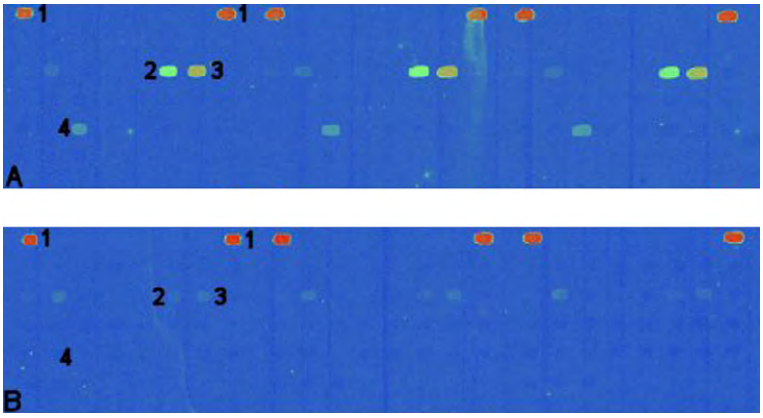


Fig. 4. Application of competitive oligonucleotides. Microarray images showing hybridization results of *Enterobacter cloacae* labeled without (A) and with (B) competitive oligonucleotides. Each array contains the same probes in triplicates: 1, internal control Msi_294; 2, Eco_1402 and 3, Eco_1404, false positive signals that could be successfully silenced with the addition of the competitive oligonucleotide CO_2; 4, Yer_1740, false positive signal that could be successfully silenced with the addition of the competitive oligonucleotides CO_7. There are no *E. cloacae*-specific probes present on the microarray. Images were scanned at 100% laser power, 750 V PMT and are displayed in rainbow color mode. Settings for both brightness and contrast were 50%.

hybridization under the conditions applied. As most of the developed probes exhibited expected hybridization results, our original guidelines established for probe design were indeed appropriate for this application. However, in silico predictions for specificity could not always be confirmed experimentally. Several probes with wMM values between 0.7 and 2.7 exhibited unexpected ("false") positive signals. Parameters for mismatch weighing can without doubt be refined by considering our results and those of further studies, which will lead to a higher success rate of in silico specificity prediction. Our results, however, also further confirm that in silico predictions of probe specificity can by no means be considered granted. Validation with pure cultures/clones is a crucial step for MDM development, which needs to be carried out for every new probe set and every alteration in the method [24,25].

*Sensitivity and specificity*

The concept of sensitivity for microbial diagnostics can be defined as the minimum amount of starting material needed or in relation to the abundance of the targeted microbe within the microbial community in the investigated sample. Depending on the nature of the sample, 1–10 ng of DNA is enough to generate sufficient PCR products for subsequent labeling and hybridization (data based on broad experience with various clinical and environmental samples for *gyrB* PCR amplification, data not shown). This amount of DNA is conveniently obtained from as little as a few hundred micrograms of sample. Thus, the amount of the sample is unlikely to be a limiting factor. The sensitivity of MDM methods is in most cases limited primarily by the relative abundance of the target group (within the analyzed community). When reported, this detection limit is found to be around 5% [2,8,11,14]. For short oligonucleotide arrays this limitation is due to the fact that the target consists of labeled nucleic acid fragments (several hundred nucleotides long) spanning the whole target gene amplified. This increases the potential for the accumulation of background signal arising from a low rate of nonspecific hybridization. In practice, using these "conventional" approaches, each probe develops a low background signal of around 1–5% with respect to the maximal signal obtainable [24]. By focusing labeling only onto the region targeted by the actual probes, the SSELO approach minimizes this background signal.

Experiments with environmental DNA from garden soil, apparently free of the bacteria targeted by our microarray, showed hardly any detectable signal on the probes of the array. The highest signal detected with any of the probes was less than 0.1% of that obtained during validation (i.e., the maximal signal of the probe). Spiking the same soil DNA with 0.1% of *Vibrio cholerae* DNA resulted in clearly detectable signals with two of the four *V. cholerae* probes (Fig. 5). Spiking with 1 and 10% of *V. cholerae* DNA resulted in clear signals on all of the *V. cholerae*-specific probes. Similar results were obtained with *Escherichia coli* mixed into soil DNA and with artificial mixtures of pure genomic DNAs (data not shown). It should also be noted that there is no signal obtained with the soil sample itself, which, considering the extremely complex nature of soil microbial communities, indicates a very high specificity.

The sensitivity of the analysis is also influenced by the inherent bias in the PCR amplification of the *gyrB* gene. Further improvements of the PCR protocol could also contribute to the overall detection sensitivity.

*Proof of concept*

The applicability of the microarray was also demonstrated by analyzing two archived veterinary samples (kindly provided by Clyde Hutchinson, Institute of Zoology, London, UK). The two samples were pathological

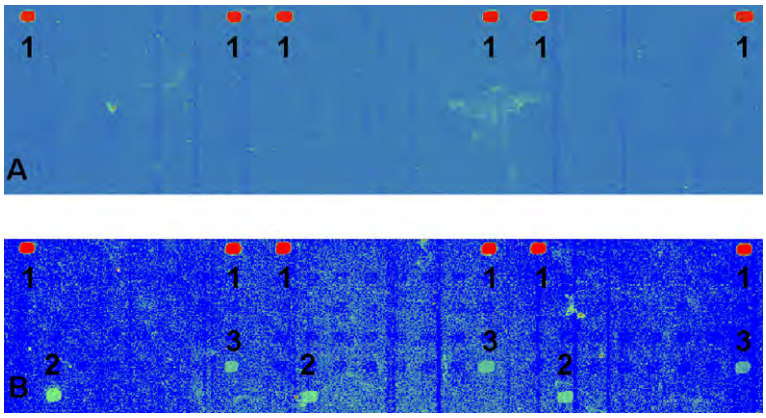


Fig. 5. Sensitivity and specificity of detection. Microarray images showing hybridization results from a soil DNA sample (A) and from the same soil DNA spiked with 0.1% *Vibrio cholerae* DNA (B). Each array contains the same probes in triplicates. Normalized signal values for Vch_1776 and Vch_1839 were 4.6 and 7.2, respectively, corresponding to around 3% of the maximal signal obtained with pure cultures during validation. 1, Internal control Msi_294; 2, Vch_1776; 3, Vch_1839. Images were scanned at 100% laser power, 750 V PMT and are displayed in rainbow color mode. Setting for brightness was 78% in both cases; settings for contrast were 78 and 81%, respectively.

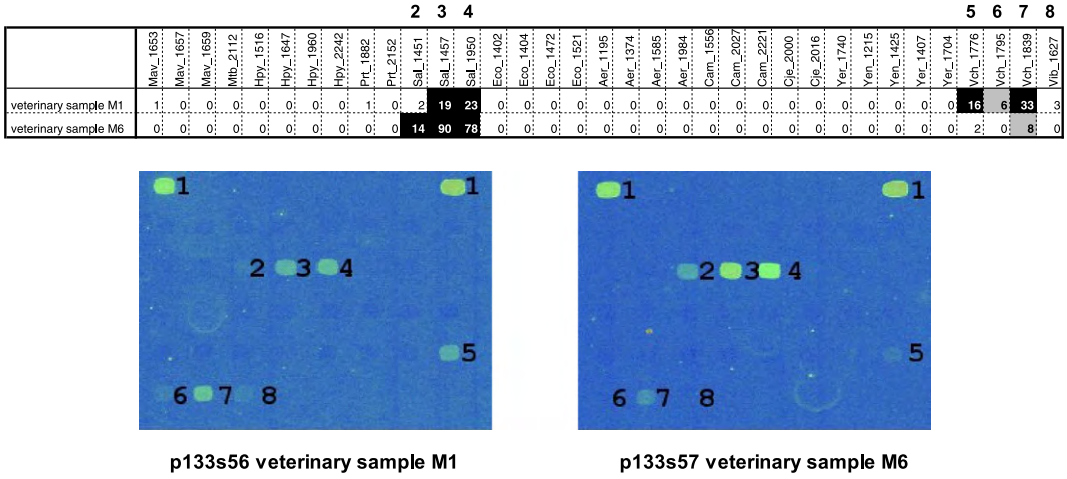


Fig. 6. Detection of pathogens in veterinary samples. Signal intensities after normalization to positive control probe (see Materials and methods for details) and microarray images showing hybridization results from archived veterinary biopsy samples M1 and M6. Positive signals obtained for *Vibrio cholerae* were also confirmed by specific PCR and sequencing. 1, Internal control Msi_294; 2, Sal_1451; 3, Sal_1457; 4, Sal_1950; 5, Vch_1776; 6, Vch_1795; 7, Vch_1839; 8, Vib_1627. Images were scanned at 100% laser power, 800 V PMT and are displayed in rainbow color mode. Setting for both brightness and contrast was 50% in both cases. Only one of three replicate subarrays per microarray is shown.

stool samples from a harbor porpoise and a greenfinch, both having shown pathological signs of *Salmonella* infection. The microarray indicated the presence of *Salmonella* in both samples, and a lower but clear signal for *V. cholerae* (Fig. 6). These findings were confirmed by successful amplification and sequencing of *V. cholerae* specific *gyrB* PCR products from both samples (data not shown). Even these preliminary results indicate the potential of parallel screening for multiple pathogens in delivering new, precious information on various aspects of the ecology, diversity, interactions, and epidemiology of bacterial pathogens.

## Conclusions

A major challenge in microbial diagnostics is the parallel detection and identification of low-abundance pathogens within a complex microbial community. Combination of a unique labeling method (sequence-specific end labeling of oligonucleotides), a housekeeping gene with a robust phylogenetic resolution at the species level (*gyrB*), microarray technology, and the concept of competitive oligonucleotides during labeling resulted in a highly specific method with significantly enhanced sensitivity (0.1% of the total microbial community analyzed), capable of addressing very complex microbial communities. An expansion of the probe set and extensive applications in pathogen surveys are planned to fully evaluate the potential of the approach. The reliability of in silico prediction of probe specificity will be further improved by the expansion of the public *gyrB* sequence database and the accumulation of hybridization results using the method (enabling a refinement of the probe design and specificity prediction parameters).

## Acknowledgments

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