Pathogen detection in milk samples by ligation detection reaction-mediated universal array method

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ABSTRACT

This paper describes a new DNA chip, based on the use of a ligation detection reaction coupled to a universal array, developed to detect and analyze, directly from milk samples, microbial pathogens known to cause bovine, ovine, and caprine mastitis or to be responsible for foodborne intoxication or infection, or both. Probes were designed for the identification of 15 different bacterial groups: *Staphylococcus aureus*, *Streptococcus agalactiae*, nonaureus staphylococci, *Streptococcus bovis*, *Streptococcus equi*, *Streptococcus canis*, *Streptococcus dysgalactiae*, *Streptococcus parauberis*, *Streptococcus uberis*, *Streptococcus pyogenes*, *Mycoplasma* spp., *Salmonella* spp., *Bacillus* spp., *Campylobacter* spp., and *Escherichia coli* and related species. These groups were identified based on the 16S rRNA gene. For microarray validation, 22 strains from the American Type Culture Collection or other culture collections and 50 milk samples were tested. The results demonstrated high specificity, with sensitivity as low as 6 fmol. Moreover, the ligation detection reaction-universal array assay allowed for the identification of *Mycoplasma* spp. in a few hours, avoiding the long incubation times of traditional microbiological identification methods. The universal array described here is a versatile tool able to identify milk pathogens efficiently and rapidly.

Key words: foodborne pathogen, ligase detection reaction, deoxyribonucleic acid array, mastitis

INTRODUCTION

Mastitis, the inflammation of the mammary gland, is a multifactorial disease usually caused by a microbial infection, which causes primarily lower milk yield, reduced milk quality, and greater production costs. Many different contagious and environmental bacteria cause

mastitis, including *Staphylococcus aureus*, *Streptococcus agalactiae*, several *Mycoplasma* spp., *Escherichia coli*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, and *Bacillus* spp. (Oviedo-Boyso et al., 2007). *Staphylococcus aureus* is one of the most significant pathogens causing IMI and mastitis in dairy ruminants in many countries, such as Slovenia, the Netherlands, and Italy (Pengov, 2006). *Streptococcus uberis*, isolated from mammary glands, is responsible for clinical and subclinical mastitis during both the nonlactating and lactation periods (Jayarao et al., 1999). There are many other streptococci that can generate unusual cases of mastitis, such as *Streptococcus bovis*, *Streptococcus canis*, *Streptococcus pyogenes*, and *Streptococcus equi* ssp. *zooepidemicus* (Watts et al., 1984; Devriese et al., 1999; Hassan et al., 2005; Blood et al., 2006; Pisoni et al., 2009). In addition, CNS, a group of staphylococcal species that are part of normal skin flora and that have been traditionally considered to be minor mastitis-related pathogens, have become the predominant pathogens causing subclinical or mild clinical mastitis in many countries (Honkanen-Buzalski et al., 1994; Pitkala et al., 2004). Finally, mastitis caused by *Mycoplasma* (especially *Mycoplasma bovis* in cattle) appears to be an emerging problem (Fox et al., 2005), because no effective treatment exists for this form of mastitis, and infected cows are considered positive for life.

At the same time, raw milk and dairy products made from nonpasteurized milk have been responsible for *Staph. aureus*, *Campylobacter*, *Salmonella*, *Bacillus*, and *E. coli* outbreaks and could also represent a hazard for consumers (Allos, 2001; Granum, 2001; Little et al., 2008). *Escherichia coli* and related species (Bottero et al., 2004), *Salmonella* (Jayarao et al., 2006), and *Bacillus cereus* (Wong et al., 1988) are well-known organisms that are potentially very dangerous for human health.

Pathogen identification has traditionally been based on microbiological techniques that are time-consuming, generally requiring at least 2 to 3 working days and, in some conditions, not having a satisfying diagnostic sensitivity. For example, isolation and microbiologi-

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cal identification of *M. bovis* require different specific growth media and long incubation times (González and Wilson, 2003).

From a clinical point of view, the limited diagnostic sensitivity of bacteriology is not sufficient to exclude any infection because a negative bacteriological result does not necessarily mean that the corresponding quarter is actually free of a pathogen infection. Consequently, a considerable number of infected cows remain undetected, and the control and eradication of pathogen mastitis in herds are therefore difficult. The development of PCR-based methods has provided a promising option for the rapid detection of bacteria, even in cases of difficult-to-detect subclinical mastitis. In recent years, DNA microarray technology has played an increasingly important role in bacterial studies, including detection and quantitative analyses (Castiglioni et al., 2004; Pingle et al., 2007). The DNA microarrays were developed for simultaneous analysis of *Staph. aureus* enterotoxin genes, *Listeria* spp., *Campylobacter* spp., and *Clostridium perfringens* toxin genes (Sergeev et al., 2004). More recently, Wang et al. (2007) described a 16S rRNA oligonucleotide microarray to identify 22 common pathogenic species, whereas Kim et al. (2008) developed a microarray for the detection of the main foodborne pathogens.

In this study, a microarray platform, based on the discriminative properties of the DNA ligation detection reaction (**LDR**), associated with a universal tag-array (**UA**), was developed and used to identify pathogens such as *Staph. aureus*, *Strep. agalactiae*, nonaureus staphylococci (**NAS**), *Strep. bovis*, *Strep. equi*, *Strep. canis*, *Strep. dysgalactiae*, *Streptococcus parauberis*, *Strep. uberis*, *Strep. pyogenes*, *Mycoplasma* spp., *Salmonella* spp., *Bacillus* spp., *Campylobacter* spp., and *E. coli* and related species.

MATERIALS AND METHODS

DNA Samples

The specificity and sensitivity of the LDR probe pairs were tested with DNA extracted from 22 American Type Culture Collection (**ATCC**) reference strains (LGC Promochem, Middlesex, UK) or isolates listed in Table 1, using the DNA extraction protocol described in Cremonesi et al. (2006). The LDR probe pairs were further evaluated with DNA extracted from a total of 50 milk samples (42 bovine, 2 ovine and 3 caprine mastitic samples plus 3 bovine milk samples from healthy cows), also collected for bacteriological examinations. For the specificity and microarray validation experiments, 50 fmol of the purified 16S rRNA PCR product

were used. For the sensitivity assays, 50, 25, 12.5, and 6 fmol of the PCR product was used.

Bacteriological Procedures

Bacteriological culturing of quarter and udder half milk samples was performed according to standards of the National Mastitis Council (1999). Briefly, 10 μ L of each milk sample was spread on blood agar plates (5% defibrinated sheep blood, Microbiol Diagnostici, Cagliari, Italy). The plates were incubated aerobically at 37°C and examined after 24 and 48 h. The colonies were provisionally identified on the basis of gram stain, morphology, and hemolysis patterns and the numbers of each colony type were recorded. The representative colonies were then subcultured on blood agar plates and incubated aerobically at 37°C for 24 h to obtain pure cultures. Production of catalase and coagulase was tested for gram-positive cocci. Specific identifications of staphylococci and streptococci were made using commercial micromethods (API Staph ID32 system and API 20 Strep, respectively, bioMérieux, Rome, Italy). Gram-negative isolates were identified by use of colony morphology, gram-staining characteristics, oxidase, and biochemical reactions on MacConkey's agar and API 20E (bioMérieux). Ten microliters of milk was also plated onto a Hayflick agar plate (Brown et al., 1990) containing 15% horse serum; agar plates were incubated at 35°C with 5% CO₂ and examined using a 40 $\times$ stereomicroscope for *Mycoplasma* growth every 48 h until such time as colonies were obtained, or for 10 d if no colonies were identified.

PCR Amplifications from DNA Samples

The 16S rRNA gene was amplified with universal primers 16S27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and R1492 (5'-TAC GGY TAC CTT GTT ACG ACT T-3'; Edwards et al., 1989). The PCR amplifications were performed with a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, CA). The reaction mixtures included 0.85 μ L of each primer (15 μ M), 10 μ L of 2 $\times$ PyroStart Fast PCR Master Mix (Fermentas, M-Medical SRL, Milan, Italy), and 10 to 15 ng of genomic DNA in a final volume of 20 μ L. Before amplification, DNA was denatured for 1 min at 95°C. Amplification consisted of 30 cycles at 94°C for 1 s, 61°C for 5 s, and 72°C for 38 s. After the cycles, an extension step (10 s at 72°C) was performed. The PCR products were purified by using a Wizard SV gel and PCR Clean-Up System purification kit (Promega Italia, Milan, Italy), according to the instructions of the manufacturer, eluted in 20 μ L of molecular grade

Table 1. Origin, signal-to-noise-ratio (SNR), and *t*-test unadjusted *P*-values for ligation detection reaction probe pair specificity tests¹

Organism	Species	Bacterial collection	Probe name	SNR-specific probe	Average SNR of other probes (n = 14)	<i>P</i> -value for the specific probe
<i>Bacillus</i>	<i>Bacillus cereus</i>	ATCC 14579	<i>Bacillus</i> spp.	45.30	0.43	3.90E-06
				238.29	0.75	1.84E-09
<i>Campylobacter</i>	<i>Campylobacter coli</i>	ATCC 1061	<i>Campylobacter</i> spp._179	6.49	0.33	1.68E-07
			<i>Campylobacter</i> spp._234	3.43	0.33	1.70E-04
	<i>Campylobacter lari</i>	ATCC BAA1060	<i>Campylobacter</i> spp._179	42.62	1.22	6.75E-06
			<i>Campylobacter</i> spp._234	19.43	1.22	1.95E-08
	<i>Campylobacter jejuni</i>	ATCC 33560	<i>Campylobacter</i> spp._179	21.26	1.11	2.36E-06
				20.12	0.71	2.58E-06
			<i>Campylobacter</i> spp._234	21.26	1.11	8.70E-04
				20.12	0.71	9.91E-05
<i>Escherichia coli</i>	<i>E. coli</i> O157	ATCC 700927	<i>E. coli</i> _et_rel	9.56	0.56	2.57E-07
				22.68	0.73	5.62E-09
<i>Mycoplasma</i>	<i>M. agalactiae</i>	DIPAE ²	<i>Mycoplasma</i> spp.	42.28	0.83	2.11E-06
				42.76	0.80	7.29E-04
	<i>M. arginini</i>	DIPAE		62.22	0.78	1.06E-04
	<i>M. bovis</i>	DIPAE		32.00	0.44	2.57E-09
				47.93	0.29	5.32E-12
	<i>M. capri</i>	DIPAE		39.46	0.83	4.00E-06
	<i>M. capricolum</i>	DIPAE		28.83	0.52	1.85E-04
	<i>M. mycoides</i>	DIPAE		7.38	0.20	4.42E-05
<i>Salmonella</i>	<i>Salmonella enteritidis</i>	ATCC 13076	<i>Salmonella</i> spp.	10.98	0.51	2.20E-08
				11.74	0.51	7.95E-11
<i>Staphylococcus</i>	<i>Staph. aureus</i>	ATCC 19095	<i>Staph. aureus</i>	5.76	0.23	1.51E-04
				10.05	0.42	4.90E-10
	<i>Staph. epidermidis</i>	ISPA-CNR ³	NAS ⁴	4.31	0.20	7.51E-03
				46.76	0.83	1.11E-06
	<i>Staph. sciuri</i>	DIPAV ⁵		9.29	0.91	7.47E-04
				10.68	0.96	8.90E-05
	<i>Staph. hemolyticus</i>	DIPAV		27.89	0.83	8.11E-08
				29.78	1.03	7.64E-05
<i>Streptococcus</i>	<i>Strep. agalactiae</i>	ATCC BAA611	<i>Str. agalactiae</i>	7.04	0.12	1.39E-07
				35.32	0.57	1.20E-07
	<i>Strep. uberis</i>	ATCC 9927	<i>Str. uberis</i>	14.46	0.42	1.24E-09
				21.16	0.48	1.48E-11
	<i>Strep. parauberis</i>	DMS 6632	<i>Str. parauberis</i>	54.68	0.47	4.04E-10
				44.41	0.31	1.54E-10
	<i>Strep. bovis</i>	DIPAV	<i>Str. bovis</i>	14.51	0.40	3.66E-05
				55.55	0.59	1.76E-06
	<i>Strep. canis</i>	DIPAV	<i>Str. canis</i>	16.32	0.53	1.15E-11
				60.46	0.88	2.10E-10
	<i>Strep. dysgalactiae</i>	DIPAV	<i>Str. dysgalactiae</i>	10.33	0.26	3.52E-07
				73.25	0.58	9.95E-08
	<i>Strep. equi</i>	DIPAV	<i>Str. equi</i>	13.09	0.38	4.22E-06
				48.30	1.11	1.94E-07
	<i>Strep. pyogenes</i>	ATCC 12344	<i>Str. pyogenes</i>	93.69	1.39	1.79E-07
				118.71	1.55	9.71E-14

¹For each sample, the bacterial collection reference, the SNR of the specific probe, and the SNR of all the remaining (nonspecific) probes are reported. *P*-values are calculated as the results of a one-sided *t*-test comparison between the intensity of fluorescence of each probe and those of the Blank spots.

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⁴NAS = nonaureus staphylococci.

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water, and quantified by capillary electrophoresis on an Agilent BioAnalyzer 2100 (Agilent Technologies, Palo Alto, CA).

LDR Probe Design for the 16S rRNA Gene

We designed specific LDR probe pairs for the 16S rRNA gene sequences of 15 different bacterial groups:

Staph. aureus, *Strep. agalactiae*, NAS, *Strep. bovis*, *Strep. equi*, *Strep. canis*, *Strep. dysgalactiae*, *Strep. parauberis*, *Strep. uberis*, *Strep. pyogenes*, *Mycoplasma* spp., *Salmonella* spp., *Bacillus* spp., *Campylobacter* spp., and *E. coli* and related species. Probe pairs for *Strep. equi* and *Strep. dysgalactiae* were designed to identify any of the 2 subspecies of each. For each of these groups, 16S

Figure 1. Schematic of the grouping and clustering of the 16S rRNA gene sequences. For each node, the number of sequences used from the Ribosomal Database Project II database (length >1,200 bp and “good” quality) is reported. The dimension of the triangles is proportional to the number of sequences that were clustered together. The phylogenetic tree was built on the 15 consensus sequences by using the neighbor-joining algorithm and Phylip tree type. The scale represents the number of differences between sequences (e.g., 0.1 means 10% differences between 2 sequences). NAS = nonaureus staphylococci.

rRNA sequences longer than 1,200 bp and flagged as being of “good quality,” chosen among those available in the Ribosomal Database Project (RDP) II, release 9.51 (<http://rdp.cme.msu.edu/html/>), were selected. A total of 744 sequences were used for the consensus determination and probe design (Figure 1). Sequences belonging to the same subgroups were then aligned together by ClustalW (Chenna et al., 2003) and imported into the Oligonucleotide Retrieving for Molecular Applications (ORMA) tool (M. Severgnini, P. Cremonesi, C. Consolandi, B. Castiglioni; and G. Careda and G. De Bellis of the Institute of Biomedical Technologies—Italian National Research Council, Segrate; unpublished data). This software is based on a set of scripts under Matlab (Mathworks, Natick, MA) to retrieve positions discriminating each sequence from the others, design optimal probes on the found positions, and quality filter the candidates, driving the user to the determination of thermodynamically optimized oligonucleotides. The consensus sequences for each subgroup were obtained according to the algorithm by Ludwig et al. (2004), setting a cutoff threshold of 40% and substituting, where necessary, multiple bases above the given threshold with the corresponding International Union of Pure and Applied Chemistry ambiguity code. The sequences were

then realigned with ClustalW and used in the ORMA tool for probe design.

We required the melting temperature to be within the 66 to 68°C range, with a length of between 25 and 50 bases; a maximum of 5 degenerated bases per oligonucleotide were allowed. Three rounds of design were performed: 1) *Salmonella* spp. was aligned against the *E. coli* sequence only; 2) *Strep. canis* and *Strep. pyogenes* were aligned against *Streptococcus* group sequences only (i.e., all *Streptococcus* species included in the LDR assay); and 3) all other probe pairs were selected considering the alignment of all the species. One probe per species or genus was designed, except for *Campylobacter* spp., for which 2 different positions were tested, to compare and verify their specificity and performances.

The specificity of the probe pairs was checked with both the Probe Match tool in RDP II database and BLAST (Basic Local Alignment Search Tool) analysis (Altschul et al., 1997). Each common probe was synthesized to have a complementary ZipCode (cZipCode) affixed to the 3' end, and a phosphate modification to the 5' end. A Cy3 label was attached to the 5' end of the discriminating probes. In LDR, if both probes are annealed to the target sequence, the fluorescent dye

and the cZipCode are linked into the same molecule. In hybridization, a cZipCode pairs with its corresponding ZipCode, addressing the fluorescent signal to a specific spot on a glass slide. All the oligonucleotides (Table 2) were synthesized by Thermo Electron GmbH (Ulm, Germany). When a probe sequence contained an ambiguity code, this base was replaced with inosine during oligonucleotide synthesis.

UA Preparation

Phenylene-diisothiocyanate-activated chitosan glass slides were used as surfaces for the preparation of microarrays (Consolandi et al., 2006). Microarrays were prepared as described in Castiglioni et al. (2004), using a MicroGrid II Compact (Biorobotics, Cambridge, UK) contact pin spotter equipped with Silicon Microarray pins (Parallel Synthesis Technologies, Santa Clara, CA). For the present study, 17 ZipCodes, randomly selected from the 49 of the entire UA layout described in Chen et al. (2000), were assigned for the recognition of bacterial group based on the 16S rRNA gene. ZipCode 66 (hybridization control) and ZipCode 63 (ligation control) were used to locate the submatrixes during the scanning and to verify the success of the ligation procedure, respectively.

LDR, UA Hybridization, and Signal Detection

All the group-specific probe pairs were added into an oligo-mix, at a concentration of 1 μ M each. The oligo-mix also contained a discriminating and a common probe specific for the synthetic oligonucleotide used as the LDR control (5'-AGC CGC GAA CAC CAC GAT CGA CCG GCG CGC GCA GCT GCA GCT TGC TCA TG-3'). The LDR was carried out in a final volume of 20 μ L containing 1 μ L of 10 $\times$ *Pfu* DNA Ligase Buffer (Stratagene, La Jolla, CA), 1 μ L of oligo-mix, 1 μ L of *Pfu* DNA ligase (Stratagene), and 0.1 μ L of the synthetic oligonucleotide. The reaction mixture was preheated for 5 min at 94°C and was then cycled for 30 rounds at 94°C for 30 s and at 65°C for 4 min. Hybridization, slide washing, and data acquisition were performed following the procedures described in Castiglioni et al. (2004). Briefly, the hybridization mixture had a total volume of 65 μ L and contained 20 μ L of LDR mixture, 5 $\times$ saline sodium citrate (SSC, Sigma-Aldrich, Milan, Italy), 0.1 μ M Cy3-labeled cZipCode 66 (complementary to ZipCode 66), and 0.1 mg/mL of salmon sperm DNA. After heating at 94°C for 2 min and chilling on ice, the hybridization mixture was applied to the slide, on which the 8 arrays were separated by Press-To-Seal silicone isolators (1.0 $\times$ 9 mm; Schleicher and Schuell BioScience, Dassel, Germany).

By using 8 subarrays per slide, associated with a multichamber hybridization system, 8 samples were run in parallel on the same slide. Hybridization was carried out in a dark chamber at 65°C for 1 h 30 min in a temperature-controlled water bath. After hybridization, the slide was washed at 65°C for 15 min in prewarmed 1 $\times$ SSC. Finally, the slide was dried by spinning at 80 $\times$ *g* for 3 min. The fluorescent signals were acquired at a 5- μ m resolution by using a ScanArray Lite laser-scanning system (PerkinElmer Life and Analytical Sciences, Boston, MA) with a green laser for Cy3 dye (λ_{ex} , 543 nm; λ_{em} , 570 nm). The power of both the laser and the photomultiplier tube was set between 70 and 95%, depending on the signal intensities, avoiding saturation in any case.

Data Analysis

Intensities of fluorescence (IF) were quantified by ScanArray Express 3.1 software (PerkinElmer), using the "adaptive circle" option, allowing diameters between 60 and 300 μ m. No normalization was performed on IF because the experiments were analyzed independently of one another. All statistical tests and data analyses were performed in Matlab. To assess whether a probe was significantly above the background, a one-sided Student *t*-test ($\alpha = 0.01$) was performed, using the IF of "Blank" spots (i.e., with no oligonucleotide spotted, $n = 6$), plus 2 times their standard deviations, as a conservative estimation of the null distribution. For each ZipCode, we considered the population of the IF of all the replicates ($n = 4$) and tested it for being significantly above the null distribution ($H_0: \mu_{\text{test}} = \mu_{\text{null}}$; $H_1: \mu_{\text{test}} > \mu_{\text{null}}$). The signal-to-noise ratio (SNR) was calculated as the ratio between the average IF of each probe and the average Blank spots for IF; coefficient of variation percentages represent the percentage ratio of the standard deviation of IF on their average values.

RESULTS

Group-Specific SNP Identification

The ORMA probe design tool (M. Severgnini, P. Cremonesi, C. Consolandi, B. Castiglioni; and G. Caredda and G. De Bellis of the Institute of Biomedical Technologies–Italian National Research Council, Segrate; unpublished data) was used in our study for SNP identification and LDR probe design on 16S rRNA, allowing the precise determination and detection of the main pathogens infecting cattle, sheep, and goats or those responsible for foodborne intoxication or infection, or both. The BLAST analysis and the "Probe Match" tool in the RDP II database were used to confirm *in silico*

Table 2. List of group-specific probes and corresponding ZipCodes

Probe pair name	Discriminating probe ¹ (5'–3')	Common probe ² (5'–3')	SNP position	ZipCode ³
<i>Staph. aureus</i>	TAA TAC CGG ATA ATA TTT TGA ACC GCA TGG TTC AAA AGT GAA AGA CGG TC	TTG CTG TCA CTT ATA GAT GGA TCC GCG CTG CAT TAG CTA GTT GGT AA	222	2
<i>Strep. canis</i>	CGT AAA GCT CTG TTG TTA GAG AAG AAC GGT AAT GGG AGT GGA AAA C	CCA TTA TGT GAC GGT AAC TAA CCA GAA AGG GAC GGC TAA CTA C	474	3
<i>Strep. dysgalactiae</i>	GGT CTA GAG ATA GGC TTT CCC TTC GGG GCA GG	AGT GAC AGG TGG TGC ATG GTT GTC GTC AGC TCG	1061	4
<i>Salmonella</i> spp.	CCA TCA GAT GTG CCC AGA TGG GAT TAG CTT GTT GGT GA	GGT AAC GGC TCA CCA AGG CGA CGA TCC CTA G	251	5
<i>Campylobacter</i> spp_179	CCC TAC ACA AGA GGA CAA CAG TTG GAA ACG ACT GCT AAT ACT	CTA TAC TCC TGC TTA ACA CAA GTT GAG TAG GGA AAG TTT TTC GGT GTA	179	8
<i>Campylobacter</i> spp_234	CTC TAT ACT CCT GCT TAA CAC AAG TTG AGT AGG GAA GGT TTT TCG G	TGT AGG ATG AGA CTA TAT AGT ATC AGC TAG TTG GTI AGG TAA TGG C	234	9
<i>Bacillus</i> spp.	CTA AGT GTT AGA GGG TTT CCG CCC TTT AGT GCT GAA GT	TAA CGC ATT AAG CAC TCC GCC TGG GGA GTA CG	880	10
<i>Strep. equi</i>	CTA ATA CCG CAT AAA AGT GGT TGA CCC ATG TTA ACI ATT TAA AAG GAG CAA CA	GCT CCA CTA TGA GAT GGA CCT GCG TTG TAT TAG CTA GTT	224	12
<i>Strep. agalactiae</i>	CGT GCC TAA TAC ATG CAA GTA GAA CGC TGA IGT TTG GTG TTT A	CAC TAG ACT GAT GAG TTG CGA ACG GGT GAG TAA CG	87	15
<i>Strep. bovis</i>	GCG TGC CTA ATA CAT GCA AGT AGA ACG CTG AAG ACT TTA GCT TGC TAA	AGT TGG AAG AGT TGC GAA CGG GTG AGT AAC GCG TAG GTA A	91	16
<i>Strep. parauberis</i>	GCC GTG GCT CAA CCA TGG TTC GCT TTG GAA ACT GGA T	AAC TTG AGT GCA GAA GGG GAG AGT GGA ATT CCA TGT GTA GC	663	18
<i>Strep. uberis</i>	AAT ACC GCA TGA CAA TAG GGT ACA CAT GTA CCC TAT TTA AAA GGG GCA AA	TGC TTC ACT ATG AGA TGG ACC TGC GTT GTA TTA GCT AGT TGG TAA GGT AA	223	19
<i>Mycoplasma</i> spp.	CGI CGC AGC TAA CGC ATT AAA TGA TCC GCC TGA GT	AGT AIG ITC GCA AGA ITI AAA CTT AAA GGA ATT GAC GGG GAI CC	906	20
NAS ⁴	CCG GAG CTA ATA CCG GAT AAT ATI TIG AAC CGC ATG GTT CIA T	AGT GAA AGA IGG TTT TGC TIT CAC TTA TAG ATG GAI CCG CGC	208	21
<i>Strep. pyogenes</i>	GCA GGC GGT TTT TTA AGT CTG AAG TTA AAG GCA TTG GCT CAA CCA A	TGT ACG CTT TGG AAA CTG GAG AAC TTG AGT GCA GAA GGG	530	22
<i>E. coli</i> _et_rel	GGG TTG TAA AGT ACT TTC AGC GGG GAG GAA GGG AGT AAA GTT AAT AC	CTT TGC TCA TTG ACG TTA CCC GCA GAA GAA GCA CCG G	484	28

¹A Cy3 label attached to the 5' end; discriminating nucleotides at the 3' end are in bold face.²Designed to anneal to the target sequence immediately adjacent to the discriminating probe.³ZipCode numbers as in Chen et al. (2000).⁴NAS = nonaureus staphylococci.

the specificity of the selected candidates. However, the NAS probe pair identified mostly the following species: *Staphylococcus epidermidis*, *Staphylococcus hemolyticus*, and *Staphylococcus lugdunensis*. Moreover, the probe pair designed for the *E. coli* group targeted some *Shigella* strains (data not shown). Because *Shigella* can also be found in milk samples and can potentially be harmful for human health, we decided to keep the probe pair in the assay, renaming it “*E. coli_et_rel*.”

Where necessary, multiple rounds of design were performed to maximize the quality of the probe pairs found. In particular, the *Salmonella* spp. probe, whose consensus resulted in values significantly different from all the other species, was obtained by comparing its consensus with only the *E. coli* and related species consensus sequence.

Specificity of the Probe Pairs

The specificity of the probe pairs was tested separately with the 16S rRNA products on a total of 22 ATCC or isolated bacterial strains (Table 1). Several examples of these results are also shown in Figure 2. No signal was detected using the *Listeria monocytogenes* strain ATCC 51780 as negative control (data not shown). All the LDR-UA experiments were replicated by duplicate independent LDR. Table 1 reports the SNR and the *P*-values of the *t*-tests of the assays for the probe pairs on single PCR product target. The procedure showed optimal specificity, with excellent signal-to-noise ratios. Results were in complete concordance with identification of ATCC strains and microbiological isolates: only probes associated with the expected species were present (*P*-values always <0.005), whereas all remaining probes were well below any acceptable *P*-value for the *t*-test. Hybridization and ligation control probes were always significantly present (data not shown).

The SNR of the probes called as significantly over the background varied from 4.31 to 238.3, with an average of 37.98; at the same time, SNR of the nonsignificantly over-background spots varied between 0.12 and 1.55, with an average of 0.65 ± 0.33 . Both *Campylobacter* spp. probes performed nearly the same in terms of specificity, with *P*-values below 10^{-4} . Reproducibility and homogeneity of hybridization was excellent as well, with an average coefficient of variation percentage among replicates for the present probes of $12.62 \pm 7.35\%$.

Sensitivity of the Probe Pairs

To test the sensitivity of the method and the correlation between signal intensity and template concentration, the assay was evaluated by separate LDR on

serial dilutions from 50 to 6 fmol of *M. bovis*, *Strep. agalactiae*, *Strep. pyogenes*, and *Staph. aureus* 16S rRNA-PCR products.

Figure 3 shows the SNR and the *P*-values (in logarithmic scale) of the serial dilutions of the 4 pathogen species. Considering a threshold value of 0.01 for significance of the presence of a probe, *M. bovis*, *Strep. agalactiae*, and *Strep. pyogenes* revealed a detection limit of 6 fmol, whereas *Staph. aureus* was limited to 12 fmol. As expected, SNR and *P*-values were inversely correlated (Pearson correlation between -0.81 and -0.97), whereas the correlation between SNR and PCR product concentration or *P*-values and PCR product concentration are slightly lower (range 0.63 to 0.79 and 0.59 to 0.85, respectively).

Evaluation of the Probe Pairs with Milk Samples

The performance of the LDR probe pairs was evaluated using DNA extracted from 50 milk samples, 47 of which were from mastitic animals. With bacteriological analysis of the 50 milk samples, 9 samples were found to be positive for *Staph. aureus*, 24 were positive for *Streptococcus* spp., 5 were positive for *Staphylococcus* spp., 9 were positive for *Mycoplasma* spp., and 3 were negative controls. The LDR results obtained from the analysis of these samples revealed a good correlation with those obtained from the microbiological assays (Figure 4), also showing the presence of *Bacillus* spp. in 2 mastitic milk samples and specifying the presence of *Strep. uberis* (8), *Strep. agalactiae* (6), *Strep. dysgalactiae* (3), *Strep. equi* (2), *Strep. bovis* (4), and *Strep. canis* (3) for the *Streptococcus* spp., and NAS (7) for the *Staphylococcus* spp. Nine samples were positive for *Staph. aureus*, whereas 11 were positive for *Mycoplasma* spp. Finally, 3 samples were negative with both bacteriological and LDR analyses.

DISCUSSION

The objective of this work was to develop an assay that could rapidly identify the main pathogens known to cause mastitis in cattle, sheep, and goats or those responsible for foodborne intoxication or infection, or both. The bacterial pathogens on the assay panel were chosen to include 1) the organisms most frequently isolated from mastitis samples, such as *Staph. aureus*, *Strep. agalactiae*, *Strep. uberis*, and some NAS; 2) those that have been described recently as a contagious cause of subclinical mastitis, such as *Mycoplasma* spp. (Fox et al., 2005); and 3) those potentially dangerous for human health, such as *E. coli* and related species (Bottero et al., 2004), *Salmonella* spp. (Jayarao et al., 2006), *Staph. aureus* (De Buyser et al., 2001), *B. cereus* (Wong et al.,

Figure 2. A) Universal array scheme. Each ZipCode is spotted in quadruplicate; oligonucleotides associated with actual probe pairs are highlighted in gray. ZipCodes 66 and 63 are the hybridization and ligation controls, respectively. Blank spots are negative controls (no oligo; only spotting buffer). B) Table specifying the bacterial groups with the corresponding ZipCode. C) Specificity results of the amplified 16S rRNA gene from *Staphylococcus aureus* (ATCC 19095, ZipCode 2), *Salmonella enteritidis* (ATCC 13076, ZipCode 5), *Campylobacter coli* (ATCC 1061, ZipCodes 8 and 9), *Bacillus cereus* (ATCC 14579, ZipCode 10), *Streptococcus agalactiae* (ATCC BAA611D, ZipCode 15), *Streptococcus uberis* (ATCC 9927, ZipCode 19), *Mycoplasma bovis* (ATCC 25523, ZipCode 20), and *Escherichia coli* O157 (ATCC 700927, ZipCode 28). Each bacterial group has 4 replicate spots. ZipCode numbers of the specific probes are reported under each replicate. Lighted spots at the corners represent the hybridization and ligation controls (spots 66 and 63), as in the universal array scheme (A). NAS = nonaureus staphylococci.

1988), and *Strep. equi* ssp. *zooepidemicus* (Bergonier et al., 1999).

In the last decade, many molecular diagnostic methods have been developed for direct detection of the specific genes of bacteria in clinical and food samples, and many species- or genus-specific assays have been developed for such detection. Some of them are PCR-based assays (Hsu and Tsen, 2001) in which the discrimination of the target species is accomplished by the specific amplification of a unique region. Recently, a commercial real-time PCR-based assay that identifies 11 major pathogen species or groups responsible for IMI and a gene coding for staphylococcal β -lactamase

production was validated for its analytical specificity and sensitivity (Koskinen et al. 2009). Other authors have integrated the PCR assay, performing a post-amplification hybridization of the target sequence to a very small number of oligonucleotides on membrane (Lin and Tsen, 1999) or glass arrays (Sergeev et al., 2004). Generally, all these assays have targeted a limited number of species (i.e., most of them focused on only 1 and never on more than 6 species) and have required careful optimization for finding the appropriate conditions to amplify the specific target, with subsequent increases in detection time and overall assay duration. These hindrances have been partially circum-

Figure 3. The signal-to-noise ratios (SNR; top, black line) and the unadjusted P -values (bottom, gray line, logarithmic scale) of the serial dilutions of *Mycoplasma bovis* (ATCC 25523), *Streptococcus pyogenes* (ATCC 12344), *Staphylococcus aureus* (ATCC 19095), and *Streptococcus agalactiae* (ATCC BAA611D). The x-axis reports the quantity of PCR product (fmol). The threshold for significance ($P < 0.01$) is drawn as a dashed line.

vented in most recent works, which have targeted 11 or 13 pathogenic species at a time by analyzing the 16S rRNA gene (Wang et al., 2007) or designing the probes by means of comparative genomics (Kim et al., 2008), respectively. However, these assays have not been able to discriminate *Streptococcus* at the species level and have required probes on different genes for detecting *E. coli* and *Salmonella* (Wang et al., 2007) or have shown some degree of aspecificity in detecting *Staphylococcus* spp., requiring at least 10 h to complete and 500 ng of genomic DNA (Kim et al., 2008).

In this work, the molecular procedure chosen to discriminate among the different pathogen groups was the LDR-UA approach. The PCR-LDR-UA assay, including DNA extraction, PCR amplification, LDR,

and data analysis, can be completed in less than 7 h. This assay is based on the properties of DNA LDR and requires 2 specific probes for each target sequence, as first described by Gerry et al. (1999). The use of the LDR in addition to PCR greatly reduces the chances of false positives and results in high specificity, because the procedure is based on the discriminating power of the thermostable ligase that joins the ends of the 2 adjacent oligonucleotides designed for a given target [discriminating probe (DS) or common probe]. The 3' end of the DS lies on the base that discriminates the target from among the others, whereas its 5' end bears a fluorescent reporter. The common probe anneals immediately downstream of the discriminating position and has a phosphorylated 5' end. Only when there is

Figure 4. Heat map of unadjusted *P*-values for ligation detection reaction experiments on 50 milk samples. The map (on the left) indicates the *P*-value of the one-sided *t*-tests. No multiple comparisons correction has been made. The white color indicates a *P*-value of 0.005 or less, whereas the black color indicates a *P*-value of 0.05 or more. Threshold value of 0.01 is highlighted in light gray. Microbiological characterization and source of the samples are provided on the right. NAS = nonaureus staphylococci.

a perfect match between the 2 oligonucleotides does a thermostable ligase join the 2 ends and can the ligation process take place; an artificial sequence (called the ZipCode; Chen et al., 2000), added at the 3' end, then drives the ligated product to a specific position on the UA, where it can be detected. Use of terminal

3'-specific DS to distinguish a species among the others maximizes the selectivity of the ligation process, and thus of the entire procedure. Another advantage of using LDR coupled with PCR in this context is the multiplexing capability of LDR. In the UA-based approach, the optimization of hybridization conditions

for each probe set is not required because the melting temperatures of the ZipCodes are nearly the same (about 68°C). New probe pairs can be added to the array without further optimization, thus reducing costs and setup time (Castiglioni et al., 2004; Chessa et al., 2007). In the current UA layout, up to 48 different species can be targeted independently, but there are no particular hindrances in increasing this number to 100. Moreover, this method can be used to analyze 8 different milk samples simultaneously, reducing the cost of analysis.

Proper design of probes is the key factor for successful oligonucleotide-based assays, relying either on hybridization or on enzymatic procedures. The 16S rRNA is the most commonly used gene for designing oligonucleotide arrays because it has large conserved regions, useful for alignment and “universal” primer design and species-specific variations, capable of distinguishing genera and well-resolved species (Plays et al., 1997) while avoiding the use of several primer sets that might result into a poor amplification efficiency, and thus generating false-positive results (Wilson et al., 2002). The RDP II and BLAST were used in our study to achieve probes that showed optimal specificity and sensitivity for the target species.

In this work, we aimed for precise discrimination of similar species based on 16S rRNA pathogen-specific sequences. Our experimental data, analyzed by the typical *P*-value criterion on a *t*-distribution pattern (<0.01) to determine hybridization differences, confirmed the high specificity of the LDR assay. In most experiments, the calculated *P*-value was well below 0.01 (Table 1), indicating that our detection platform was statistically effective for discriminating between target pathogens.

Significantly, the designed probes correctly differentiated the *Streptococcus* spp. causing mastitis: 1 probe was designed for the following species of the *Streptococcus* group (*Strep. agalactiae*, *Strep. equi*, *Strep. dysgalactiae*, *Strep. uberis*, *Strep. parauberis*, *Strep. bovis*, *Strep. canis*) to determine the prevalence of different species in dairy herds. The PCR-LDR-UA assay allowed for discrimination between *Strep. uberis* and *Strep. parauberis* on the basis of a single PCR on the 16S rRNA gene, avoiding the use of species-specific PCR primers (Hassan et al., 2005). Probe pairs for *Strep. equi* and *Strep. dysgalactiae* were designed to identify any of the 2 subspecies of each. The discrimination of *Strep. equi* ssp. *zooepidemicus* from *Str. equi* ssp. *equi* and *Str. dysgalactiae* ssp. *equisimilis* from *Str. dysgalactiae* ssp. *dysgalactiae* is not possible with the present set of LDR probe pairs. A further refinement of the assay would be the design and testing of specific probe pairs for the zoonotically important subspecies. Moreover, the diagnosis of *Mycoplasma* spp., known to cause mastitis

in cattle, sheep, and goats, is most commonly made by microbiological procedures, using different specific growth media and long incubation times (González and Wilson, 2003). Thus, isolation and identification of *Mycoplasma* spp. are notoriously difficult and time-consuming. Strains of *M. bovis*, *Mycoplasma agalactiae*, *Mycoplasma arginini*, *Mycoplasma capri*, *Mycoplasma capricolum*, and *Mycoplasma mycoides* can be clearly identified as belonging to the *Mycoplasma* genus by the specific probe pair in the PCR-LDR-UA assay, providing an improved diagnostic method for this pathogen.

In our study, we showed that carefully designed specific oligonucleotide probes can distinguish *Salmonella* spp. from *E. coli* and related species (Table 1), which share large stretches of homologous regions within the amplified segments of the 16S rRNA gene. However, our probe pair, initially designed to be specific only for *E. coli* strains, was found in silico to recognize some *Shigella* strains as well, confirming that they cannot be distinguished on the basis of the 16S rRNA gene, as stated by Wang et al. (2007).

From the probe sensitivity experiments, the PCR-LDR-UA assay revealed a detection limit for *Staph. aureus* down to 12 fmol of PCR product, well below the limit imposed by the European Union [Commission Regulation (EC) No. 2073/2005; <http://eur-lex.europa.eu/JOIndex.do?ihmlang=en>; i.e., 500 cfu/mL, corresponding to approximately 150 fmol]. The sensitivity for 3 other species (*M. bovis*, *Strep. pyogenes*, *Strep. agalactiae*) was even better, resulting in 6 fmol.

Generally, the presence of typical endogenous microflora might mask the detection of foodborne pathogens. To determine the ability of the PCR-LDR-UA assay to identify pathogens in mixed bacterial populations, we prepared and analyzed artificially unbalanced DNA mixtures of different microbial species, obtaining efficient and highly specific detections (data not shown).

Finally, this method was evaluated using 50 bovine, ovine, and caprine milk samples; all the obtained bacterial identifications were in agreement with the result of the microbiological methods; moreover, the presence of other pathogens was assessed. For example, sample number 30, which was found to be positive for *Staph. aureus* by microbiological test, also revealed the presence of *Mycoplasma* spp., *Strep. uberis*, and *Strep. agalactiae* by PCR-LDR-UA.

In conclusion, we demonstrated the great potential of the DNA chip described herein for rapid, sensitive, and reliable identification, directly from milk samples, of the main pathogens known to cause mastitis in cattle, sheep, and goats or those responsible for foodborne intoxication or infection, or both. Further, the specific target spectra produced by this gene chip may be gradually expanded through addition of newly designed

oligonucleotide probes into the microarray, without the optimization of hybridization conditions. In microbiological contexts, pathogen detection by means of DNA microarrays could be integrated into routine milk monitoring. The estimated unit cost is approximately 1 € per sample, including the oligonucleotide probes and all the reagents. The initially high costs for the eventual setup of a microarray laboratory (because of the spotting robot and the laser scanner) can be amortized over the medium term (approximately 3 yr). Moreover, many facilities offer the spotting and scanning service for reasonable costs. The LDR-UA assay, offering the power of a microarray format, will be expected to facilitate the analyses of several pathogens at the same time in the near future.

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