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(54) Title: DETECTION OF *LISTERIA MONOCYTOGENES, LISTERIA SPP.*, AND *RHODOCOCCUS COPROPHILUS*

(57) Abstract

The invention relates to methods of detecting the: genus *Listeria*; species *Listeria monocytogenes*; and species *Rhodococcus coprophilus*. All primers that react with the named species, but do not cross react with related or unrelated species of bacteria are claimed. The preferred primers for the: genus species *Listeria monocytogenes*; *Listeria*; and species *Rhodococcus coprophilus*; come from the: Listeriolysin O (HylA); 23s rRNA subunit; and 16s rRNA, genes respectively.

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DETECTION OF *LISTERIA MONOCYTOGENES, LISTERIA SPP.*, AND *RHODOCOCCUS COPROPHILUS*

TECHNICAL FIELD

The invention relates to a method of detecting *Listeria* and *Listeria monocytogenes* and in particular to a PCR method for the detection of *Listeria* and *Listeria monocytogenes*. It also relates to primers for use in detecting *Listeria* and *Listeria monocytogenes*.

The invention also relates to a method of detecting *Rhodococcus coprophilus*. In particular the invention relates to a PCR method of detecting *Rhodococcus coprophilus*. It also relates to primers for use in detecting *Rhodococcus coprophilus*.

BACKGROUND ART

Listeria is a bacterial pathogen found in contaminated food.

Current microbiological culture procedures for the detection of *Listeria* are labourious and time consuming. Many authors in the literature have laboured this point. The recent developments in molecular biology have raised the possibility of detecting pathogens in foods and other samples. For this reason we decided to investigate the use of DNA techniques for the detection of *Listeria monocytogenes*.

A DNA method used by the Australian Molecular Microbiological Laboratory, Division of Analytical Laboratories, NSW Health Department, Lidcombe, Sydney has been found to produce too many non specific products causing a problem in positive identification of *L.monocytogenes*. For this reason we decided to go back to basics using DNA sequences from Genbank and designing our own primers and establish our own PCR system.

Some *L.monocytogenes* primers have been disclosed previously (Mengaud 1988). However the specificity is low and nonspecific binding is relatively high.

The development of a simple and convenient assay for *Listeria monocytogenes* would be useful. It would, for example, enable one skilled in the art to determine whether a sample, for example, a food sample, was contaminated with *L.monocytogenes*.

Contamination of New Zealand's aquatic environments by faecal material degrades them for use as a drinking supply or for recreation because of the potential presence of pathogenic microbes. New Zealand with its high animal to human ratio would be expected to have a high proportion of animal faecal material in rural waterways. The main international opinion is that human faecal pollution constitutes a greater disease risk than animal pollution, however this has not been conclusively established. Whatever their relative quantities and risk factors, management of human effluents is generally accorded a higher priority than animal, since they are more likely to contain human pathogens than animal faeces. Current methods used for faecal indication do not allow the sources of contamination to be differentiated.

Rhodococcus coprophilus (Gram positive to Gram variable) was recognised almost 20 years ago as a potential indicator of domesticated herbivores (Rowbotham and Cross 1977; Mara and Oragui, 1981) but traditional methods of culturing (2 weeks incubation) have limited its application. Traditional culturing procedures take up to 14 days to establish and speeding up identification would also be an advantage. Enumeration of *R. coprophilus* by traditional culturing methods also

presents potential problems due to the possibility of break-up of actinomycete hyphae into coccoid elements.

Initial searches on databases for sequence data yielded very little information on *Rhodococcus* species. Some sequence data has been entered into the Genbank database by F. A. Rainey *et al.* 1995. Complete sequences for many *Rhodococcus* species (F. A. Rainey *et al.* 1995) and *Rhodococcus* related species (F. A. Rainey *et al.* 1995) are available. The part of the DNA targeted by the primers is the 16S rRNA DNA which has shown promise in its ability to differentiate at a genus and species level (Kreader 1995). Since the 16S rRNA DNA has multiple copies, theoretically fewer cells should be required for initial PCR reactions. As there is much information published on the 16S rRNA already it enables a large variety of organisms to be compared which makes selecting a *R. coprophilus* specific area of the DNA for primer annealing much simpler.

The development of a simple and convenient assay for *R. coprophilus* would be useful and would, for example, enable one skilled in the art to determine whether a sample such as a water sample was polluted with faecal material animal in origin.

Accordingly, it is an object of this invention to go at least some way in overcoming problems with known methods of detecting *L.monocytogenes* or *R.coprophilus* and to provide an assay for the detection of *L.monocytogenes* and to provide an assay for the detection of *R.coprophilus*, or to at least provide the public with a useful choice.

DISCLOSURE OF THE INVENTION

The invention provides a primer which reacts with *Listeria monocytogenes* but which does not react with related or unrelated species of bacteria.

Preferably the primer is a DNA primer. More preferably it is targeted against the Listeriolysin O gene.

Preferably the primer is selected from the group comprising 310F, 1016R, 715F and 1183R. A combination of two primers is especially preferred.

In another aspect the invention provides a method for detecting *Listeria monocytogenes* in a sample comprising the use of a primer or combination of primers above in a polymerase chain reaction (PCR) method.

The invention also provides a primer which reacts with *Listeria* but which does not react with related or unrelated species of bacteria.

Preferably the primer is a DNA primer. More preferably it is targeted against the 23S rRNA DNA.

Preferably it is selected from the group comprising L318F, L1541F, L1993F, L559R, L2038R and L2534R.

Any combination of two of the primers selected from the group comprising L318F, L1541F, L1993F, L559R, L2038R, and L2534R is preferred.

In another aspect the invention provides a method for detecting *Listeria* in a sample comprising the use of a primer or combination of primers selected from the group comprising L318F, L1541F, L1993F, L559R, L2038R, and L2534R in a PCR method.

The invention also provides a method of detecting *L.monocytogenes* in a sample comprising the step of using a nucleotide primer which reacts specifically with *L.monocytogenes* and which does not react with related species of bacteria to detect the presence or absence of *L.monocytogenes* wherein two primers selected from the group comprising 310F, 1016R, 715F and 1183R are used together with two primers selected from the group comprising L318F, L1541F, L1993F, L559R, L2038R and L2534R.

In another aspect, the invention provides a primer which reacts with *Rhodococcus coprophilus* but which does not react with related or unrelated species of bacteria.

Preferably the primer is a DNA primer and more preferably it is targeted against a 16S rRNA DNA sequence.

More preferrably the primer is selected from the group comprising 143F, 568R, 419F, 443F, 467R and 1124R.

In another aspect, the invention provides a method for detecting *Rhodococcus coprophilus* in a sample comprising the step of using a DNA primer which reacts specifically with *R.coprophilus* and which does not react with related species of bacteria to detect the presence or absence of *R.coprophilus* in the sample in a PCR method.

Preferably the primer is of a DNA primer and more preferably is targeted against a 16S rRNA DNA sequence. Preferably the DNA primer is selected from the group comprising 143F, 568R, 419F, 443F, 467R and 1124R.

In a further aspect, the invention provides a method of producing a primer which is able to react with *R.coprophilus* but which does not react with related or unrelated bacterial species.

Preferably the method may comprise selecting suitable, specific primers on the basis of a 16S rRNA DNA sequence alignment of *R.coprophilus* and related genera. Preferably a suitable primer giving a specific reaction with *R.coprophilus* are tested against related and unrelated species.

The invention also provides a method of producing a primer which is able to react with *L.monocytogenes* but which does not react with related or unrelated bacterial species.

In the method for detecting *Listeria monocytogenes* the denaturation step is preferably carried out at 92-95°C, annealing at 52-65°C and extension at 65-80°C.

In the methods for detecting *Listeria* and *Rhodococcus coprophilus* the denaturation step is preferably carried out at 92-98°C, annealing at 60-70°C and extension at 65-80°C.

With respect to *L.monocytogenes* the method is preferably used for the detection of *L.monocytogenes*cytogenes in a food sample. With respect to *R.coprophilus*, the method is preferably used for the detection of *R.coprophilus* in a water sample.

BRIEF DESCRIPTION OF DRAWINGS

Embodiments of the invention will now be described, by way of example only, with reference to the drawings, in which:

Figure 1 shows *R.coprophilus* 16S rRNA and associated primers; *R.coprophilus* specific primers are shown in bold;

Figure 2 shows PCR using new primesr 419F-1124R and 419F-568R;

Figure 3 shows PCR using new primers, 143F-568R;

Figure 4 shows primers 419F-568R with non related genera;

Figure 5 shows primers 143F-568R with non related genera;

Figure 6 shows primers 143F-568R and 419F-568R with some *Rhodococcus* species;

Figure 7 shows primers 143F-568R and 419F-568R with some *Rhodococcus* species at 60 degree Celcius;

Figure 8 shows the effect of temperature on *R.coprophilus* and *R.zopfii*;

Figure 9 shows the effect of MgC12- wide range of concentrations;

Figure 10 shows the effect of MgC12- narrow range of concentrations;

Figure 11 shows the effect of primer concentration;

Figure 12 shows a narrower range of primers;

Figure 13 shows the effect of DNA concentration;

Figure 14 shows the effect of DNA concentration (lanes 9-17 only);

Figure 15 shows dNTP Optimisation;

Figure 16 shows fine tuning dNTP concentration;

Figure 17 shows Taq Optimisation

Figure 18 shows 143F-568R with *Rhodococcus* and *Rhodococcus* related genera;

Figure 19 shows DNA from extraction 2 run on a 1% agarose gel;

Figure 20 shows Phylogenetic dendrogram based on the comparison of 16S rRNA sequences of *Rhodococcus* and *Rhodococcus* related genera;

Figure 21 shows results of PCR with R. coprophilus DNA and different sets of primers;

Figure 22 shows *L. monocytogenes* listeriolysin O gene and associated specific primers;

Figure 23 shows *Listeria* 23S rRNA gene and associated Listeria specific primers;

Figure 24 shows *Listeria monocytogenes* with all *L. monocytogenes* specific primers;

Figure 25 shows positive and negative food samples with all *L. monocytogenes* specific primers;

Figure 26 shows two positive food samples with all *L. monocytogenes* specific primer pairs;

Figure 27 shows *Listeria* species against the four different primer pairs;

Figure 28 shows *Shigella flexneri, Shigella sonnei* and *Salmonella menston* with *L. monocytogenes* specific primers;

Figure 29 shows two *L. monocytogenes* positive food samples (298 and 297), *Yersinia enterolitica, Campylobacter jejuni* with *L. monocytogenes* specific primers;

Figure 30 shows the effect of BSA and DNA concentration on PCR product in food samples;

Figure 31 shows *Listeria* specific primers against four *Listeria* species and two closely related bacteria;

Figure 32 shows the effect of temperature on the specificity of the *Listeria* specific primers;

Figure 33 shows the effect of $MgCl_2$ on L1541F & L2038R using *L. monocytogenes* and *B.subtilis*;

Figure 34 shows Multiplex (310F & 1016R, L318F & L559R);

Figure 35 shows Optimisation of $MgCl_2$ (lanes 2-9). Different muliplexes (lanes 10-12);

Figure 36 shows *L.monocytogenes* DNA extracted using the current procedure and tested using the different multiplex systems;

Figure 37 shows the use of the new primers 310R and 1016R;

Figure 38 shows a PCR using known primers;

Figure 39 shows the use of new primers;

Figure 40 shows the use of known primers;

Figure 41 shows food samples using the new primers;

Figure 42 shows food samples using known primers;

Figure 43 shows food samples using the new primers;

Figure 44 shows food samples using known primers;

Figure 45 shows food samples using new primers;

Figure 46 shows food samples using known primers;

Figure 47 shows the names given to the *Listeria* species and related genera used in the phylogenetic dendrogram;

Figure 48 shows a phylogenetic dendrogram based on the comparison of 23S rRNA sequences of *Listeria* and related genera;

Figure 49 shows the specificity of primer pairs with *E. coli* and *B. subtilis;*

Figure 50 shows *Bacillus cereus* with *L. monocytogenes* specific primers;

Figure 51 shows *Staphlococcus aureus*;

Figure 52 shows *Enterococcus faecalis* (lanes 2-10) and *Aeromonas hydrophila* (lanes 11-19);

Figure 53 shows a list of the bacterial species tested.

MODES OF CARRYING OUT THE INVENTION

A) Development of a PCR method for the detection of *Rhodococcus coprophilus*

Experimental

Bacterial Strains & Cultivation

The bacterial strains included in this specification are listed in Table 1.

Table 1

Bacterial Strain	Source
Rhodococcus Species	
R. coprophilus	ATCC 29080, NCTC 10994, DSM 43347T
R. equi	ATCC 6939, NCTC 1621, DSM 20307
R. fascians	ATCC 12974, DSM 20669
R. marinonascens	DSM 43752 T
R. rhodnii	DSM 43959
R. rhodochrous	ATCC 13808, NCTC 10210, DSM 43241 T
R. rhodochrous	ATCC 271, DSM 43274
R. ruber	DSM 43338 T
R. zopfii	ATCC 51349 T
Rhodococcus Related Species	
Actinomyces naeslundii	ATCC 12104, NCTC 10301
Corynebacterium xerosis	ATCC 373
Gordona bronchialis	NZ isolate MY 89/0484
Nocardia brasiliensis	ATCC 19295, NCTC 10300
Streptomyces griseus	NCTC 7807
Other Bacteria	
Aeromonas hydrophila	ATCC 7966, NCTC 8049, NCIB 9240
Bacillus cereus	ATCC 10702, NCTC 8035, NCIB 8122
Bacillus subtilis	ATCC 6051, NCTC 3610, NCIB 3610
Enterobacter aerogenes	ATCC 13048, NCTC 10006
Enterococcus faecalis	ATCC 19433, NCTC 775, NCDO 581
Escherichia coli	ATCC 25922
Morganella morganii	ATCC 25830, NCTC 235
Pseudomonas aeruginosa	ATCC 25668, NCTC 10662
Staphylococcus aureus	ATCC 25923
Staphylococcus epidermidis	ATCC 12228

ATCC American Type Culture Collection. NCTC National Collection of Type Cultures. NCIB National Collection of Industrial Bacteria. DSM Deutsche Sammlung von Mikroorganismen. NCDO National Collection of Dairy Organisms.

Table 2. Growth conditions for all bacterial strains used.

Bacterial Strain	Broth/ agar	Days Growth	Temp °C
Rhodococcus Species			
R. coprophilus	BHI broth	8	30
R. equi	BHI broth	5	35
R. fascians	TSA	5	30
R. marinonascens	TSA	5	30
R. rhodnii	TSA	6	30
R. rhodochrous	TSA	6	30
R. rhodochrous	TSA	6	30
R. ruber	TSA	6	30
R. zopfii	TSA	6	30
Rhodococcus Related Species			
Actinomyces naeslundii	BHI broth	6	37
Corynebacterium xerosis	BHI broth	6	37
Gordona bronchialis	BHI broth	6	37
Nocardia brasiliensis	BHI agar	6	37
Streptomyces griseus	BHI broth	6	37
Other Bacteria			
Aeromonas hydrophila	BHI broth	2	35
Bacillus cereus	BHI broth	2	35
Bacillus subtilis	BHI broth	2	35
Enterobacter aerogenes	BHI broth	1	35
Enterococcus faecalis	BHI broth	2	35
Escherichia coli	BHI broth	1	35
Morganella morganii	BHI broth	2	35
Pseudomonas aeruginosa	BHI broth	2	35
Staphylococcus aureus	BHI broth	2	35
Staphylococcus epidermidis	BHI broth	2	35

BHI - Brain Heart Infusion. TSA - Tryptic Soy Agar

BHI agar: 5.3 g BHI agar in 100 mls distilled water. Autoclave 121°C for 15 mins.

BHI broth: 3.8 g BHI broth in 100 mls distilled water. Autoclave 121 °C for 15 mins.

Tryptic soy agar: 4 g in 100 ml distilled water . Autoclave 121°C for 15 mins.

4% Acrylamide Gel

Gel plate preparation

Both plates were washed with Jif and rinsed with dd H_2O and then with 95% ethanol. The good side of small plate was covered with repel silane and the good side of the large plate with bind silane. 4mm plastic spacers were placed on the sides of the large plate and a strip of 4mm paper along the bottom. The plates were sandwiched together with gel tape.

Gel preparation

The urea was prepared while the plates were taped. 42.0 g urea was added to 36 mls of dd H_2O and warmed to dissolve. To the urea the following was added:

10 ml 10x Sanger TBE
16.5 mls 40 % acrylamide
36µl TEMED

and made up to 100 mls with dd H_2O. 350 µl of 10 % ammonium persulphate was added, and the acrylamide mixture drawn up into a syringe and poured slowly down one side of the tilted plate. Any bubbles were tapped out and the comb positioned. The plates were clamped, covered with Gladwrap and left overnight to polymerise.

The gel was run with loading buffer in 1 x Sanger TBE for 30 mins at 1800V, 40 mA, 50W). 8 µl of sample was added to 6 µl of loading buffer and denatured for 4 minutes at 94 °C. The wells of the gel were flushed with 1 x Sanger TBE to remove urea and 8 µl of sample was added per well. The gel was soaked in ethidium bromide/Sanger TBE (200 µl in 1 litre) but the bands were too faint to visualise and the gel was silver stained instead.

Silver staining

The gel was first fixed in 2L of 10 % glacial acetic acid for 30 minutes. After 3 washes in dd H_2O it was agitated in staining solution (2 g silver nitrate and 3 ml formaldehyde in 2 L water) for 30 minutes. The gel was placed in 1L of developing solution (60 g sodium carbonate in 2 L water and chilled to 10 °C. Immediately before use 3 ml of 37% formaldehyde was added and 400 µl sodium thiosulphate 10 mg/ml) for 2-3 minutes. The developing solution was then replaced with the remaining 1 L and the gel agitated for another 2-3 minutes. It was then rinsed twice in water and dried vertically over night.

PCR Assay

PCR amplification was performed in 0.5 ml tubes in a total reaction volume of 100 µl using 50 mM KCl, 10 mM Tris and 2.5 mM $MgCl_2$ pH 8.4, 5 pmoles of each primer (0.05 µM), 2.5 Units of Taq and 200 µM of each dNTP.

Premix	1 Tube

10x PCR Buffer (500mM KCl, 100 mM Tris, 25 mM $MgCl_2$)	10.0 µl
Forward primer	1.0 µl
Reverse primer	1.0 µl
dNTP's (200 µM each)	0.8 µl
Taq (2.5 units)	0.5 µl
DNA	2.0 µl
dd H_2O	84.7 µl
Total volume	100.0 µl

The reaction mixture was overlaid with 50 µl of nujol oil and 2 µl of DNA was added (200 ng/100 µl). The tubes were then briefly centrifuged and then they were placed in a programmable DNA thermal cycler (Perkin-Elmer Thermal Cycler 480).

The thermal profile was 94°C denaturing for 1 min, 55°C annealing for 1 min, 72°C extension for 1 min, over 30 cycles followed by a final 8 min extension step at 72°C.

Detection of PCR products

PCR products were analysed by gel electrophoresis using 2% agarose gels in TBE buffer.

10 X TBE Buffer:	0.9M Tris 0.9 M Boric acid 0.02 M EDTA pH 8.0.
Working TBE (1 X TBE) :	100 mls 10 X TBE 900 mls ddH_2O 25 µl EtBr (10 mg/ml)
2% Agarose gel:	10 g agarose 50 mls 10 X TBE 450 mls ddH_2O 25 µl EtBr (10 mg/ml)

Gels were run for 75 mins at 100 V in 1 x TBE buffer containing ethidium bromide to enable visualisation of the PCR products by u.v. transillumination. Molecular weight markers were included on each gel (123 bp DNA ladder Life Technologies).

APC film development

The gel was placed on the light box gel side up and the film placed onto the gel glossy white (emulsion) side down and exposed for 20 secs. Grey side up the film was placed in developer until the bands appeared. It was washed in water, fixer and again in water.

Results

See next page for gel results.

Lane 1 123 bp ladder

2	143F-467R *R. coprophilus*
3	143F-467R *N. brasiliensis*
4	143F-1124R *R. coprophilus*
5	143F-1124R *G. bronchialis*
6	143F-1124R *C. xerosis*
7	143F-1124R *N. brasiliensis*
8	143F-926R *R. coprophilus*
9	143F-926R *S.griseus*
10	143F-926R *G. bronchialis*
11	143F-926R *N. brasiliensis*
12	143F-1220R *R. coprophilus*
13	123 bp ladder
14	143F-1220R *S.griseus*
15	143F-1220R *G. bronchialis*
16	143F-1220R *C. xerosis*
17	143F-1220R *N. brasiliensis*
18	443F-1124R *R. coprophilus*
19	443F-1124R *R.equi*
20	443F-1124R *M. morganii*
21	443F-1124R *S.griseus*
22	443F-1124R *G. bronchialis*
23	443F-1124R *C. xerosis*
24	443F-1124R *N. brasiliensis*
25	123 bp ladder

From this it was determined that no significant difference in band size could be seen and that 1124R would not be suitable for use as a primer.

DNA Extraction

Crude Extraction

10 ml cultures were centrifuged for 40 mins at 3300 g. The supernatant was removed and 300 µl extraction buffer (25 mM Tris, 10 mM EDTA, 50 mM glucose, pH 8.0. Autoclaved before use) added. The contents were transferred to a microcentrifuge tube and 20 µl lysozyme (50 mg/ml in dd H_2O) was added and incubated for 5 minutes at room temperature. 12 µl 20 % SDS and 4 µl proteinase K (10 mg/ml in ddH_2O) were then added and incubated at 37°C for 30 mins.

Phenol chloroform preparation

To 500 g phenol (BDH), 500 ml chloroform with 20 ml iso-amylalcohol and 0.5 g 8-hydroxyquinoline was added and left overnight at room temperature. Sufficient 0.1 M Tris HCl (pH 8.0) was added to nearly fill the container and the container shaken gently to equilibrate. The layers were allowed to separate and the top aqueous layer discarded. The mixture was washed twice more with 0.1 M Tris HCl (pH 8.0) and the pH of the supernatant checked to be between 7.5 and 8.0. The phenol/chloroform was store at 4°C under 0.1M Tris HCl (pH 8.0).

Nucleic acid extraction

300 µl of the above phenol chloroform was added to the lysed culture and gently mix end over end for 10 mins. It was then centrifuge for 15 mins at 13,000 rpm and the top aqueous layer transferred to another tube. A further 300 µl of phenol chloroform was added and mixed end over end for 10 mins. The extract was then centrifuged as before and the top aqueous phase transferred to a new tube. 300 µl of chloroform was added and mixed end over end for 10 mins. It was centrifuge as before, the top aqueous phase transferred to a new tube and 25 µl of 3M sodium acetate pH 5.2 was added with 600 µl of absolute ethanol. Tubes were then stored either overnight at -20°C or for at least 1 hour at -70°C. The tubes were centrifuged again at 13,000 rpm for 15 mins and the supernatant discarded. 600 µl of 70 % ethanol was added and centrifuged again as before. As much as possible of the supernatant was removed with a pipette and any remaining ethanol evaporated by placing the tube on a 100°C hot block until the tube was dry. The DNA was then resuspended in 20µl of dd H_2O and stored at -20°C.

DNA Quantitation

To quantitate the amount of DNA present two absorbances were necessary, one at 260 nm and the other at 280 nm. 2 µl of the stock DNA solution was added to a quartz cuvette with 2 ml of ddH_2O. Using ddH_2O as a blank the absorbances at 260 nm and at 280 nm were read.

From the A260 the concentration of DNA was determined:

1 OD unit A260 = 50 µg/ml double stranded DNA
[DNA] µg/ml = A260 x 50 x 1000 (dilution)

From the A260/A280 ratio an estimate of the purity of the DNA was determined. Pure preparations of DNA have A260/A280 ratios of 1.8.

Table 3. Extraction 1. *R. coprophilus.*

Bacteria	A260	A280	$\frac{A260}{A280}$	mg/ml
R. coprophilus	0.044	0.025	11.76	2.2

Table 4. Extraction 2. Unrelated species.

Bacteria	A260	A280	$\frac{A260}{A280}$	mg/ml
S. Epidermidis	-0.034	-0.037		
E. faecalis	0.001	-0.055		
A. hydrophila	-0.007	-0.019		
E. aerogenes	0.077	0.045	1.71	3.85
E. coli	0.063	0.023	2.74	3.15
B. subtilis	-0.036	-0.037		
M. morganii	0.044	0.008	5.5	2.2
P. aeruginosa	-0.027	-0.032		
B. cereus	-0.019	-0.024		
S. aureus	-0.023	-0.029		

Some of the above samples were too dilute too measure the amount of DNA present spectrophotometrically and they were run on a 1% agarose gel to check that there was DNA present. (See Figure 19) These samples were used directly in PCR and not diluted.

Table 5. Extraction 3.

Bacteria	A260	A280	A260/A280	mg/ml
S. aureus	0.045	0.030	1.5	2.25
S. epidermidis	0.010	0.009	1.1	0.5
B. cereus	0.173	0.105	1.6	8.65
B. subtilis	0.074	0.050	1.5	3.7
E. faecalis	0.019	0.015	1.3	0.95
A. hydrophila	0.098	0.070	1.4	4.9
R. equi	0.030	0.020	1.5	1.5
P. aeruginosa	0.006	0.007	0.86	0.3
M. morganii	0.012	0.010	1.2	0.6
A. naeslundii	0.022	0.020	1.1	1.1
G. bronchialis	0.010	0.007	1.4	0.5
C. xerosis	0.010	0.007	1.4	0.5
N. brasiliensis	0.014	0.003	4.7	0.7
S. griseus	0.067	0.055	1.2	3.35

Table 6. Extraction 4. *Rhodococcus* species

Bacteria	A260	A280	A260/A280	mg/ml
R. rhodochrous DSM43241	0.014	0.008	1.75	0.7
R. rhodochrous DSM 43274	0.034	0.019	1.8	1.7
R. ruber	0.012	0.005	2.4	0.6
R. rhodnii	0.008	0.002	4.0	0.4
R. zopfii	0.031	0.018	1.7	1.6

Table 7. Extraction 5. *Rhodococcus* species

Bacteria	A260	A280	A260/A280	mg/ml
R. fascians	0.009	0.003	3.0	0.45
R. marinonascens	0.057	0.040	1.4	2.85

Preparation of DNA for PCR

If the stock sample of DNA was greater than 100 ng/ml(0.1 mg/ml) a working solution was prepared by dilution of the stock to 100 ng/ml. 2 µl of this working solution was added to each PCR assay to give a final concentration of 200 ng/100 µl. If the DNA was more dilute than this it was used neat in the PCR reaction, 2 µl being added.

Oligonucleotide primers

Previous searches for DNA sequence data for *R. coprophilus* have yielded very little information. Since then however a large number of genetic sequences has been entered onto the Genbank database (http://ncbi.nlm.nih.gov/genbank) largely following an extensive study by Rainey F. A. *et al* 1995 on the 16S rRNA sequences for 32 strains of 26 species of the genera *Rhodococcus* and *Nocardia.*

Having collated all the 16S rRNA sequence data possible from Genbank the sequences were aligned using the DNAMAN sequence alignment package. From the alignment, areas specific to *R. coprophilus* could be chosen and PCR primers designed around these areas. (See appendix 2 for sequence data related to the primers used)

Eight primers were chosen, 4 selected to be specific for *R. coprophilus* and another four which were specific for all bacteria to be used to determine which of the *R. coprophilus* specific primers were working.

Table 8 showing details of 16S primers chosen

Primer	Specificity	Sequence	bp	Tm *	Tm **	% GC	µg	ΔG
143F	*R.coprophilus*	GGGTCTAATACCGGATATGACCAT	24	56	67	45	666	- 0.8
443F	*R.coprophilus*	TGTACCTGCAGAAGAAGCACCGGCT	25	66	71	56	753	- 0.9
467R	*R.coprophilus*	AGCCGGTGCTTCTTCTGCAGGTACA	25	66	71	56	535	- 0.9
1124R	*R.coprophilus*	CCGGCAGTCTCTTACGAGTCCCCC	24	66	74	66	665	+ 0.9
530F	Bacteria	GTGCCAGCAGCCGCGG	16	62	70	81	457	+ 1.0
926R	Bacteria	TCCGCCGCTTGTGCGGGC	18	69	72	77	481	- 5.7
942F	Bacteria	GGGCCCGCACAAGCGG	16	63	70	81	465	- 3.9
1220R	Bacteria	ATTGTAGCATGTGTGAAGCC	20	50	63	45	551	+ 1.1

* Tm calculated by Oligo ** Tm calculated by manufacturer Life Technologies
bp = base pairs

Figure 1 shows *R. coprophilus* 16S rRNA and associated primers.

ddH_2O was added to the primers to give a stock concentration of 100 nmoles/ml. A further 1/20 dilution of each primer was done to produce a working solution for PCR of 5 pmoles/µl.

From the sequence we used (Rainey F *et al*, 1995), the primer positions are as follows:

- 5' end of the 143F primer starts on base pair 143
- 5' end of the 419F primer starts on base pair 419
- 5' end of the 443F primer starts on base pair 443
- 5' end of the 467R complement starts on base pair 467
- 5' end of the 568R complement starts on base pair 568
- 5' end of the 1124R complement starts on base pair 1124.

PCR

R.coprophilus. R. equi and unrelated genera

Initially the first step was to test the different combinations of primers with *R. coprophilus* to determine that bands would be obtained. *R. coprophilus* was grown as stated in 'Bacteria and Cultivation' and the DNA extracted as in 'DNA extraction' extraction 1. The PCR was carried out according to 'PCR assay' above. The procedure was then repeated for all the other species outlined in Table 9, using the DNA from extraction 2. All PCR products were detected using agarose gels and visualised using ethidium bromide as outlined in 'Detection of PCR products'. The results are summarised below.

Table 9. Summary of PCR products (bp) obtained with different combinations of primers with *R. coprophilus, R. equi* and some commonly found bacteria.

	143F 467R	143F 1124R	143F 926R	143F 1220R	443F 926R	443F 1124R	443F 1220R	530F 926R	530F 1124R	530F 1220R	942F 1124R	942F 1220R
Rhodococcus coprophilus	220	980	760	1060	460	690	750	460	650	710	295	335
Rhodococcus equi	-	-	-	-	500 4000	680	-	430	680 1120 >4000	740, 550 1120,1600 4000	250 4000	260 350
Staphylococcus epidermidis	-	-	-	-	-	-	-	430	250, 550 670, 4000	ND	250 4000	ND
Enterococcus faecalis	-	-	-	-	490	1600	-	430	615	ND	120	ND
Aeromonas hydrophila	-	-	-	-	430	310 620	310 740	370	615	ND	250	ND
Enterobacter aerogenes	-	-	-	-	490	-	740	430	-	680	-	310
Escherichia Coli	-	-	-	-	450	-	740	430	-	740	-	310
Bacillus subtilis	-	-	-	-	-	-	310	430 860 1350	615, 860 980, 1230 1350, 4000	ND	250 4000	ND
Morganella morganii	-	-	-	-	490	680	740	430	-	ND	-	ND
Pseudomonas aeruginosa	-	-	-	-	490	-	-	430	620	730	-	310
Bacillus subtillis	-	-	-	-	-	-	-	430	620	ND	120	ND
Staphylococcus aureus	-	-	-	-	-	-	-	430	620	ND	120	ND

ND = Not determined

From Table 9 it can be seen that primer 143F can distinguish between *R. coprophilus* and all the bacteria tested including the closely related *R. equi*, by the absence of a band whether it is used with or without a specific primer. From the DNA sequence alignment, for 143F to be able to distinguish between *R. coprophilus* and *R. equi* it must be able to pick up a difference of 3 bp on the 3' end.

Effect of temperature.

It was thought that the two sets of primers may bind more specifically if the annealing temperature was raised from 55°C to 60°C. The PCR was repeated for these three sets of primers with all the bacterial DNA that produced a band and all the related genera. The PCR conditions were as outlined in 'PCR assay' with the exception that the annealing temperature was raised from 55°C to 60 °C

Table 12. Effect of temperature.

Primers	Bacteria	55°C	60°C	
143F-467R	*R.coprophilus*	320	320	No change
	S. griseus	-	320	Worse
	A naeslundii	-	-	No change
	G. bronchialis	-	320	Worse
	C. xerosis	-	-	No change
	N. brasiliensis	350	320	No change
	R. equi	-	-	No change
143F-1124R	*R.coprophilus*	980	980	No change
	S. griseus	-	-	No change
	A naeslundii	-	-	No change
	G. bronchialis	1000	980	No change
	C. xerosis	980	-	Improved
	N. brasiliensis	1050	980	No change
	R. equi	-	-	No change
443F-1124R	*R.coprophilus*	690	680	No change
	E. faecalis	1600	-	Improved
	A hydrophila	310, 620	310, 680	No change
	M. morganii	680	680	No change
	R. equi	680	680	Fainter, improved
	S. griseus	700	680	No change
	A. naeslundii	-	680	Worse
	G. bronchialis	680	680	Fainter, improved
	C. xerosis	620	680	No change
	N. brasiliensis	680	680	No change

Temperature improved some of the specificity but was reduced in others.

R. coprophilus and related genera

The next set of experiments was aimed at testing more closely related species of bacteria. DNA was extracted as before from five more closely related species (extraction 3), *Actinomyces naeslundii, Gordona bronchialis, Corynebacterium xerosis, Norcardia brasiliensis & Streptomyces griseus.*

Table 11. PCR results for *Rhodococcus coprophilus* and 5 related genera.

	143F 467R	143F 1124R	143F 926R	143F 1220R	443F 926R	443F 1124R	443F 1220R	530F 926R	530F 1124R	530F 1220R	942F 1124R	942F 1220R
Rhodococcus coprophilus	320	980	760	1060	460	690	750	460	650	710	295	335
Streptomyces griseus	-	-	750	1050	480	700	740 550	430	-	750	-	370
Actinomyces naeslundii	-	-	-	-	480	-	800	-	650	700	250	300
Gordona bronchialis	-	1000	800	1100	490	680	- -	430	680	ND	-	ND
Corynebacterium xerosis	-	980	-	980	430	600	720	400	610	ND	250	ND
Nocardia brasiliensis	350	1050	800	1100	490	680	740	430	620	ND	250	ND

From Table 11. it can be seen that the primers are having more difficulty distinguishing between *R. coprophilus* and the more closely related species. It was decided to concentrate on the most likely sets of primers that would work, 143F-467R, 143F-1124R and 443F-1124R.

Sequences of the DNA at the position of the 143F primer in some closely related *Rhododcoccus* species.

	5' 3'
R. coprophilus DSM 43357T	GGGTCTAATACCGGATATGACCAT
R. equi ATCC 6939	GGGTCTAATACCGGATATGAGCTC
R. marinonascens DSM 43752T	GGGTCTAATACCGGATACGACCTT
R. fascians DSM 20669	GGGTCTAATACCGGATATGACCAC

If 143F is to be used it must be capable of picking up smaller differences such as those shown above for *R. marinonascens* and *R. fascians*. Both these cultures were ordered as these should be some of the most difficult to differentiate.

Primer 467R can only be used in conjunction with 143F and as 143F to date has always worked it can't be determined if 467F is working or not. Therefore another non specific forward primer was ordered, 27F, to test whether it is working.

Primer 443F is unable to distinguish *R. coprophilus* from other species and therefore 143F is preferred as the forward primer. Another forward primer was ordered to replace it, 419F.

Primer 1124R can't always distinguish between *R. coprophilus* and other species although it often produces several bands which could be a way of distinguishing or it may be that the bands it does produce are of different lengths. All the PCR products from 1124R and 443F were run on a 4% acrylamide gel to determine if there is any difference in band size. From this it was determined that no significant difference in band size could be seen and that 1124R would not be suitable for use as a primer. A replacement primer for 1124R was ordered, 568R.

Table 10. Details of new primers

Primer	Specificity	Sequence	bp	Tm *	Tm **	% GC	µg	ΔG
27F	Bacteria	AGAGTTTGATCCTGGCTCAG	20	50	65	50	610	+0.6
419F	*R. coprophilus*	CAGCAGGGACGAAGCGCAAGTGACT	25	68	72	60	688	+1.1
568R	*R. coprophilus*	GCAGTTGAGCTGCGGGATTTCACAC	25	66	71	56	582	-2.2

bp = base pairs

New Primers (419F and 568R) - Related genera/species

The new primers were diluted to give a stock concentration of 100 nmoles/ml. A further 1/20 dilution was carried out to give a working solution of 5 pmoles/µl.

PCR amplification was performed as outlined in 'PCR assay' with the following exception. The thermal profile on the Perkin-Elmer was: 94°C denaturing for 1 min, 60°C annealing for 1 min, 72°C extension for 1 min, over 30 cycles followed by a final 8 min extension step at 72°C.

Figure 2 shows PCR using new primers, 419F - 1124R and 419F - 568R.

Figure 3 shows PCR using new primers, 143F - 568R.

Large amounts of primer dimer are seen in 419F-1124R. No primer dimer is seen with 419F-568R or 143F-568R. The latter two primers show good strong bands with *R. coprophilus* but not with any other of the related families tested. As 419F-1124R gave bands with *N. brasiliensis* work was continued with the other two sets of primers(143F-568R and 419F-568R).

New Primers (143F-568R; 419F-568R) - unrelated genera

The next step was to test the two new sets of primers with the non related species.
A PCR was set up for both sets of primers using the same conditions as for the last experiment.

Figure 4 shows Primers 419F-568R with non related genera.

Figure 5 shows Primers143F-568R with non related genera.

Both sets of the primers perform well with all non related species. The only band formed was one with *B. cereus* with 419F-568R, which was of a different size and easily distinguishable from the *R. coprophilus* band. *R . coprophilus* DNA was also tested in a multiplex of 143F, 419F and 568R which gave two strong bands at 425 and 149 base pairs (lane 14).The next step of the assay is to test both sets of primers with closely related *Rhodococcus* species to determine if there is any non specific bands.

New Primers (143F-568R; 419F-568R) - *Rhodococcus* species

The DNA was extracted as before (extraction 4) and a PCR set up using the same conditions as used previously.

Figure 6 shows Primers 143F - 568R and 419F - 568R with some *Rhodococcus* species.

143F -568R showed a strong band with *R.coprophilus* and only a faint band with *R. zopfii* all other species tested showed a negative reaction. 419F-568R showed a strong band with *R. coprophilus* but weak bands with all others tested. It was thought that these weak bands may be removed by increasing the temperature even further to 65°C or by changing the $MgCl_2$ concentration. Temperature was thought to be the main effector and so the experiment was repeated exactly as above except at an annealing temperature of 65°C and not 60°C.

Figure 7 shows Primers 143F - 568R and 419F - 568R with some *Rhodococcus* species at 60 °C.

The increase in temperature removed the non specific *R. zopfii* band with 143F-568R primers, however bands could still be seen with *R. rhodochrous* DSM 43241 and *R. zopfii* with primers 419F-568R. As temperature was thought to be the major factor to effect specificity, work was now only continued with primer set 143F-568R.

Optimisation of the PCR protocol - using 143F and 568R as primers

Having established a basic PCR assay that was specific for *R. coprophilus*, it was necessary to optimise the assay conditions to further improve the specificity of the reaction.

Temperature

An increase in temperature from 60°C to 65°C had a dramatic effect on the specificity. However it was thought possible that the temperature may be too high. Usually an annealing temperature of less than 5°C of the Tm for each primer should be used. (Tm 67°C for 143F and 71°C for 568R) A range of temperatures was tried between 60°C and 65°C to determine the optimum, ie the sharpest and most intense *R. coprophilus* band and no *R. zopfii* band.

PCR's for *R. coprophilus* and *R. zopfii* were carried out at annealing temperatures of 61°C, 62°C, 63°C and 64°C. Apart from the annealing temperature the conditions were identical to the previous experiments. (PCR products for 60°C and 65°C from the previous experiment were run on the gel also)

Figure 8 shows the effect of temperature on *R. coprophilus* and *R. zopfii.*
The *R. coprophilus* band starts decreasing in intensity at 64°C so 63°C would be optimum, but there is possibly a faint band of *R. zopfii* at this temperature. To allow for changes in temperature on other machines and for the possibility of other *Rhodococcus* species having a stronger band the temperature is to be kept at 65°C.

$MgCl_2$ concentration

10x PCR buffer with no $MgCl_2$ was prepared (500 mM KCl, 100 mM Tris) and autoclaved. 25 mM $MgCl_2$ was also prepared and autoclaved. A range of $MgCl_2$ concentrations was tried around the concentration already being used.

Figure 9 shows the effect of $MgCl_2$- Wide range of concentrations.

A narrower range was then tried at 2.0, 2.2, 2.4, 2.6, 2.8 & 3.0 mM

Figure 10 shows the effect of $MgCl_2$ - Narrow range of concentrations.

It was decided that the original concentration of 2.5 mM $MgCl_2$ was optimum.

Primer concentration

The primer concentration used throughout the experiments was 5 pmoles/100 µl. To optimise the primers a range was tried of 2, 4, 5, 6, 8 & 10 pmoles/100 µl.

Figure 11 shows the effect of primer concentration

From the above results a narrower range was tried of 2, 2,5, 3.0, 3.5, 4.0, 4.5 and 5 pmoles.

Figure 12 shows a narrower range of primers.

DNA concentration

Initially concentration of DNA is 200 ng/100 µl. A range was tried from 400 ng to 20 ng as shown below:

Figure 13 shows the effect of DNA concentration.

A lower range was then tried from 200 ng / 100µl to 0.2 ng /100 µl

Figure 14 shows the effect of DNA concentration (lanes 9-17 only)

dNTP concentration

The usual dNTP concentration of 25mM (final conc. of 200 µM/100 µl) was further diluted 1/10 to allow a range of concentrations to be tried from 50, 100, 150, 200, 250, 300 µM.

Figure 15 shows dNTP Optimisation.
It was thought that no DNA was added to the PCR tube for the sample in lane 3. The PCR was repeated to narrow down the range and to repeat 150 µM.

Figure 16 shows fine tuning dNTP concentration.

150 µM was chosen as the optimum.

Taq polymerase concentration

Taq is currently used at 2.5 Units/100 µl and therefore a range was tried around this at, 3, 2.5, 2.0, 1.5, 1.0 and 0.5 Units.

Figure 17 shows Taq Optimisation.

2.5 Units is to be used.

Final PCR *Rhodococcus* and related genera.

From the optimisation experiment the initial conditions were found to be optimum and the only change added was a decrease in the level of dNTP's in the final amplication mix to a 150 μM solution.

PCR amplification was performed in 0.5 ml tubes in a total reaction volume of 100 μl using 50 mM KCl, 10 mM Tris and 2.5 mM $MgCl_2$ pH 8.4, 5 pmoles of each primer (0.05 μM), 2.5 Units of Taq and 150 μM of each dNTP.

Premix	1 Tube
10x PCR Buffer (500mM KCl, 100 mM Tris, 25 mM $MgCl_2$)	10.0 μl
Forward primer (5 pmoles/μl)	1.0 μl
Reverse primer (5 pmoles/μl)	1.0 μl
dNTP's (25mM each)	0.6 μl
Taq (2.5 units)	0.5 μl
DNA (100 ng/μl)	2.0 μl
dd H_2O	84.7 μl
Total volume	100.0 μl

The reaction mixture was overlaid with 50 μl of nujol oil and 2 μl of DNA was added (200 ng/100 μl). The tubes were then briefly centrifuged and then they were placed in a programmable DNA thermal cycler (Perkin-Elmer Thermal Cycler 480).

The thermal profile was 94°C denaturing for 1 min, 65°C annealing for 1 min, 72°C extension for 1 min, over 30 cycles followed by a final 8 min extension step at 72°C.

All *Rhodococcus* and *Rhodococcus* related species were run including *R. fascians* and *R. marinonascens* that had not been tested before.

Discussion and Conclusions

Of all the eleven primers ordered only two reproducibly gave a *R. coprophilus* specific amplification, these two were 143F and 568R. All the other primer set combinations produced bands either with the related genera or with the other *Rhodococcus* species. All the amplification conditions have been optimized so that the resultant band is very strong.

The related genera were chosen from dendrogram family trees based on the 16S rRNA homology and similarly the *Rhodococcus* species were chosen in the same manner. The species chosen were ones that were either very closely related in DNA homology to *R. coprophilus* or selected species or genera indicative of other branches from the dendrogram. The extreme genera (little or no homology) were also tested. Obviously not all genera or species were tested, however we believe we have selectively chosen a good representation of the field and have covered the species/genera most likely to cause false positives (most closely related). An example of a genera not tested was *Gordona terrae* (since renamed *R. terrae*) however the DNA homology of this genus and *R.*

coprophilus would indicate that there would be no reaction with the selected primers (143F and 568R). This conclusion is strengthened by the experimental observation that *R. zopfii* which is more closely related to *R. coprophilus* than *R.terrae* did not amplify with these primers.

Primers :

	5' 3'
R. coprophilus at site of 143F:	GGGTCTAATACCGGATATGACCAT
R. terrae:	GGGTCTAATACCGGATATGACCAA
R. zopfii:	GGGTCTAATACCGGATATGACCAA
R. coprophilus at site of 568R:	GCAGTTGAGCTGCGGGATTTCACAC
R. terrae:	GCAATTGAGTTGCAGAATTTCACAG
R. zopfii:	GCAGTTGAGCTGCGGGTTTTCACAG

We believe that the mix of genera and species chosen, based on the published work of Rainey *et al* 1995 which includes most of the *Rhodococcus* and related genera, has included any potential species which may cause false positives.

The *Rhodococcus* related genera not tested are shown below with the DNA sequences at the priming sites used (143F and 568R):

Rhodococcus coprophilus #	143F GGGTCTAATACCGGATATGACCAT
Rhodococcus marinonascens **	143F GGGTCTAATACCGGATACGACCTT
Rhodococcus fascians **	143F GGGTCTAATACCGGATATGACCAC
Tsukamurella paurometabolum	143F GGGTCTAATACCGGATATGACCTT
Nocardia brasiliensis **	143F GGGTCTAATACCGGATATGACCTT
Nocardia transvalensis	143F GGGTCTAATACCGGATATGACCAC
Nocardia otitidis-caviarum	143F GGGTCTAATACCGGATATGACCTT
Nocardia farcinica	143F GGGTCTAATACCGGATATGACCTT
Nocardia calcarea	143F GGGTCTAATACCGGATATGACCTC
Nocardia corynebacteroides	143F GGGTCTAATACCGGATAGGACTGC
Nocardia carnea	143F GGGTCTAATACCGGATATGACCTG
Nocardia asteroides	143F GGGTCTAATACCGGATATGACCTT
Nocardia restrica	143F GGGTCTAATACCGGATATGAGCTC
Mycobacterium chlorophenolicum	143F GGGTCTAATACCGAATAGGACCAC
Dietzia maris	143F GGGTCTAATACCGGATATGAACTC
Corynebacterium glutamicum	143F GGGTCTAATACCGAATATTCACAC
Gordona sputi	143F GGGTCTAATACCGAATATTCATTT
Gordona rubropertinctus	143F GGGTCTAATACCGGATATGACCTT
Gordona amarae.	143F GGGTCTAATACCGGATATGACCTG

Rhodococcus coprophilus #	568R GCAGTTGAGCTGCGGGATTTCACAC
Rhodococcus marinonascens **	568R ACAGTTGAGCTGTGAGTTTTCACAA
Rhodococcus fascians **	568R GAAGTTGAGCCCCGGGTTTTCACAA
Tsukamurella paurometabolum	568R GAGGTTAAGCCTCGGGTTTTCACAG
Nocardia brasiliensis **	568R GGGGTTGAGCCCCAAGTTTTCACGG
Nocardia transvalensis	568R GGGGTTGAGCCCCAAGTTTTCACGA
Nocardia otitidis-caviarum	568R GGGGTTGAGCCCCAAGTTTTCACGG

Nocardia farcinica	568R GGGGTTGAGCCCCAAGTTTTCACGG
Nocardia calcarea	568R GCAGTTGAGCTGCTGGTTTTCACAA
Nocardia corynebacteroides	568R ACAGTTGAGCTGCTGGTTTTCACAG
Nocardia carnea	568R GGGGTTGAGCCCCGAGTTTTCACGA
Nocardia asteroides	568R GGGGTTGAGCCCCAAGTTTTCACGA
Nocardia restrica	568R GGGGTTGAGCCCCAAGTTTTCACGG
Mycobacterium chlorophenolicum	568R ACAGTTAAGCTGTGAGTTTTCACGA
Dietzia maris	568R CCGGTTAAGCCGAGGGATTTCACAG
Corynebacterium glutamicum	568R GAAGTTAAGCCCNGGGATTTCAAAG
Gordona sputi	568R GCAGTTAAGCTGCAGAATTTCACAG
Gordona rubropertinctus	568R ACAATTGAGTTGCAGAATTTCACAG
Gordona amarae	568R ACAATTGAGTTGCAGAATTTCACAG

** Denotes genera or *Rhodococcus* species that have been tested with this method and found not to give false positives. They have been included as markers to allow comparison of sequence differences which can be tolerated in this method.
# Denotes *R. coprophilus* and the DNA sequence for which this method has been designed at the primer sites.

All tested species with closely related DNA sequences were found not to react. From the above list and Appendix 2 most bacteria not tested have more mismatches in the primer regions than those tested. Therefore these species are highly unlikely to cause false positives. All of the genera and *Rhodococcus* species not tested were positioned closely in the DNA dendrogram to other bacteria that tested negative. For this reason we believe that false positives will not occur. For example *Tsukamurella paurometabolum* has a close DNA sequence to *R. coprophilus* at the 143F primer site. However this sequnce is very similar to *N. brasiliensis* which was found not to give a product.

Of the *Nocardia, N. transvaliensis,* only has one mismatch on the DNA sequence at the 143F primer site, however within the DNA sequence in the 568R primer region many mismatches occur and for this reason we do not believe amplification is possible. Other bacteria such as *N. calcarea* and *N. corynebacteroides* have few mismatches in the 568R primer region but more in the 143F region. Of the remainding genera *Gordona rubropertinctus* is the most similar to *R. coprophilus.* However all of the genera have more mismatches than *R. zopfii* and therefore highly unlikely to amplify.

The following list contains those genera and *Rhodococcus* species which have not been tested but potentially need to be investigated if any problems occur:

R. opacus	DSM 43206T	R. *erythropolis*	ATCC 4277T
R. luteus	DSM 43673	*R. globerulus*	DSM 4954T
G. terrae	DSM 43249	*N. calcarea*	DSM 43188T
N. transvalensis	DSM 43405T	*N. corynebacteroides*	DSM 20151T
T. paurometabolum	DSM 20162T	*R. chubuensis*	DSM 44019T
G. rubropertinctus	DSM 43197T		

From the above it can be seen that a reliable assay has been developed producing strong bands with *R. coprophilus.* From the DNA sequences obtained this assay is highly unlikely to react with any other bacteria. No amplification products have been found with any other species tested to date other than *R. coprophilus.*

Figure 18 shows 143F-568R with *Rhodococcus* and *Rhodococcus* related genera

Figure 19 shows DNA extraction 2 run on a 1% agarose gel

Figure 20 shows phylogenetic dendrogram based on the comparison of 16S rRNA sequences of *Rhodococcus* and *Rhodococcus* related genera.

Figures 21 shows the results of the PCRs with *Rhodococcus coprophilus* DNA and different sets of primers.

B. Development of a PCR method for the detection of *Listeria monocytogenes*

Experimental

Bacterial Strains & Cultivation

The bacterial strains included in this study are listed in Table 1.

Table 1

Bacterial Strain	Source
Listeria Species	
Listeria monocytogenes	ATCC 19111
Listeria innocua	ATCC 33090
Listeria ivanovii	CDC 797
Listeria seeligeri	ATCC 35967
Listeria Related Species	
Bacillus cereus	ATCC 10702, NCTC 8035, NCIB 8122
Bacillus subtilis	ATCC 6051, NCTC 3610, NCIB 3610
Staphylococcus aureus	ATCC 25923
Other Bacteria	
Aeromonas hydrophila	ATCC 7966, NCTC 8049, NCIB 9240
Campylobacter jejuni	ATCC 33560
Enterobacter aerogenes	ATCC 13048, NCTC 10006
Enterococcus faecalis	ATCC 19433, NCTC 775, NCDO 581
Escherichia coli	ATCC 25922
Morganella morganii	ATCC 25830, NCTC 235
Rhodococcus coprohilus	ATCC 29080, NCTC 10994, DSM 43347T
Pseudomonas aeruginosa	ATCC 25668, NCTC 10662
Salmonella menston	CDC 383
Shigella flexneri	CDC 972
Shigella sonnei	CDC 86
Staphylococcus epidermidis	ATCC 12228
Yersinia enterolitica	ATCC 9610

ATCC American Type Culture Collection. NCTC National Collection of Type Cultures. NCIB National Collection of Industrial Bacteria. DSM Deutsche Sammlung von Mikroorganismen. NCDO National Collection of Dairy Organisms. CDC Communicable Disease Centre New Zealand.

Table 2. Growth conditions for all bacterial strains used.

Bacterial Strain	Broth/ agar	Days Growth	Temp °C
Listeria Species			
Listeria monocytogenes	BHI broth	1	35
Listeria innocua	BHI broth	1	35
Listeria ivanovii	BHI broth	1	35
Listeria seeligeri	BHI broth	1	35
Listeria Related Species			
Bacillus cereus	BHI broth	2	35
Bacillus subtilis	BHI broth	2	35
Staphylococcus aureus	BHI broth	2	35
Other Bacteria			
Aeromonas hydrophila	BHI broth	2	35
Campylobacter jejuni	BHI broth	1	35
Enterobacter aerogenes	BHI broth	1	35
Enterococcus faecalis	BHI broth	2	35
Escherichia coli	BHI broth	1	35
Morganella morganii	BHI broth	2	35
Rhodococcus coprophilus	BHI broth	8	30
Pseudomonas aeruginosa	BHI broth	2	35
Salmonella menston	BHI broth	1	35
Shigella flexneri	BHI broth	1	35
Shigella sonnei	BHI broth	1	35
Staphylococcus epidermidis	BHI broth	2	35
Yersinia enterolitica	BHI broth	1	35

BHI - Brain Heart Infusion. TSA - Tryptic Soy Agar

BHI agar: 5.3 g BHI agar in 100 mls distilled water. Autoclave 121°C for 15 mins.

BHI broth: 3.8 g BHI broth in 100 mls distilled water. Autoclave 121 °C for 15 mins.

Tryptic soy agar: 4 g in 100 ml distilled water . Autoclave 121°C for 15 mins.

DNA Extraction

Crude Extraction

10 ml cultures were centrifuged for 40 mins at 3300 g. The supernatant was removed and 300 µl extraction buffer (25 mM Tris, 10 mM EDTA, 50 mM glucose, pH 8.0. Autoclaved before use) added. The contents were transferred to a microcentrifuge tube and 20 µl lysozyme (50 mg/ml in

dd H_2O) was added and incubated for 5 minutes at room temperature. 12 µl 20 % SDS and 4 µl proteinase K (10 mg/ml in ddH_2O) were then added and incubated at 37°C for 30 mins.

Phenol chloroform preparation

To 500 g phenol (BDH), 500 ml chloroform with 20 ml iso-amylalcohol and 0.5 g 8-hydroxyquinoline was added and left overnight at room temperature. Sufficient 0.1 M Tris HCl (pH 8.0) was added to nearly fill the container and the container shaken gently to equilibrate. The layers were allowed to separate and the top aqueous layer discarded. The mixture was washed twice more with 0.1 M Tris HCl (pH 8.0) and the pH of the supernatant checked to be between 7.5 and 8.0. The phenol/chloroform was store at 4°C under 0.1M Tris HCl (pH 8.0).

Nucleic acid extraction

300 µl of the above phenol chloroform was added to the lysed culture and gently mix end over end for 10 mins. It was then centrifuge for 15 mins at 13,000 rpm and the top aqueous layer transferred to another tube. A further 300 µl of phenol chloroform was added and mixed end over end for 10 mins. The extract was then centrifuged as before and the top aqueous phase transferred to a new tube. 300 µl of chloroform was added and mixed end over end for 10 mins. It was centrifuge as before, the top aqueous phase transferred to a new tube and 25 µl of 3M sodium acetate pH 5.2 was added with 600 µl of absolute ethanol. Tubes were then stored either overnight at -20°C or for at least 1 hour at -70°C. The tubes were centrifuged again at 13,000 rpm for 15 mins and the supernatant discarded. 600 µl of 70 % ethanol was added and centrifuged again as before. As much as possible of the supernatant was removed with a pipette and any remaining ethanol evaporated by placing the tube on a 100°C hot block until the tube was dry. The DNA was then resuspended in 20µl of dd H_2O and stored at -20°C.

DNA Quantitation

To quantitate the amount of DNA present two absorbances were necessary, one at 260 nm and the other at 280 nm. 2 µl of the stock DNA solution was added to a quartz cuvette with 2 ml of ddH_2O. Using ddH_2O as a blank the absorbances at 260 nm and at 280 nm were read.

From the A260 the concentration of DNA was determined:

1 OD unit A260 = 50 µg/ml double stranded DNA
[DNA] µg/ml = A260 x 50 x 1000 (dilution)

From the A260/A280 ratio an estimate of the purity of the DNA was determined. Pure preparations of DNA have A260/A280 ratios of 1.8.

Table 3. Extraction 1. *L. monocytogenes*

Bacteria	A260	A280	$\frac{A260}{A280}$	mg/ml
L. monocytogenes	0.051	0.027	1.88	2.55

Table 4. Extraction 2.

Bacteria	A260	A280	A260/A280	mg/ml
S. aureus	0.045	0.030	1.5	2.25
S. epidermidis	0.010	0.009	1.1	0.5
B. cereus	0.173	0.105	1.6	8.65
B. subtilis	0.074	0.050	1.5	3.7
E. faecalis	0.019	0.015	1.3	0.95
A. hydrophila	0.098	0.070	1.4	4.9
E. coli	0.063	0.023	2.74	3.15
P. aeruginosa	0.006	0.007	0.86	0.3
M. morganii	0.012	0.010	1.2	0.6
E. aerogenes	0.077	0.045	1.71	3.85
R. coprophilus	0.044	0.025	1.76	2.2

Table 5. Extraction 3.

Bacteria	A260	A280	A260/A280	mg/ml
300	0.0335	0.017	2.06	1.75
307	0.034	0.017	2.0	1.70
298	0.054	0.026	2.08	2.70
297	0.035	0.016	2.19	1.75
382	0.018	0.006	3.0	0.90
385	0.029	0.013	2.23	1.45
L. ivanovii	0.066	0.035	1.89	3.25
L. innocua	0.102	0.061	1.67	5.10
L. seeligeri	0.057	0.036	1.58	2.85
S. flexneri	0.122	0.07	1.74	6.10
S. sonnei	0.044	0.022	2.0	2.20
S. menston	0.113	0.061	1.85	5.65
Y. enterolitica	0.143	0.083	1.72	7.15
C. jejuni	0.092	0.077	1.19	4.60

Preparation of DNA for PCR

If the stock sample of DNA was greater than 100 ng/ml(0.1 mg/ml) a working solution was prepared by dilution of the stock to 100 ng/ml. 2 µl of this working solution was added to each PCR assay to give a final concentration of 200 ng/100 µl. If the DNA was more dilute than this it was used neat in the PCR reaction, 2 µl being added.

Oligonucleotide primers

Two pairs of primers were needed for the PCR, one pair to be specific for *L. monocytogenes* the other pair specific for all *Listeria* species.

***Listeria monocytogenes* specific primers**

For the *L. monocytogenes* specific primers the listeriolysin O gene was chosen as it is only found in haemolytic bacteria and would narrow down the amount of organisms that the primers would cross react with. The DNA sequence for the few *Listeriolysin* sequences that could be found on the Genbank database (http://ncbi.nlm.nih.gov/genbank) were aligned using the GCG software package and regions specific for *L. monocytogenes* were chosen for the priming sequences. Four primers were chosen that were thought to be specific *for L. monocytogenes*. Two other primer sequences, LF and LR that have already been published (Bansal 1996) were also selected. Details of each primer are given in Table 6.

Table 6 showing the details of the listeriolysin O primers chosen

Primer	Specificity	Sequence 5 3''	bp	Tm *	% GC	µg	ΔG
310F	*L.monocytogenes*	GCCTGCAAGTCCTAAGACGCCAATC	25	71	56	515	+ 1.8
715F	*L.monocytogenes*	CGCAGTAAATACATTAGTGGAAAGA	25	64	36	592	+ 1.6
LF	*L.monocytogenes*	CAAACGTTAACAACGCAGTA	20	61	40	482	- 0.5
1016R	*L.monocytogenes*	CTTGCAACTGCTCTTTAGTAACAGC	25	67	44	517	- 0.6
1183R	*L.monocytogenes*	ATTTGTCAGTTCTACATCACCTGAG	25	65	40	750	- 0.6
LR	*L.monocytogenes*	TCCAGAGTGATCGATGTTAA	20	61	40	549	+ 2.4

* Tm calculated by manufacturer Life Technologies
bp = base pairs

Figure 22 shows *L.monocytogenes listeriolysin* O gene and associated specific primers.

Most favoured primers shown in bold giving a 706 bp product.

The primer positions are as follows:

- the 5' end of 310F starts on base pair 310,
- the 5' end of 715F starts on base pair 715,
- the 5' end of 1016R complement starts on base pair 1016,
- the 5' end of 1183R complement starts on base pair 1183.

Listeria specific primers

For the *Listeria* specific primers the 16S rRNA was compared from a large number of bacteria but there were very few regions that were specific to *Listeria*. Having collated all the 23S rRNA sequence data possible from Genbank the sequences were aligned using the GCG sequence alignment package. From the alignment, areas specific to *Listeria* could be chosen and PCR primers designed around these areas.

Table 7 showing details of 23S rRNA primers chosen

Primer	Specificity	Sequence 5' 3'	bp	Tm *	% GC	µg	ΔG
L318F	*Listeria*	GGGGAACCCACTATCTTTAGTC	22	67	50	503	+ 1.2
L1541F	*Listeria*	GTGAGAATCCCTTCCACCGAATAT	24	67	45	487	+ 1.3

L1993F	*Listeria*	GTGCTCTATTAGGGTGCAAGTCCGA	25	71	56	556	- 1.9
L559R	*Listeria*	GGGCCTTTCCAGACCGCTTCA	21	70	61	432	+ 1.2
L2038R	*Listeria*	GGCTCTCTCGGGCTTGCACCCTAAT	25	72	60	589	- 1.9
L2534R	*Listeria*	CTGCCCACCTGACACTGTCTCCCCA	25	73	64	510	+ 2.2

* Tm calculated by manufacturer Life Technologies
bp = base pairs

Figure 23 shows Listeria 23S rRNA gene and associated Listeria specific primers.

Most favoured primers shown in bold giving a 241 bp product

The primer positions are as follows:

- the 5' end of L318F starts on base pair 318,
- the 5' end of L1541F starts on base pair 1541,
- the 5' end of L1993F complement starts on base pair 1993,
- the 5' end of L559R complement starts on base pair 559,
- the 5' end of L2038R complement starts on base pair 2038,
- the 5' end of L2534R complement starts on base pair 2534.

All primers were diluted with ddH_2O to give a stock concentration of 100 nmoles/ml. A further 1/20 dilution of each primer was done to produce a working solution for PCR of 5 pmoles/µl.

PCR Assay

PCR amplification was performed in 0.5 ml tubes in a total reaction volume of 100 µl using 50 mM KCl, 10 mM Tris and 2.5 mM $MgCl_2$ pH 8.4, 5 pmoles of each primer (0.05 µM), 2.5 Units of Taq and 200 µM of each dNTP.

Premix	**1 Tube**
10x PCR Buffer (500mM KCl, 100 mM Tris, 25 mM $MgCl_2$)	10.0 µl
Forward primer	1.0 µl
Reverse primer	1.0 µl
dNTP's (200 µM each)	0.8 µl
Taq (2.5 units)	0.5 µl
DNA	2.0 µl
dd H_2O	84.7 µl
Total volume	100.0 µl

The reaction mixture was overlaid with 50 µl of nujol oil and 2 µl of DNA was added (200 ng/100 µl). The tubes were then briefly centrifuged and then they were placed in a programmable DNA thermal cycler (Hybaid Omnigene).

The thermal profile was 95°C denaturing for 1 min, 55°C annealing for 1 min, 72°C extension for 1 min, over 30 cycles followed by a final 8 min extension step at 72°C.

Detection of PCR products

PCR products were analysed by gel electrophoresis using 2% agarose gels in TBE buffer.

10 X TBE Buffer:	0.9M Tris 0.9 M Boric acid 0.02 M EDTA pH 8.0.
Working TBE (1 X TBE) :	100 mls 10 X TBE 900 mls ddH_2O 25 µl EtBr (10 mg/ml)
2% Agarose gel:	10 g agarose 50 mls 10 X TBE 450 mls ddH_2O 25 µl EtBr (10 mg/ml)

Gels were run for 75 mins at 100 V in 1 x TBE buffer containing ethidium bromide to enable visualisation of the PCR products by u.v. transillumination. Molecular weight markers were included on each gel (123 bp DNA ladder Life Technologies).

PCR - All *L. monocytogenes* specific primers against *L. monocytogenes*

Initially the first step was to test the nine different combinations of *L. monocytogenes* specific primers against *L. monocytogenes* to determine that they all give positive results. *L. monocytogenes* was grown as stated in 'Bacteria and Cultivation'and the DNA extracted as in 'DNA extraction' extraction 1. The PCR was carried out according to 'PCR assay' above.

Figure 24 shows *Listeria monocytogenes* with all *L.monocytogenes* specific primers.

Lane	Primer 1	Primer 2
1	123 bp	ladder
2	LF	1016R
3	LF	1183R
4	LF	LR
5	715F	1016R
6	715F	1183R

Lane	Primer 1	Primer 2
7	715F	LR
8	310F	1016R
9	310F	1183R
10	310F	LR
11	123 bp	Ladder

All primer combinations gave bands with *L. monocytogenes* and all gave the theoretical size of product. The primer pairs that gave the strongest bands were those with 310F. 310F and 1016R were chosen to be run as a positive control for later gels. LF and LR gave a fainter band than the others but this could be due to them not being used at the optimum conditions specified by Bansal (1996) ie 1.5 mM MgCl2 at 51°C annealing temperature.

The procedure was then repeated with *E. coli, B. subtilis, B. cereus, S. aureus, E. faecalis* and *A. hydrophila* to determine the specificity of the primers. *B. subtilis* is quite closely related to *L. monocytogenes* and it was thought that it may react. The DNA used was prepared from extraction

2. For all photographs of the gels see Figures 49-52. No product was formed with any of the primer pairs tested against any of the bacteria stated above.

PCR - All *L.monocytogenes* specific primers against some food samples

In an attempt to narrow down the number of primers used two food samples were tested. These two food samples had been previously tested in the PHL lab and one was found to be positive for *Listeria monocytogenes* (sample 382) and the other negative (sample 385). These two samples were tested with all nine different sets of primers. The results are shown below:

Figure 25 shows Positive and negative food samples with all *L. monocytogenes* specific primers.

Lane	Sample	Primer1	Primer2
1	Ladder	123 bp	
2	382	LF	1016R
3	382	LF	1183R
4	382	LF	LR
5	382	715F	1016R
6	382	715F	1183R
7	382	715F	LR
8	382	310F	1016R
9	382	310F	1183R
10	382	310F	LR

Lane	Sample	Primer1	Primer 2
11	385	LF	1016R
12	385	LF	1183R
13	385	LF	LR
14	385	715F	1016R
15	385	715F	1183R
16	385	715F	LR
17	385	310F	1016R
18	385	310F	1183R
19	385	310F	LR
20	L. mono	310F	1016R
21	No DNA	310F	1016R
22	ladder	123 bp	

The above experiment was repeated with two other food samples 300 and 307, both positive for *L. monocytogenes*.

Figure 26 shows two positive food samples with all *L. monocytogenes* specific primer pairs.

Lane	Sample	Primer1	Primer2
1	ladder	123 bp	
2	300	LF	1016R
3	300	LF	1183R
4	300	LF	LR
5	300	715F	1016R
6	300	715F	1183R
7	300	715F	LR
8	300	310F	1016R
9	300	310F	1183R

Lane	Sample	Primer1	Primer 2
11	307	LF	1016R
12	307	LF	1183R
13	307	LF	LR
14	307	715F	1016R
15	307	715F	1183R
16	307	715F	LR
17	307	310F	1016R
18	307	310F	1183R
19	307	310F	LR

10	300	310F	LR

20	L. mono	310F	1016R
21	No DNA	310F	1016R
22	ladder	123 bp	-

From the above two experiments it was found that all the *L. monocytogenes* specific primers chosen were working, forming PCR products with the three positive food samples and no PCR products with the negative food sample. Any of these pairs could be used at this stage but it was decided to narrow the number of primer pairs down to the four sharpest and most intense bands. The primer pairs chosen were:

715F & 1016R 301 bp. See lane 5, 14 Figure 48
715R & 1183R 468 bp. See lane 6, 15 Figure 48
310F & 1016R 706 bp. See lane 8, 17 Figure 48
310F & 1183R 873 bp. See lane 9, 18 Figure 48

PCR - Other *Listeria* species with *L. monocytogenes* specific primers

Three other *Listeria* species were tested against the four primer pairs, *L. ivanovi, L. innocua* and *L. seeligeri.*

Figure 27 shows *Listeria* species against the four different primer pairs.

Lane	Sample	Primer1	Primer2
1	Ladder	123 bp	
2	*L. ivanovi*	715F	1016R
3	*L. ivanovi*	715F	1183R
4	*L. ivanovi*	310F	1016R
5	*L. ivanovi*	310F	1183R
6	*L. innocua*	715F	1016R
7	*L. innocua*	715F	1183R
8	*L. innocua*	310F	1016R

Lane	Sample	Primer1	Primer2
9	*L. innocua*	310F	1183R
10	*L. seeligeri*	715F	1016R
11	*L. seeligeri*	715F	1183R
12	*L. seeligeri*	310F	1016R
13	*L. seeligeri*	310F	1183R
14	No DNA	310F	1016R
15	*L.mono.*	310F	1016R
16	ladder	123 bp	

No PCR product was formed with any of the three *Listeria* species tested with any of the four primer pairs.

PCR - Other food related bacteria with *L. monocytogenes* specific primers

Five food related bacteria and two *L. monocytogenes* positive samples were tested with each of the four primer pairs:

Figure 28 shows *Shigella flexneri, Shigella sonnei* and *Salmonella menston* with *L. monocytogenes* specific primers.

Lane	Sample	Primer1	Primer2
1	Ladder	123 bp	
2	*S. flexneri*	715F	1016R

Lane	Sample	Primer1	Primer2
9	*S. sonnei*	310F	1183R
10	*S. menston*	715F	1016R

3	*S. flexneri*	715F	1183R
4	*S. flexneri*	310F	1016R
5	*S. flexneri*	310F	1183R
6	*S. sonnei*	715F	1016R
7	*S. sonnei*	715F	1183R
8	*S. sonnei*	310F	1016R

11	*S. menston*	715F	1183R
12	*S. menston*	310F	1016R
13	*S. menston*	310F	1183R
14	No DNA	310F	1016R
15	*L.mono.*	310F	1016R
16	ladder	123 bp	

Figure 29 shows two *L. monocytogenes* positive food samples (298 & 297), *Yersinia enterolitica, Campylobacter jejuni* with *L. monocytogenes* specific primers

Lane	Sample	Primer1	Primer2
1	Ladder	123 bp	
2	298	715F	1016R
3	298	715F	1183R
4	298	310F	1016R
5	298	310F	1183R
6	297	715F	1016R
7	297	715F	1183R
8	297	310F	1016R
9	297	310F	1183R
10	*Y. enterolitica*	715F	1016R
11	*Y. enterolitica*	715F	1183R

Lane	Sample	Primer1	Primer2
12	*Y. enterolitica*	310F	1016R
13	*Y. enterolitica*	310F	1183R
14	*C. jejuni*	715F	1016R
15	*C. jejuni*	715F	1183R
16	Blank		
17	Blank		
18	*C. jejuni*	310F	1016R
19	*C. jejuni*	310F	1183R
20	*L.mono.*	310F	1016R
21	No DNA	310F	1016R
22	ladder	123 bp	

It was found that the positive *L. monocytogenes* samples 298 and 297 gave either faint bands or in two cases (lanes 2 & 3) gave no visible band at all. This was thought to be due to either inhibition by a contaminant in the food sample which may be eliminated by adding BSA to the premix, or a too dilute sample of DNA.

All species results with both sets of primers and final PCR procedure are summarised on page 42.

PCR - Effect of BSA in the premix

The two food samples from the previous experiment were retested using BSA in the PCR premix. A 2 mg/ml solution of BSA was prepared in water. 10 µl of this solution was added to each tube to give a final concentration of 0.2 mg/ml The concentration of DNA was also tested by adding double the usual volume with no BSA using 310F & 1016R as primers.(Lanes 6 & 12). A control of no BSA and a normal 1x concentration of DNA was run using 310F & 1016R as primers. (Lanes 7 & 13)

Figure 30 shows Effect of BSA and DNA concetration on PCR product in food samples

Lane	Sample	Primer1	Primer2
1	Ladder	123 bp	
2	298+BSA	715F	1016R

Lane	Sample	Primer1	Primer2
9	297+BSA	715F	1183R
10	297+BSA	310F	1016R

3	298+BSA	715F	1183R
4	298+BSA	310F	1016R
5	298+BSA	310F	1183R
6	298x2DNA	310F	1016R
7	298x1 DNA	310F	1016R
8	297+BSA	715F	1016R

11	297+BSA	310F	1183R
12	297x2 DNA	310F	1016R
13	297x1 DNA	310F	1016R
14	No DNA	310F	1016R
15	*L.mono.*	310F	1016R
16	ladder	123 bp	

It was found that all PCR products were more intense and sharper with the addition of BSA. BSA at 0.2 mg/ml is to be added to the premix in the future. For one sample the increase in DNA had no effect but the other there was a marked increase in the intensity of the band.

PCR - Listeria specific primers

The first step was to test the three sets of *Listeria* specificprimer pairs aginst the four *Listeria* species and two closely related bacteria, *B. subtilis* and *S. aureus.*

Figure 31 shows *Listeria* specific primers against four *Listeria* species and two closely related bacteria.

Lane	Sample	Primer1	Primer2
1	Ladder	123 bp	
2	L. monocytogenes	L318F	L559R
3	L. monocytogenes	L1541F	L2038R
4	L. monocytogenes	L1993F	L2534R
5	*L. ivanovii*	L318F	L559R
6	*L. ivanovii*	L1541F	L2038R
7	*L. ivanovii*	L1993F	L2534R
8	*L. innocua*	L318F	L559R
9	*L. innocua*	L1541F	L2038R
10	*L. innocua*	L1993F	L2534R
11	*L.seeligeri*	L318F	L559R

Lane	Sample	Primer1	Primer2
12	*L.seeligeri*	L1541F	L2038R
13	*L.seeligeri*	L1993F	L2534R
14	*B. subtilis*	L318F	L559R
15	*B. subtilis*	L1541F	L2038R
16	*B. subtilis*	L1993F	L2534R
17	*S. aureus*	L318F	L559R
18	*S. aureus*	L1541F	L2038R
19	*S. aureus*	L1993F	L2534R
20	No DNA	L1541F	534R
21	ladder	123 bp	
22			

The first primer pair L318F & L559R gave bands with all the *Listeria* species (lanes 2, 5, 8 and 11) and no bands with the other two related species *B. subtilis*(lane 14) and *S. aureus*(lane 17). The second primer pair L1541F & L2038R gave a false positive with *B. subtilis*(lane 15) but not with *S. aureus*(lane 18). The final pair, L1993F & L2534R gave false positives with both related bacteria(lanes 16 & 19). It was thought that an increase in the annealing temperature from 55°C to 62°C may improve the specificity.

Effect of temperature

It is thought that 5°C below the Tm of the primer is an optimum annealing temperature. The lowest Tm for the *Listeria* specific primers is 67°C (see Table 7)and it was therefore decided that the annealing temperature could be raised to 62°C The previous experiment was repeated as before but with an annealing temperature of 62°C.

Figure 32 shows the effect of temperature on the specificity of the *Listeria* specific primers.

Lane	Sample	Primer1	Primer2
1	ladder	123 bp	
2	*L. monocytogenes*	L318F	L559R
3	*L. monocytogenes*	L1541F	L2038R
4	*L. monocytogenes*	L1993F	L2534R
5	*L. ivanovii*	L318F	L559R
6	*L. ivanovii*	L1541F	L2038R
7	*L. ivanovii*	L1993F	L2534R
8	*L. innocua*	L318F	L559R
9	*L. innocua*	L1541F	L2038R
10	*L. innocua*	L1993F	L2534R
11	*L.seeligeri*	L318F	L559R

Lane	Sample	Primer1	Primer2
12	*L.seeligeri*	L1541F	L2038R
13	*L.seeligeri*	L1993F	L2534R
14	*B. subtilis*	L318F	L559R
15	*B. subtilis*	L1541F	L2038R
16	*B. subtilis*	L1993F	L2534R
17	*S. aureus*	L318F	L559R
18	*S. aureus*	L1541F	L2038R
19	*S. aureus*	L1993F	L2534R
20	No DNA	L1541F	534R
21	ladder	123 bp	
22			

It was found that increasing the annealing temperature to 62°C from 55°C eliminated the PCR products with *S. aureus* but two bands were still produced with *B. subtilis* although the L1541F & L2038R band was fainter.

Effect of Magnesium Chloride

One pair of *Listeria* specific primers (L318F & L559R) picks only the *Listeria* strains and could be used in the PCR assay. L1541F & L2038R could potentially be improved with a change in the $MgCl_2$ concentration. No further work was to be carried out on L1993F & L2038R. A PCR was set up using 10x PCR buffer with no $MgCl_2$ A 25 mM solution of Mg Cl_2 was prepared and a range of concentrations set up between 0.5 mM and 5.0 mM. Two organisms were tested *L. monocytogenes* and *B subtilis*, a concentration was looked for that would produce a band with *L. monocytogenes* but not with *B. subtilis*. The PCR was run at an annealing temperature of 62°C.

Figure 33 shows the effect of $MgCl_2$ on L1541F & L2038R using *L.monocytogenes* and *B.subtilis*.

Lane	Sample	[$MgCl_2$]
1	Ladder 123 bp	
2	*L. monocytogenes*	0.5 mM
3	*L. monocytogenes*	1.0 mM
4	*L. monocytogenes*	1.5 mM
5	*L. monocytogenes*	2.0 mM
6	*L. monocytogenes*	2.5 mM
7	*L. monocytogenes*	3.0 mM
8	*L. monocytogenes*	3.5 mM
9	*L. monocytogenes*	4.0 mM
10	*L. monocytogenes*	4.5 mM
11	*L. monocytogenes*	5.0 mM

Lane	Sample	[$MgCl_2$]
12	*B. subtilis*	0.5 mM
13	*B. subtilis*	1.0 mM
14	*B. subtilis*	1.5 mM
15	*B. subtilis*	2.0 mM
16	*B. subtilis*	2.5 mM
17	*B. subtilis*	3.0 mM
18	*B. subtilis*	3.5 mM
19	*B. subtilis*	4.0 mM
20	*B. subtilis*	4.5 mM
21	*B. subtilis*	5.0 mM
22	ladder 123bp	

It was found that 1.5 mM $MgCl_2$ gave a faint band with *L.monocytogenes* (lane 4) the minimum $MgCl_2$ would be 2.0 mM. At this concentration there was no band with *B. subtilis.* Primer pair L1541F & L2038R could be used for *Listeria* detection if used at

2.0 mM $MgCl_2$ concentration as a back up for L318F & L559R.

The *B. subtilis* 23S rRNA was retrieved from the Genbank database and the 3 sets of primers compared to it using Oligo.It was found that:

- L318F & L559R had no matches and would not bind the *B. subtilis* DNA and therefore not produce any amplification prdoducts.
- L1541F had 12 matches on the 3' end but a lot of mismatches on the 5'end. L2038R had 24 of its 25 bp matching the *B. subtilis* DNA. This would indicate that they are quite likely to form a PCR product.
- L1993F had 1 mismatch which was not near the 3' end and L2534R had 1 mismatch near the 3; end indicating that these as a pair would almost certainly bind to *B. subtilis* and produce amplification products.

The theoretical findings using Oligo matched exactly with what was found experimentally, indicating that Oligo can be a very useful tool for the initial selection of primer sequences.

PCR - Multiplex

Two primer pairs have now been established individually, one specific for *L. monocytogenes* (310F & 1016R), the other for *Listeria* (L318F & L559R) The next step is to determine if the two primer pairs can be used together in the same PCR reaction and can distinguish between *L. monocytogenes*, other *Listeria* species and other bacteria. PCR was carried out accordong to 'PCR Assay' except that BSA was used at 0.2 mg/ml per tube and the annealing temperature was at 62°C.

Figure 34 shows multiplex (310F & 1016R, L318F & L559R)

Lane	Sample
1	ladder 123 bp
2	*L. monocytogenes*
3	300 Sliced Ham
4	307 Sliced Ham
5	298 Sliced Ham
6	297 Sliced Ham
7	382
8	385
9	*L. ivanovii*
10	*L. innocua*

Lane	Sample
11	*L. seeligeri*
12	*S. flexneri*
13	*S. menston*
14	*Y. enterolitica*
15	*C. jejuni*
16	*B. cereus*
17	*B. subtilis*
18	*S. aureus*
19	Blank

It was found that the primer pairs worked as expected. They showed the first six samples to be *L. monocytogenes* positive. The seventh sample (lane 8) was a negative food sample and gave no bands on the gel. The next three were *Listeria* species and only the expected *Listeria* band formed.. The last seven organisms were not related and no PCR product was obtained. In lane 5 the bands were quite weak. The gel was rerun to see if it was a loading problem.The gel was run as before but at 70V.

The gel still showed faint bands therefore they are not due to under loading.

Optimisation of $MgCl_2$ for the multiplex

The next step was to determine the optimum concentration of $MgCl_2$ for the multiplex. A range of $MgCl_2$ concentrations was tried between 0.5 and 4.0 mM for primers 310F, 1016R, L318F and L559R (lanes 2-9) Other multiplex combinations were also tried (lanes 10-12):

- 310F, 1183R, L318F and L559R producing bands at 873 and 241
- 310F, 1016R, L1541F and L2038R producing bands at 706 and 497
- 310F, 1183R, , L1541F and L2038R producing bands at 873 and 497

Figure 35 shows optimisation of $MgCl_2$ (lanes 2-9). Different multiplexes (lanes 10-12)

Lane	
1	123 bp ladder
2	0.5 mM
3	1.0 mM
4	1.5 mM
5	2.0 mM
6	2.5 mM
7	3.0 mM
8	3.5 mM
9	4.0 mM

Lane	Primers
10	310F, 1183R, L318F and L559R
11	310F, 1016R, L1541F and L2038R
12	310F, 1183R, , L1541F and L2038R
22	123 bp ladder

From the gel it can be seen that there is no PCR product formed at 0.5 and 1.0 mM $MgCl_2$. The concentration is to be kept at 2.5 mM $MgCl_2$. It was found that two of the other primer combinations worked lane 10 (310F, 1183R, L318F and L559R) and lane11 (310F, 1016R, L1541F and L2038R).Lane 12 (310F, 1183R, , L1541F and L2038R) gave a faint *L.monocytogenes* band and therefore may not be as good as the other three combinations.

PCR - To test the current DNA extraction procedure.

To be able to directly compare the current PCR procedure with the new one the extraction methods used must be the same. It was therefore decided to run a pure culture of *L. monocytogenes* DNA having been extracted using the current DNA extraction procedure. The PCR was then run according to 'PCR assay' with added BSA and an annealing temperature of 62°C. Four different primer combinations were tested (lanes 2-5). When primer pair L1541F & L2038R were used the $MgCl_2$ concentration had to be 2.0 mM.

Figure 36 shows *L. monocytogenes* DNA extracted using the current procedure and tested using the different mulitplex systems.

Lane	Sample	Extraction procedure	*L. mono* Primer 1	*L. mono* Primer 2	*Listeria* Primer 1	*Listeria* Primer2	[$MgCl_2$]
1 & 8	Ladder						
2	*L. mono*	Current	310F	1016R	L318F	L559R	2.5 mM

3	*L. mono*	Current	310F	1183R	L318F	L559R	2.5 mM
4	*L. mono*	Current	310F	1016R	L1541F	L2038R	2.0 mM
5	*L. mono*	Current	310F	1183R	L1541F	L2038R	2.0 mM
6	No DNA		310F	1016R	L318F	L559R	2.5 mM
7	*L. mono*	New	310F	1016R	L318F	L559R	2.5 mM

Even when using the current extraction procedure, which doesn't give such a pure preparation of DNA, only two bands occur. No non specific bands are present. All combinations of the primers shown could be used. However the one in lane 2 is favoured as it gives the strongest bands.When the L.monocytogenes specific primers are used with L1541F and L2038R (lanes 4 and 5) they give a weaker band than with the other combinations. This makes them less suitable for use than the primer combination in lane 2.

PCR – Comparing known primers and primers according to the present invention

Viable and non viable *L. monocytogenes* cells from 3 samples had been stored frozen. The samples were defrosted and treated in three different ways (according to appendix 4 section 7.5, 7.6 and 7.7) before running the new PCR:

Viable cells: resuspended and diluted in water (7.5)
denatured (7.6)
centrifuged (7.7) - sample PCR'd(A)

Non viable: centrifuged (7.7)- supernatant PCR'd (C)
resuspended, denatured,(7.6) centrifuged (7.7)- supernatant PCR'd (B)

Figure 37 shows a procedure using primers 310F and 1016R.

Sample No.	Lane	Sample
1	13	L.monocytogenes (A)
2	14	L.monocytogenes (B)
3	15	L.monocytogenes (C)
4	16	Ham 10^{-6}(A)
5	17	Ham 10^{-6}(B)

Sample No.	Lane	Sample
6	18	Ham 10^{-6}(C)
7	19	Ham 10^{-9}(A)
8	20	Ham 10^{-9}(B)
9	21	Ham 10^{-9}(C)

A number of conclusions can be drawn from this experiment:
1. DNA can be extracted from frozen cells and still give a reliable positive result.
2. Heat denatured DNA is able to be frozen and reused with no adverse effect on the results. i.e.the expected bands still amplify and there are no non specific bands.
3. There is no need to redenature DNA, it is sufficient to recentrifuge the cells and use the supernatant.

The results from the new primers were then compared to the results from the known primers using a PCR procedure.

Figure 38 shows the results of a PCR using known primers

Sample No.	Lane	Sample
1, 2, 3	1	*L. monocytogenes*
	2	10^{-2}
4, 5, 6	3	10^{-6}
	4	10^{-8}
7, 8, 9	5	10^{-9}
	6	123 bp ladder
	7	Negative Ham sample

The known *Listeria* primers gives weak amplification products when the sample is at 10^{-9}(lane 5 figure 38) however the new procedure gives strong bands at 10^{-9}. The new primers appear more sensitive.

PCR - Comparison of new and current PCR procedures with food samples

Four food samples were run using the new procedure and compared to the results obtained using the current method. In Figure 39 the upper band (241 bp) is the *Listeria* band and the lower (706 bp) is the *L. monocytogenes*. In Figure 40 the upper band in sample 1 is the *L. monocytogenes* band and the lower one the *Listeria* band.

Figure 39 shows a method using new primers.

Figure 40 shows a method using known primers.

Sample No.	Sample.
1	*L. monocytogenes*
2	300 Sliced Ham
3	307 Sliced Ham
4	298 Sliced Ham
5	297 Sliced Ham

It is clear from the above results that the known primers give a lot of non specific amplification products that can cause confusion in reading the result. The new primers according to the present invention amplify more specifically and only gives two bands with *L. monocytogenes.*

It was thought that the non specific bands could be due to the extraction procedure used for the current method which only crudely purifies the DNA. To determine if this was so a further 34 food samples were tested having had the DNA extracted using the current procedure. The following results therefore are from samples that have been extracted using the current DNA extraction procedure and the only variation in the results is caused by the difference in the PCR procedures.

Figure 41 shows food samples using the new primers.

Figure 42 shows food samples using the known primers.

Sample No.	Sample
1	436 sandwich ham
2	437 sandwich ham
3	438 sandwich ham
4	439 sandwich ham
5	440 sandwich ham
6	011 ice cream
7	012 ice cream
8	013 ice cream
9	015 luncheon
L	123 bp ladder
B	Blank

Sample No.	Sample
10	016 luncheon
11	017 luncheon
12	018 luncheon
13	019 luncheon
14	020 luncheon
15	021 sandwich ham
16	022 sandwich ham
17	023 sandwich ham
18	024 sandwich ham
C	*L. monocytogenes* control

From the above threee of the samples (1, 2, and 3) were found to be *Listeria* positive but not *L. monocytogenes* positive. A much clearer result is obtained using the primers according to the present invention.

Figure 43 shows food samples using the primers according to the invention.

Figure 44 shows food samples using known primers.

Sample No.	Sample
1	055 salami & salad sandwich
2	056 ham sandwich
3	057 ham & pickle sandwich
4	058 ham & egg sandwich
5	059 ham & egg sandwich
6	060 ham & salad sandwich
7	061 ham, salad, egg filled roll
8	062 ham sandwich
9	063 ham sandwich
10	064 ham sandwich

Sample No.	Sample
11	065 ham & egg sandwich
12	066 ham sandwich
13	067 ham,salad,cheese filled roll
14	068 ham, pickle,salad sandwich
15	069 ham,egg,tomato sandwich
16	070 ham & egg sandwich
L	123 bp ladder
C	*L. monocytogenes* control
B	Blank

PCR -Comparison of more food samples and some unrelated bacteria

Figure 45 shows food samples using new primers.

L C B 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 C L

Figure 46 shows food samples using known primers.

Sample No.	Sample
1	*E. coli*

Sample No.	Sample
10	071 corned beef & lettuce sandwich

2	*E. faecalis*
3	*A. hydrophila*
4	*S. sonnei*
5	*S. epidermidis*
6	*P. aeruginosa*
7	*M. morganii*
8	*E. aeruginosa*
9	*R. coprophilus*

11	072 ham & mustard sandwich
12	073 ham sandwich
13	074 corned beef & pickle sandwich
14	075 ham sandwich
15	076 ham sandwich
L	123 bp ladder
B	Blank
C	*L.monocytogenes* control

One sample showed *Listeria* positive but *L. monocytogenes* negative (sample 11). This result is a lot clearer using the new primers than it is using known primers.

SUMMARY

Table 8. Summary of all the pure cultures tested with the two sets of primers.

	Listeria primers L318F & L559R	*L. monocytogenes* primers 310F & 1016R
***Listeria* Species**		
Listeria monocytogenes	+	+
Listeria innocua	+	-
Listeria ivanovii	+	-
Listeria seeligeri	+	-
***Listeria* Related Species**		
Bacillus cereus	-	-
Bacillus subtilis	-	-
Staphylococcus aureus	-	-
Other Bacteria		
Aeromonas hydrophila	-	-
Campylobacter jejuni	NT	-
Enterobacter aerogenes	-	-
Enterococcus faecalis	-	-
Escherichia coli	-	-
Morganella morganii	-	-
Pseudomonas aeruginosa	-	-
Rhodococcus coprophilus	-	-
Salmonella menston	NT	-
Shigella flexneri	NT	-
Shigella sonnei	-	-
Staphylococcus epidermidis	-	-
Yersinia enterolitica	NT	-

NT = Not tested.

PCR procedure according to the present invention:

The final PCR procedure for the detection of *Listeria monocytogenes* is outlined below. PCR amplification was performed in 0.5 ml tubes in a total reaction volume of 100 µl (This can be scaled down to 20 µl to economise on materials.) using 50 mM KCl, 10 mM Tris and 2.5 mM $MgCl_2$ pH 8.4, 5 pmoles of each primer (0.05 µM), 2.5 Units of Taq and 200 µM of each dNTP. It has also been determined that the addition of BSA at a final concentration of 0.2 mg/ml helps to prevent inhibiton of amplification by any contaminants in the samples. The annealing temperature has also been raised from 55°C to 62°C to increase the specificty of the assay.

Premix	**1 Tube**
10x PCR Buffer(500mM KCl, 100 mM Tris, 25 mM $MgCl_2$)	10.0 µl
BSA (2 mg/ml)	10.0 µl
Forward primer 310F	1.0 µl
Reverse primer 1016R	1.0 µl
Forward primer L318F	1.0 µl
Reverse primer L559R	1.0 µl
dNTP's (200 µM each)	0.8 µl
Taq (2.5 units)	0.5 µl
DNA	2.0 µl
dd H_2O	72.7 µl
Total volume	100.0 µl

The reaction mixture was overlaid with 50 µl of nujol oil and 2 µl of DNA was added (200 ng/100 µl). The tubes were then briefly centrifuged and then they were placed in a programmable DNA thermal cycler (Hybaid Omnigene).

The thermal profile was 95°C denaturing for 1 min, 62°C annealing for 1 min, 72°C extension for 1 min, over 30 cycles followed by a final 8 min extension step at 72°C.

Discussion and Conclusions

Listeria

When the primer sequences for the two *Listeria* specific primers, known previously, were checked against the 16S rRNA DNA alignment, primer UI was found (region 528 -545) not to be *Listeria* specific but a universal primer and will bind to any bacteria. UII, the second primer (region 1566-1587) was found to be *Listeria* specific on the last base pair on the 3' end with a few other mismatches throughout the sequence. The 3' end of a primer is the most important region for specificity to occur. The significance of only one mismatch on the 3' end is, the assay may have great difficulty in distinguishing between *Listeria* and other species particularly at the low annealing temperature used (49°C) and the higher magnesium concentration used (3 mM) both of which would promote non specific binding. This may well account for all the extra bands that have been formed during this current PHL PCR.

PCR method according to the present invention using new primers: When all the 16S rRNA sequences were taken from Genbank and aligned using the GCG package very few regions common to all *Listeria* species, but not found in any other species were found. This is why the 23S rRNA DNA was checked for possible *Listeria* specific sites. A number of *Listeria* specific sites were found on the 23S rRNA which have several mismatches on the 3' end with other species and were therefore highly likely to bind specifically to *Listeria*. This has been confirmed with the work carried out to date

Of the three *Listeria* specfic primer pairs that were chosen one pair gave better amplification than the others (L318F and L559R). This primer pair gave a 241 bp product with all *Listeria* species tested but not with any other bacteria tested. A second primer pair (L1541F & L2038R) could be used if for any reason the first cannot but only if the $MgCl_2$ concentration is at 2.0 mM

Listeria monocytogenes

PCR method according to the present invention using new primers: The listeriolysin gene which codes for the protein causing haemolysis was chosen as the region of DNA specific for the *L. monocytogenes* species DNA sequences were taken from Genbank for all lysin genes. We obtained sequences from 5 *L. monocytogenes* species, *L. ivanovii* and *L. seeligeri* and using the GCG package aligned these to examine for potential primer sites. We chose four sites and in addition included the two new sites used by Dr Bansal, LF and LR (Bansal 1996 in press). All sites (9 combinations) amplified with no cross reactivity occuring with other *Listeria* species. However we chose one combination based on the strength and length of the product from primers 310F and 1016R.

Of the nine primer pairs specific for *L. monocytogenes* all of them gave bands against *L. monocytogenes* and didn't cross react with any of the three other *Listeria* cultures or the closely related species tested. The primer pair that was selected (310F and 1016R) gave a 706 bp amplification product.

We were concerned that very few DNA sequences had been found for the lysin gene. We downloaded all the Listeriolysin sequences for all the *L. monocytogenes* strains listed on Genbank, 11 *L. monocytogenes*, 2 *Listeria* species 9 other non *Listeria* species:

Aeromonas hydrophila
Escherichia coli
Proteus vulgaris
Streptococcus canis
Streptococcus equisimilis
Streptococcus pneumoniae
Streptococcus pyogenes
Vibrio cholerae
Vibrio parahaemolyticus

The DNA was checked against the new primers (310F and 1016R) using the DNA program Oligo. All the *L. monocytogenes* species showed complete binding of both primers and all produced a 706 base pair fragment. All the other species and genera tested showed no or only an extremely small degree of potential primer binding. Theoretically the chosen primers should not bind with this DNA and certainly will not cause ambiguity of the results.

When the *L. monocytogenes* primers (LMI and LMII) that are currently used were checked against the DNA GCG alignment package they were found to be in regions that had degrees of specificity for *L. monocytogenes*. The new *L.monocytogenes* primers 310F and 1016R are in the same area as LMI and LMII but are upstream. The 310F primer is three bases and the 1016R primer is twelve

bases upstream. Both these differences incorporate more mismatches at the 3' ends and along the whole template. In addition the primers are 25 bases not 18 bases in length. The effects of both are to increase specificity of binding to the *L. monocytogenes* species and obviously reducing the non specific binding observed.

The melting temperatures (TMs) of our new primers and known, published primers are shown in the following Table 9.

TABLE 9

Primer	Life Technologies formula	Oligo method
310F	71	63
LM1	63	47
1016R	67	54
LM11	64	52
715F	64	51
LF	61	47
1183R	65	52
primer 2(Fluit)	63	41

This shows that our new primers have different TMs to known published primers.

A known DNA method based on earlier work of Dr Bansal used the *Listeria* specific primers of UI and UII combined with *the L. monocytogenes* primers LMI and LMII. This combination of primers has been found to cause many non specific bands. The *Listeria* primers UI and UII (UII is also known as LII in Bansal 1996) have been combined with *L. monocytogenes* primers LF and LR. These latter two primers are used at a lower annealing temperature of 51°C. The LR primer has no *L. monocytogenes* sites on the last three bases at the 3' end and the LF primer has only one specific site on the 3' end. The LF primer has therefore fewer specific sites on this 3' end than our corresponding primer and is potentially more likely to mistype than the primers according to the present invention.

The primers according to the present invention have been designed to maximise the nucleotide differences between all the existing *Listeria* sequences. Preexisting primers do not. The present primers are superior and significantly different to any known primers.

Of the *Listeria* specific primers UI and UII only UII is *Listeria* specific placing all the specifitcity on one primer and not two which is preferable and which we have achieved.

When testing some food samples it was noted that some of the bands were very faint when there should have been a strong positive result. This was thought to be due to inhibiton by a contaminant in the food sample. It was found that the PCR products were more intense and sharper with the addition of BSA in the premix. BSA should now be added at
0.2 mg/ml to the premix to decrease the inhibtion by food samples.

The two primer pairs have been tested in a multiplex system against forty food samples. Both DNA methods have typed these samples similarly however the majority of the current PHL DNA results were difficult to interpret with many non specific amplification bands being formed. These same samples when run in the new DNA method produced either the expected one or two bands or nothing in non *Listeria* samples. The absence of the non specific bands greatly increase the ease of reading the results and makes it less likely for errors to occur. It was thought that some of the non specific bands in the current PHL assay could be due to the DNA extraction which only crudely purifies the DNA. However the forty food samples tested with the new primers were extracted using the crude method and therefore the improvement seen is due solely to the new PCR method and not the extraction procedure.

The new PCR has been tested against three closely related bacteria and a number of other unrelated organisms. The extreme genera (little or no homology) were tested particularly if they were food related pathogens that may be present in the types of samples for which the PCR will ultimately be applied. A larger field of related organisms are still to be tested (see future work)

From the above it can be seen that an assay has been developed producing two strong bands one specific for *L.monocytogenes* and the other specific for *Listeria* species. No amplification products have been found with any other species tested to date other than those that should cause amplification.

Figure 53 shows a list of bacterial species tested to date.

CURRENT POLYMERASE CHAIN REACTION(**PCR**) METHOD FOR DETECTION OF *LISTERIA MONOCYTOGENES* IN FOOD SAMPLES WITH KNOWN PRIMERS

1. OBJECTIVE

This current PCR method is based on amplification of certain sequences of DNA on the *Listeria monocytogenes* genome. It is a multiplex method where two pairs of primers are used in one PCR reaction. One pair of primers is designed specifically for genus **Listeria** identification and another pair for species **monocytogenes** identification.

Primer pair for Listeria genus:

U1 **5'- CAG CAG CCG CGG TAA TAC** Lane et al (1985)

UII **5'- CTC CAT AAA GGT GAC CCT** Stackenbrandt & Curiale (1988)

Primer pair for monocytogenes species:

LM1 **5'- CCT AAG ACG CCA ATC GAA** Mengaud et al (1988)

LMII **5'- AAG CGC TTG CAA CTG CTC** Mengaud et al (1988)

Carry out a PCR reaction using the above primers and cycling times of 95°C for 30 seconds, 49°C for 20 seconds and 72°C for 30 seconds, for 29 cycles with a final extension of 72 °C for 8 minutes.

DNA is extracted from the bacterial cells by heat blasting the cells and adding aliquots to the PCR reaction.

INDUSTRIAL APPLICABILITY

Listeria is a contaminant of food samples and is pathogenic to humans.

The new, specific primers will enable the detection of *Listeria* and *L.monocytogenes* in food samples.

R.coprophilus is a contaminant of water and is also pathogenic to humans.

The invention provides new, specific primers allowing for a simple and convenient assay for its detection. This would enable one to determine whether a sample is polluted with faecal material.

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CLAIMS

1. A primer which reacts with *Listeria monocytogenes* but which does not react with related or unrelated species of bacteria.

2. A primer according to claim 1 which is a DNA primer.

3. A primer according to claim 1 or claim 2 which is targeted against the Listeriolysin O gene.

4. A primer according to any one of the preceding claims which is selected from the group comprising 310F, 1016R, 715F and 1183R.

5. A primer according to claim 4 which is 310F or 1016R.

6. A combination of two primers selected from the group comprising 310F, 1016R, 715F and 1183R.

7. The combination of the two primers 310F and 1016R.

8. A method for detecting *Listeria monocytogenes* in a sample comprising the use of a primer or combination of primers according to any one of the preceding claims in a polymerase chain reaction (PCR) method.

9. A primer which reacts with *Listeria* species but which does not react with related or unrelated species of bacteria.

10. A primer according to claim 9 which is a DNA primer.

11. A primer according to claim 9 or 10 which is targeted against the 23S rRNA DNA.

12. A primer according to any one of claims 9 to 11 which is selected from the group comprising L318F, L1541F, L1993F, L559R, L2038R and L2534R.

13. A combination of two primers selected from the group comprising L318F, L1541F, L1993F, L559R, L2038R and L2534R.

14. A primer according to claim 12 which is L318F or L559R.

15. The combination of the two primers L318F and L559R.

16. A method for detecting *Listeria* in a sample comprising the use of a primer or combination of primers according to any one of claims 9 to 15 in a polymerase chain reaction method.

17. A method of detecting *Listeria monocytogenes* in a sample comprising the use of two primers selected from the group comprising 310F, 1016R, 715F and 1183R together with two primers selected from the group comprising L318F, L1541F, L1993F, L559R, L2038R and L2534R.

18. A method according to claim 17 in which the four primers are 310F, 1016R, L318F and L559R.

19. A primer which reacts with *Rhodococcus coprophilus* but which does not react with related or unrelated species of bacteria.

20. A primer according to claim 19 which is a DNA primer.

21. A primer according to any one of claims 19 or 20 which is targeted against a 16S rRNA DNA sequence.

22. A primer according to any one of claims 19 to 21 which is selected from the group comprising 143F, 568R, 419F, 443F, 467R and 1124R.

23. A primer according to claim 22 which is 143F or 568R.

24. A combination of two primers selected from the group comprising 143F, 568R, 419F, 443F, 467R and 1124R.

25. The combination of the two primers 143F and 568R.

26. A method of detecting *Rhodococcus coprophilus* in a sample comprising the use of a primer or combination of primers according to any one of claims 19 to 25 in a PCR method.

27. A method according to claim 8 or claim 16 wherein denaturation is carried out at 92-98°C, annealing is carried out at 52-70°C and extension is carried out at 65-80°C.

28. A method according to claim 26 wherein denaturation is carried out at 92-98°C, annealing is carried out at 55-70°C and extension is carried out at 65-80°C.

29. A method according to claim 27 or 28 wherein the repetitive cycle is carried out 30-50 times.

30. A method according to any one of claims 27-29 wherein the denaturation, annealing and extension steps are carried out for 30-60 seconds each.

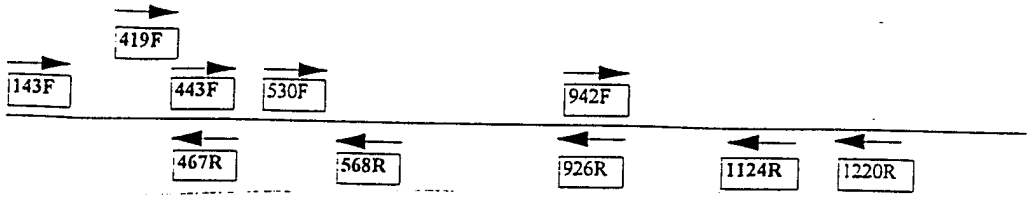
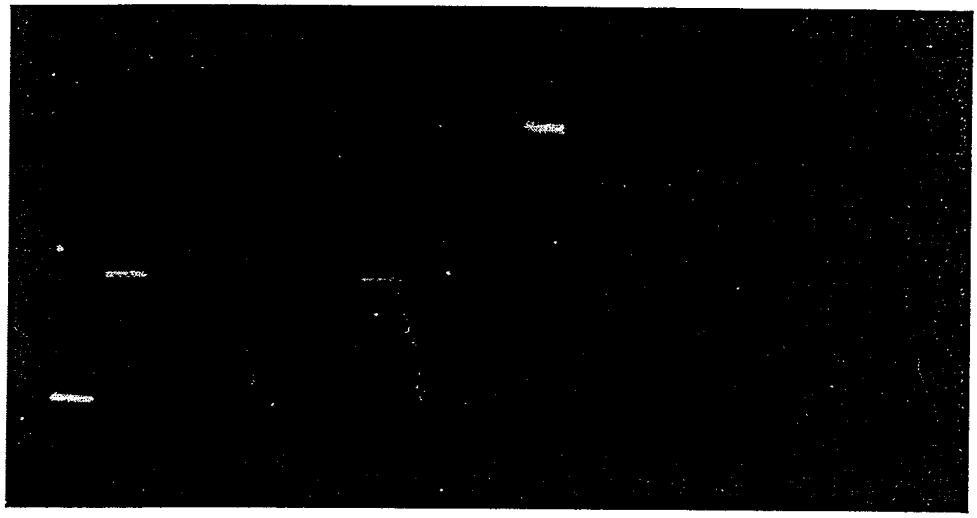
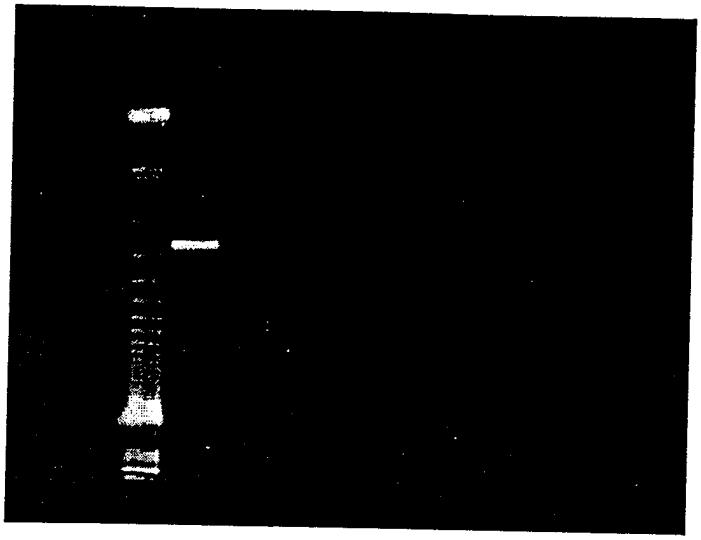


FIGURE 1



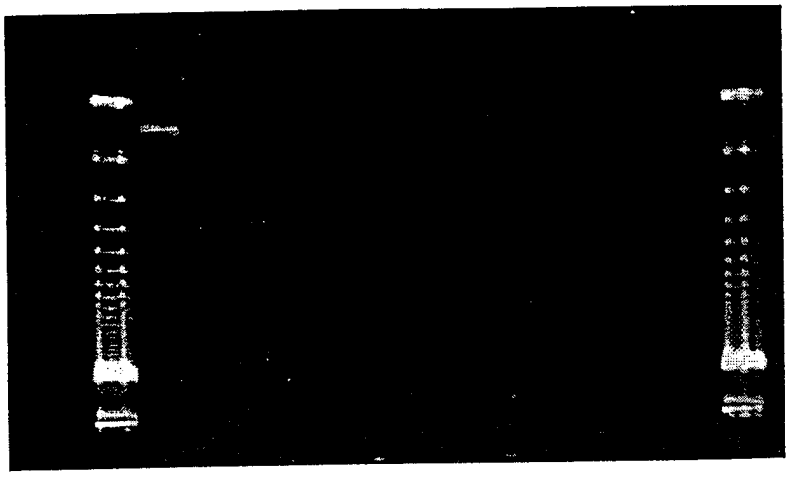
Lane			
	1	123 bp ladder	
	2	419F-1124R	*R. coprophilus*
	3	419F-1124R	*S. griseus*
	4	419F-1124R	*A. naeslundii*
	5	419F-1124R	*G. bronchialis*
	6	419F-1124R	*C. xerosis*
	7	419F-1124R	*N. brasiliensis*
	8	419F-1124R	*R. equi*
	9	419F-1124R	No DNA
	10	419F-568R	*R. coprophilus*
	11	419F-568R	*S. griseus*
	12	419F-568R	*A. naeslundii*
	13	419F-568R	*G. bronchialis*
	14	419F-568R	*C. xerosis*
	15	419F-568R	*N. brasiliensis*
	16	419F-568R	*R. equi*
	17	419F-568R	No DNA

FIGURE 2



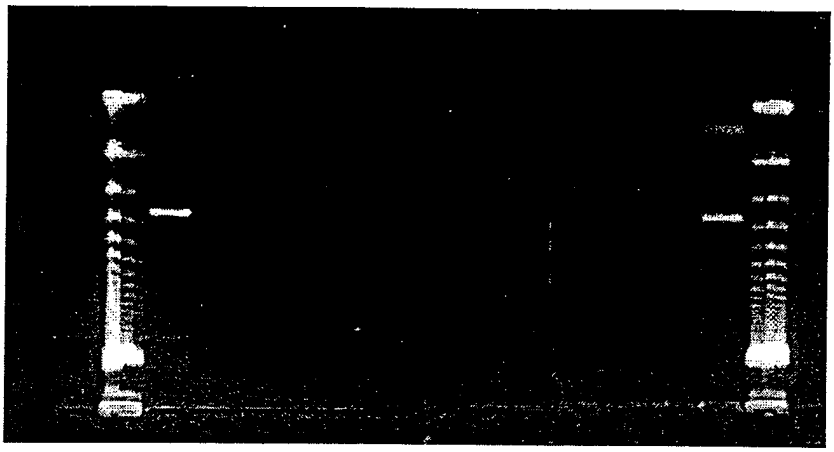
Lane			
	1	123 bp ladder	
	2	143F-568R	*R. coprophilus*
	3	143F-568R	*S. griseus*
	4	143F-568R	*A. naeslundii*
	5	143F-568R	*G. bronchialis*
	6	143F-568R	*C. xerosis*
	7	143F-568R	*N. brasiliensis*
	8	143F-568R	*R. equi*
	9	143F-568R	No DNA

FIGURE 3



Lane		
	1	123 bp ladder
	2	*R.coprophilus*
	3	*S. aureus*
	4	*S. epidermidis*
	5	*B. cereus*
	6	*B. subtilis*
	7	*E. faecalis*
	8	*A. hydrophila*
	9	*P. aeruginosa*
	10	*M. morganii*
	11	*E. coli*
	12	*E. aerogenes*
	13	no DNA
	14	123 bp ladder

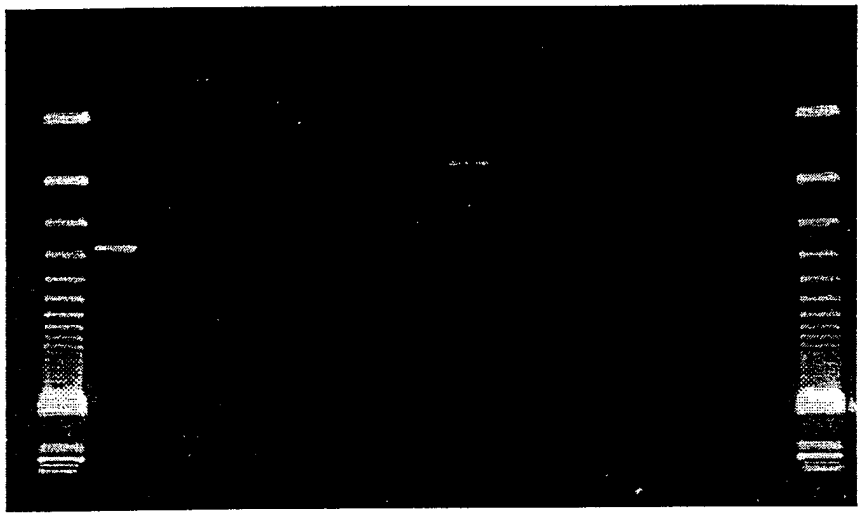
FIGURE 4



Lane 1& 15 123 bp ladder

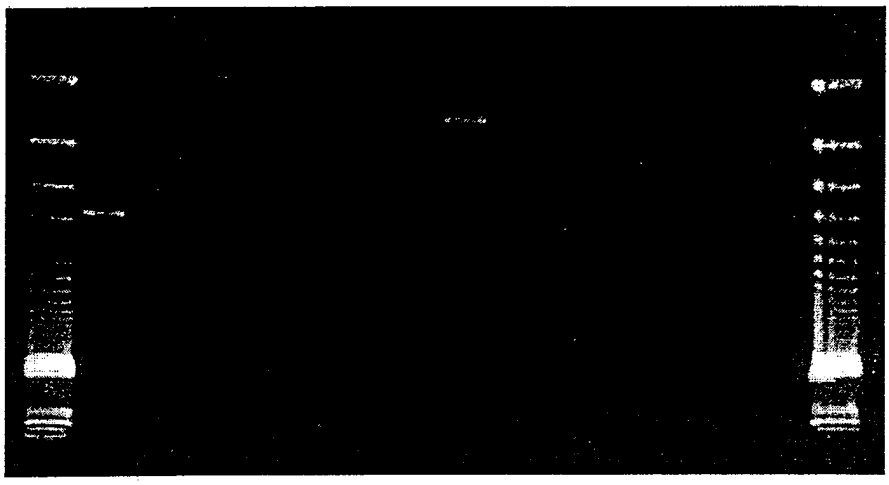
2	*R.coprophilus*
3	*S. aureus*
4	*S. epidermidis*
5	*B. cereus*
6	*B. subtilis*
7	*E. faecalis*
8	*A. hydrophila*
9	*P. aeruginosa*
10	*M. morganii*
11	*E. coli*
12	*E. aerogenes*
13	no DNA
14	*R. coprophilus* with 143F, 419R & 568R

FIGURE 5



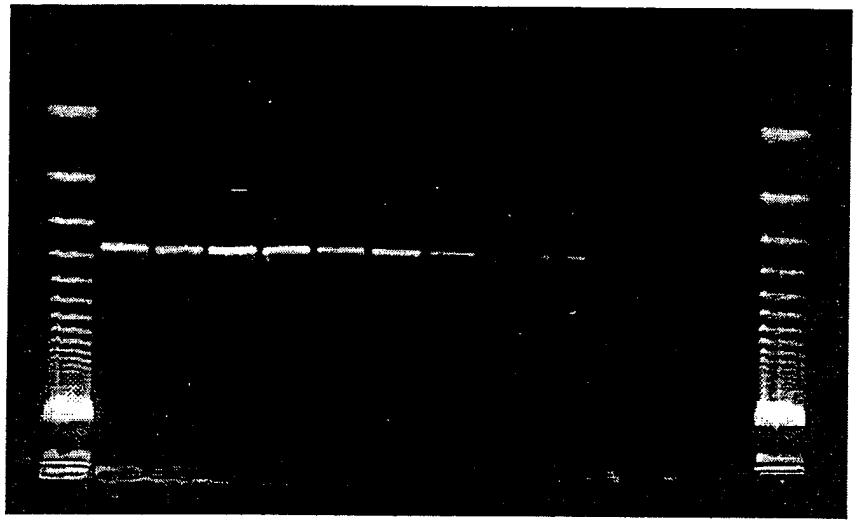
Lane 1 & 16 123 bp ladder
2 143F-568R *R. coprophilus*
3 143F-568R *R. rhodochrous* DSM43241
4 143F-568R *R. rhodochrous* DSM43274
5 143F-568R *R. ruber*
6 143F-568R *R. rhodnii*
7 143F-568R *R. zopfii*
8 Blank
9 419F-568R *R. coprophilus*
10 419F-568R *R. rhodochrous* DSM43241
12 419F-568R *R. rhodochrous* DSM43274
13 419F-568R *R. ruber*
14 419F-568R *R. rhodnii*
15 419F-568R *R. zopfii*

FIGURE 6



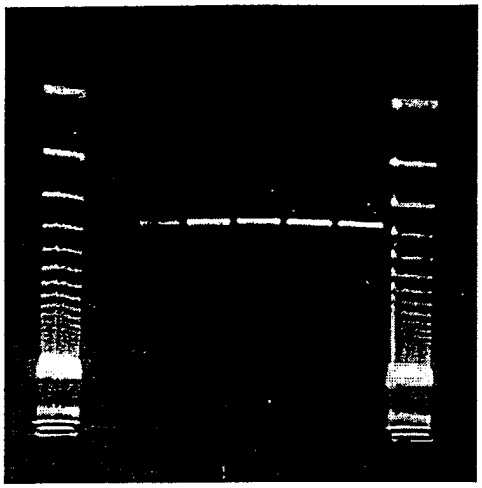
Lane 1 & 16 123 bp ladder
2 143F-568R *R. coprophilus*
3 143F-568R *R. rhodochrous* DSM43241
4 143F-568R *R. rhodochrous* DSM43274
5 143F-568R *R. ruber*
6 143F-568R *R. rhodnii*
7 143F-568R *R. zopfii*
8 Blank
9 419F-568R *R. coprophilus*
10 419F-568R *R. rhodochrous* DSM43241
11 419F-568R *R. rhodochrous* DSM43274
12 419F-568R *R. ruber*
13 419F-568R *R. rhodnii*
14 419F-568R *R. zopfii*
15 Blank

FIGURE 7



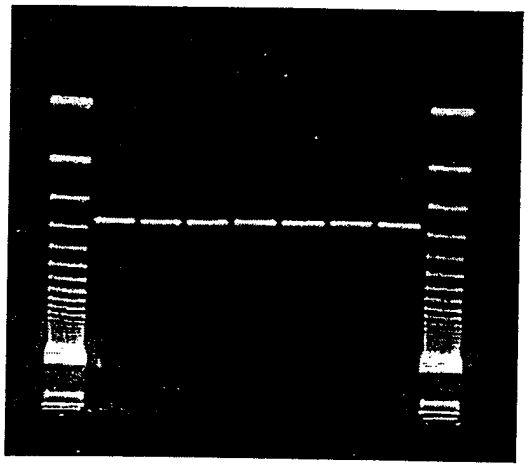
Lane				
	1 & 14	123 bp ladder		
	2	*R. coprophilus* 60°C	8	*R. zopfii* 60°C
	3	*R. coprophilus* 61°C	9	*R. zopfii* 61°C
	4	*R. coprophilus* 62°C	10	*R. zopfii* 62°C
	5	*R. coprophilus* 63°C	11	*R. zopfii* 63°C
	6	*R. coprophilus* 64°C	12	*R. zopfii* 64°C
	7	*R. coprophilus* 65°C	13	*R. zopfii* 65°C

FIGURE 8



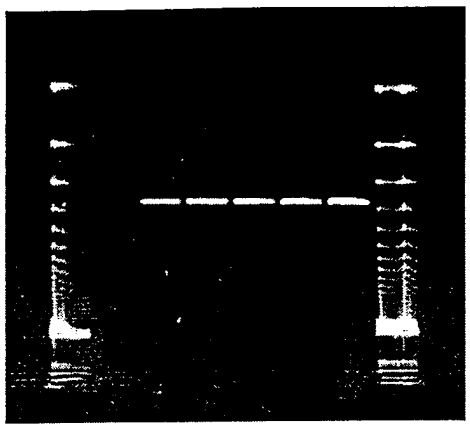
Lane	1	123 bp ladder	5	2.5 mM
	2.	1.0 mM	6	3.0 mM
	3	1.5 mM	7	3.5 mM
	4	2.0 mM	8	123 bp ladder

FIGURE 9



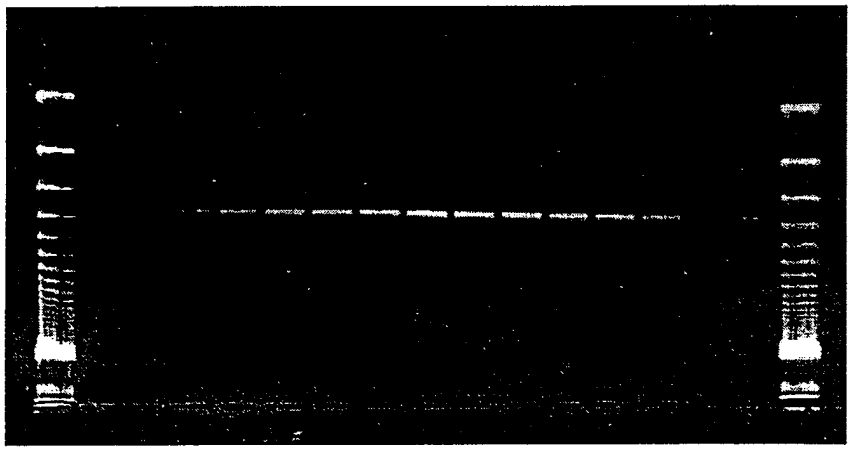
Lane	1 & 9	123 bp ladder	5	2.5 mM
	2.	2.0 mM	6	2.6 mM
	3	2.2 mM	7	2.8 mM
	4	2.4 mM	8	3.0 mM

FIGURE 10



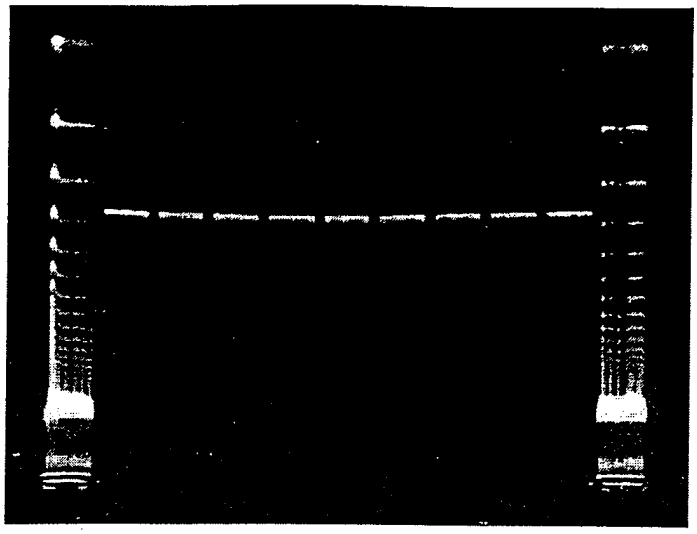
Lane	1	123 bp ladder	5	6.0 pmoles
	2	2.0 pmoles each primer	6	8.0 pmoles
	3	4.0 pmoles	7	10.0 pmoles
	4	5.0 pmoles	8	123 bp ladder

FIGURE 11



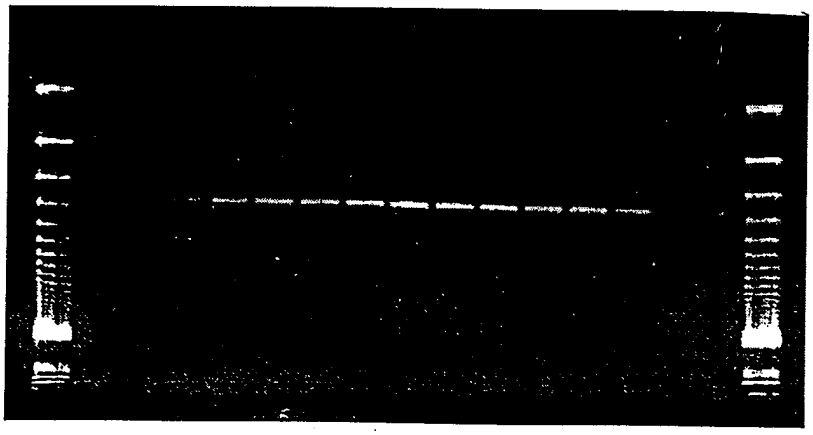
Lane	1	123 bp ladder	5	3.5 pmoles
	2	2.0 pmoles each primer	6	4.0 pmoles
	3	2.5 pmoles	7	4.5 pmoles
	4	3.0 pmoles	8	5.0 pmoles

FIGURE 12



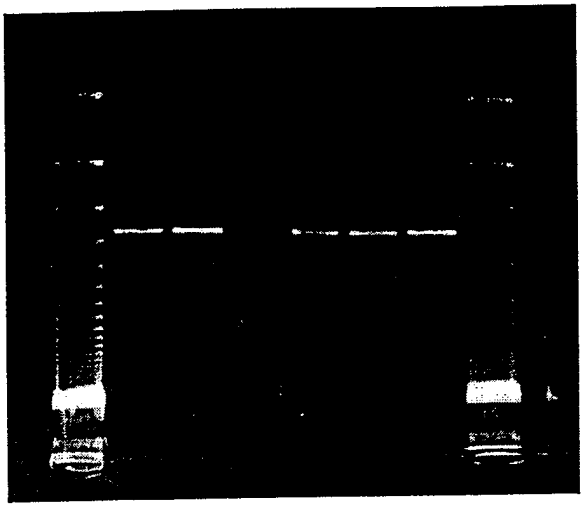
Lane	1 & 11	123 bp ladder	6	200 ng / 100µl
	2	400 ng / 100µl	7	150 ng / 100µl
	3	350 ng / 100µl	8	100 ng / 100µl
	4	300 ng / 100µl	9	50 ng / 100µl
	5	250 ng / 100µl	10	20 ng / 100µl

FIGURE 13



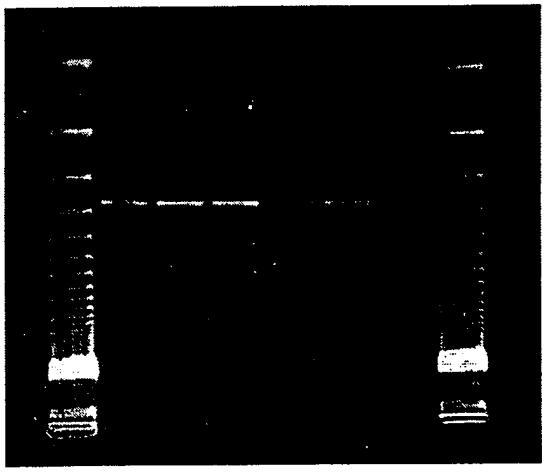
Lane	9	200 ng / 100µl	14	1 ng / 100µl
	10	20 ng / 100µl	15	0.5 ng / 100µl
	11	10 ng / 100µl	16	0.2 ng / 100µl
	12	5 ng / 100µl	17	123 bp ladder
	13	2 ng / 100µl		

FIGURE 14



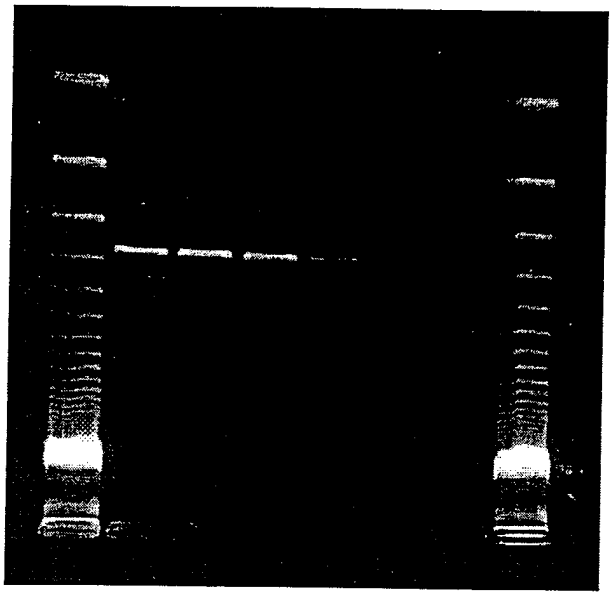
Lane	1	50 µM	4	200 µM
	2	100 µM	5	250 µM
	3	150 µM	6	300 µM

FIGURE 15



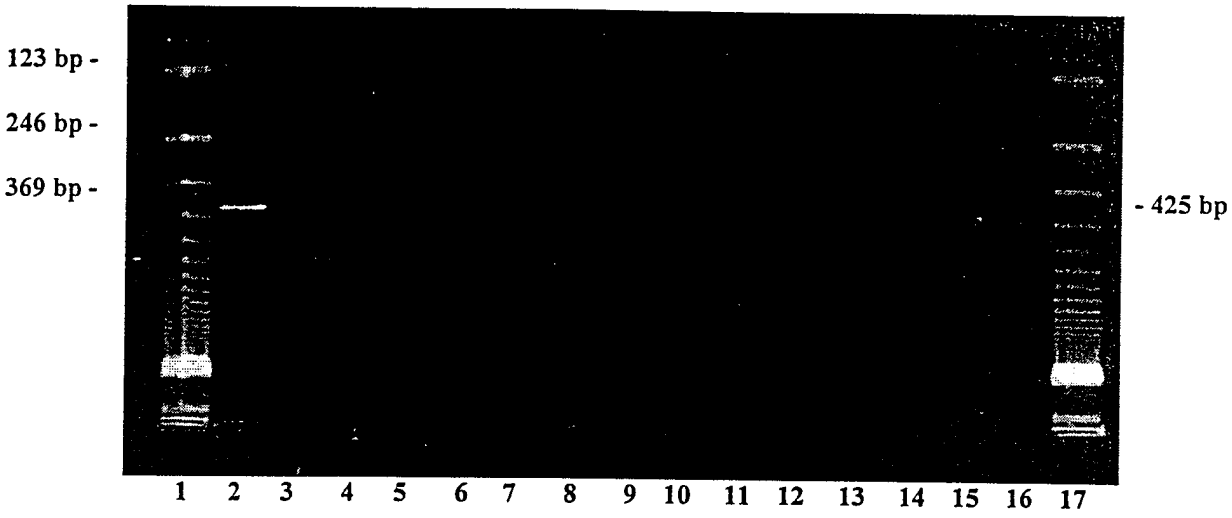
Lane	1	100 µM	4	175 µM
	2	125 µM	5	200 µM
	3	150 µM	6	150 µM (from previous experiment)

FIGURE 16



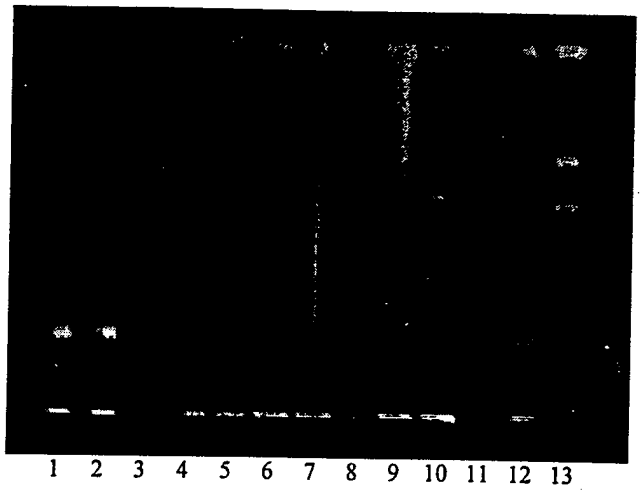
Lane 1 & 8 123 bp ladder
2 3.0 Units
3 2.5 Units
4 2.0 Units
5 1.5 Units
6 1.0 Units
7 0.5 Units

FIGURE 17



Lane 1 & 17 123 bp ladder
2 *R. coprophilus*
3 *R. zopfii*
4 *R. fascians*
5 *R. marinonascens*
6 *R. rhodococcus* DSM 43241
7 *R. rhodococcus* DSM 43274
8 *R. ruber*
9 *R. rhodnii*
10 *R. equi*
11 *A. naeslundii*
12 *G. bronchialis*
13 *C. xerosis*
14 *N. brasiliensis*
15 *S. griseus*
16 No DNA

FIGURE 18



Lane 1 Calf Thymus 1µl + 1µl water
2.Calf Thymus 1µl
3. *S. epidermidis* 4 µl
4. *E. faecalis* 4 µl
5. *A. hydrophila* 4 µl
6. *E. aerogenes* 2 µl
7. *E coli* 2 µl
8. *B. subtilis* 4 µl
9. *M. morganii* 2 µl
10 *P. aeruginosa* 4 µl
11. *B. cereus* 4 µl
12. *S. aureus* 4 µl
13. *R. coprophilus* 2 µl

FIGURE 19

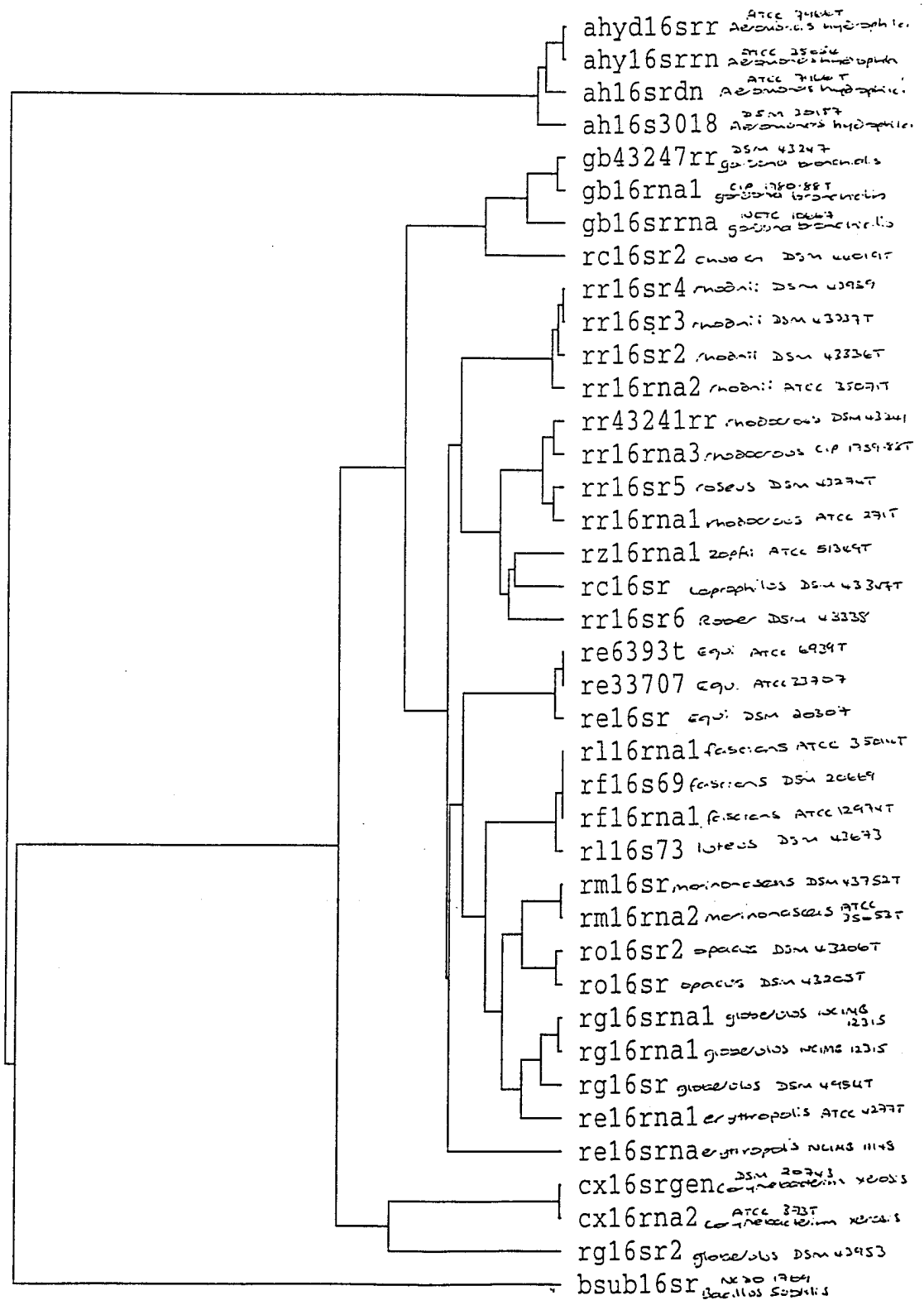
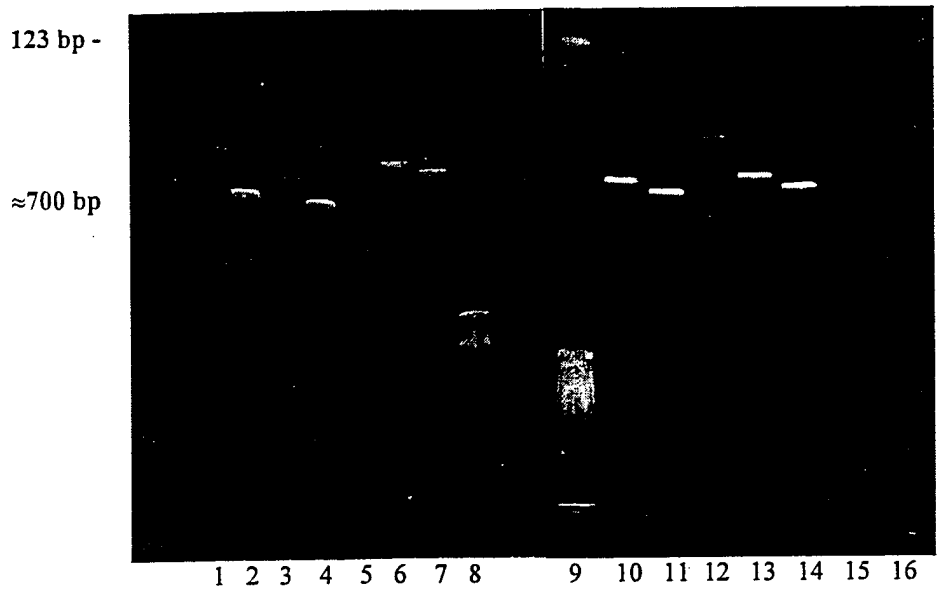
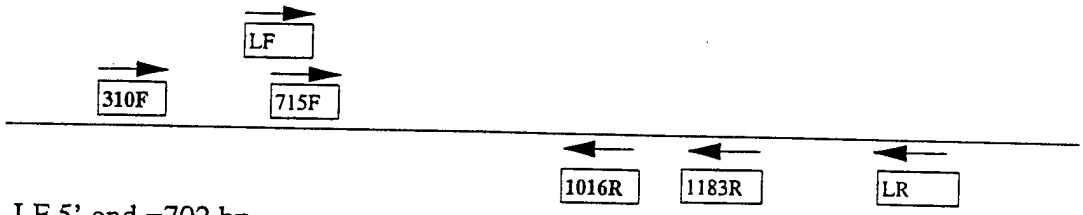

ahyd16srr
ahy16srrn
ah16srdn
ah16s3018
gb43247rr
gb16rna1
gb16srrna
rc16sr2
rr16sr4
rr16sr3
rr16sr2
rr16rna2
rr43241rr
rr16rna3
rr16sr5
rr16rna1
rz16rna1
rc16sr
rr16sr6
re6393t
re33707
re16sr
rl16rna1
rf16s69
rf16rna1
rl16s73
rm16sr
rm16rna2
ro16sr2
ro16sr
rg16srna1
rg16rna1
rg16sr
re16rna1
re16srna
cx16srgen
cx16rna2
rg16sr2
bsub16sr

FIGURE 20



Lane	1.	143F - 467R	9.	123 bp ladder
	2.	143F - 1124R	10.	443F - 1124R
	3.	143F - 926R	11.	443F - 1220R
	4.	143F - 1120R	12.	530F - 926R
	5.	443F - 926R	13.	530F - 1124R
	6.	443F - 1124R	14.	530F - 1220R
	7.	443F - 1220R	15	942F - 1124R
	8.	123 bp ladder	16.	942F - 1220R

FIGURE 21



LF 5' end =702 bp
LR 5' end = 1450 bp

FIGURE 22

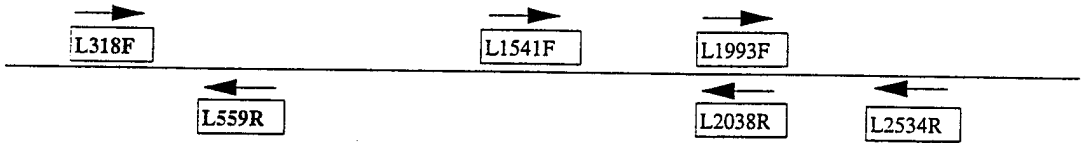
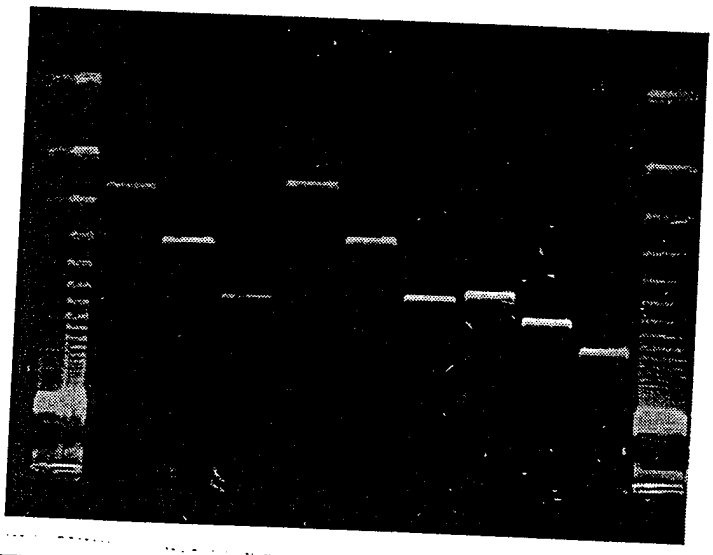


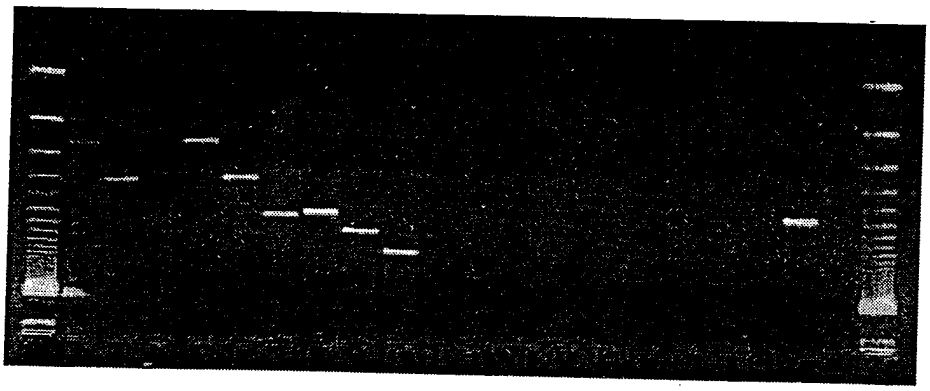
FIGURE 23



Lane	Primer 1	Primer 2
1	123 bp	ladder
2	LF	1016R
3	LF	1183R
4	LF	LR
5	715F	1016R
6	715F	1183R

Lane	Primer 1	Primer 2
7	715F	LR
8	310F	1016R
9	310F	1183R
10	310F	LR
11	123 bp	ladder

FIGURE 24



Lane	Sample	Primer1	Primer2
1	ladder	123 bp	
2	382	LF	1016R
3	382	LF	1183R
4	382	LF	LR
5	382	715F	1016R
6	382	715F	1183R
7	382	715F	LR
8	382	310F	1016R
9	382	310F	1183R
10	382	310F	LR

Lane	Sample	Primer1	Primer 2
11	385	LF	1016R
12	385	LF	1183R
13	385	LF	LR
14	385	715F	1016R
15	385	715F	1183R
16	385	715F	LR
17	385	310F	1016R
18	385	310F	1183R
19	385	310F	LR
20	L. mono	310F	1016R
21	No DNA	310F	1016R
22	ladder	123 bp	

FIGURE 25

Lane	Sample	Primer1	Primer2
1	ladder	123 bp	
2	300	LF	1016R
3	300	LF	1183R
4	300	LF	LR
5	300	715F	1016R
6	300	715F	1183R
7	300	715F	LR
8	300	310F	1016R
9	300	310F	1183R
10	300	310F	LR

Lane	Sample	Primer1	Primer 2
11	307	LF	1016R
12	307	LF	1183R
13	307	LF	LR
14	307	715F	1016R
15	307	715F	1183R
16	307	715F	LR
17	307	310F	1016R
18	307	310F	1183R
19	307	310F	LR
20	L. mono	310F	1016R
21	No DNA	310F	1016R
22	ladder	123 bp	

FIGURE 26

Lane	Sample	Primer1	Primer2
1	ladder	123 bp	
2	*L. ivanovi*	715F	1016R
3	*L. ivanovi*	715F	1183R
4	*L. ivanovi*	310F	1016R
5	*L. ivanovi*	310F	1183R
6	*L. innocua*	715F	1016R
7	*L. innocua*	715F	1183R
8	*L. innocua*	310F	1016R

Lane	Sample	Primer1	Primer2
9	*L. innocua*	310F	1183R
10	*L. seeligeri*	715F	1016R
11	*L. seeligeri*	715F	1183R
12	*L. seeligeri*	310F	1016R
13	*L. seeligeri*	310F	1183R
14	No DNA	310F	1016R
15	*L.mono.*	310F	1016R
16	ladder	123 bp	

FIGURE 27

Lane	Sample	Primer1	Primer2
1	ladder	123 bp	
2	*S. flexneri*	715F	1016R
3	*S. flexneri*	715F	1183R
4	*S. flexneri*	310F	1016R
5	*S. flexneri*	310F	1183R
6	*S. sonnei*	715F	1016R
7	*S. sonnei*	715F	1183R
8	*S. sonnei*	310F	1016R

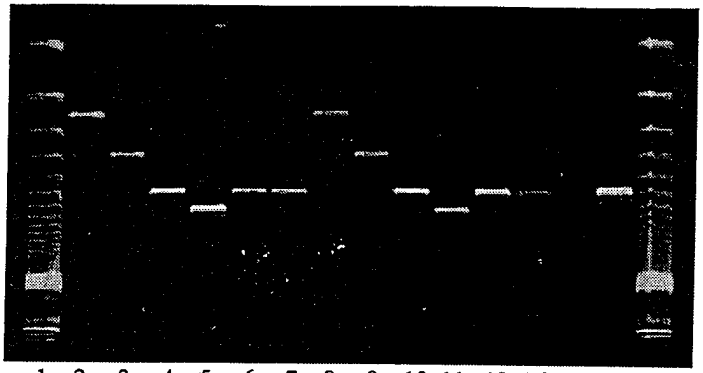
Lane	Sample	Primer1	Primer2
9	*S. sonnei*	310F	1183R
10	*S. menston*	715F	1016R
11	*S. menston*	715F	1183R
12	*S. menston*	310F	1016R
13	*S. menston*	310F	1183R
14	No DNA	310F	1016R
15	*L.mono.*	310F	1016R
16	ladder	123 bp	

FIGURE 28

Lane	Sample	Primer1	Primer2
1	ladder	123 bp	
2	298	715F	1016R
3	298	715F	1183R
4	298	310F	1016R
5	298	310F	1183R
6	297	715F	1016R
7	297	715F	1183R
8	297	310F	1016R
9	297	310F	1183R
10	*Y. enterolitica*	715F	1016R
11	*Y. enterolitica*	715F	1183R

Lane	Sample	Primer1	Primer2
12	*Y. enterolitica*	310F	1016R
13	*Y. enterolitica*	310F	1183R
14	*C. jejuni*	715F	1016R
15	*C. jejuni*	715F	1183R
16	Blank		
17	Blank		
18	*C. jejuni*	310F	1016R
19	*C. jejuni*	310F	1183R
20	*L.mono.*	310F	1016R
21	No DNA	310F	1016R
22	ladder	123 bp	

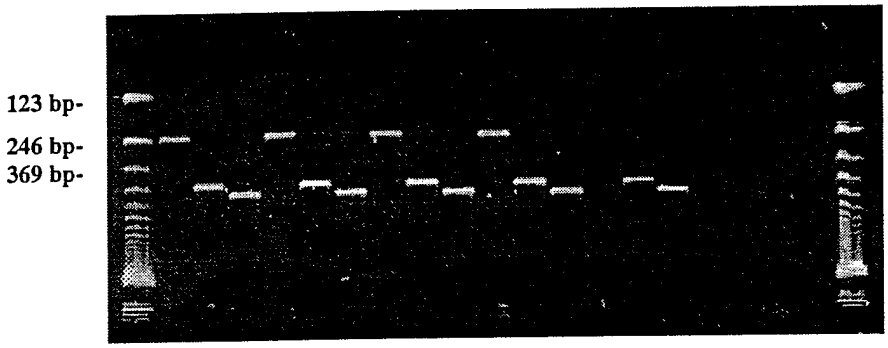
FIGURE 29



Lane	Sample	Primer1	Primer2
1	ladder	123 bp	
2	298+BSA	715F	1016R
3	298+BSA	715F	1183R
4	298+BSA	310F	1016R
5	298+BSA	310F	1183R
6	298x2DNA	310F	1016R
7	298x1 DNA	310F	1016R
8	297+BSA	715F	1016R

Lane	Sample	Primer1	Primer2
9	297+BSA	715F	1183R
10	297+BSA	310F	1016R
11	297+BSA	310F	1183R
12	297x2 DNA	310F	1016R
13	297x1 DNA	310F	1016R
14	No DNA	310F	1016R
15	*L.mono.*	310F	1016R
16	ladder	123 bp	

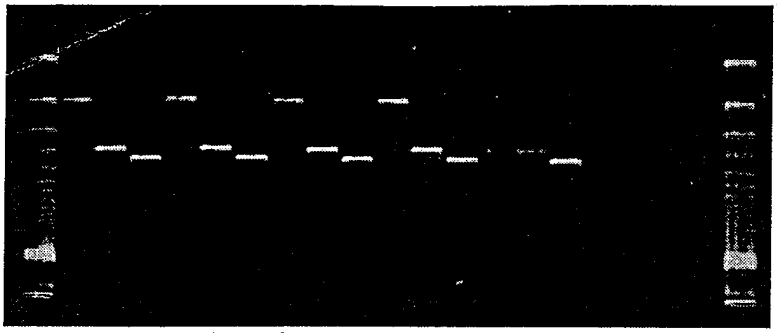
FIGURE 30



Lane	Sample	Primer1	Primer2
1	ladder	123 bp	
2	L. monocytogenes	L318F	L559R
3	L. monocytogenes	L1541F	L2038R
4	L. monocytogenes	L1993F	L2534R
5	*L. ivanovii*	L318F	L559R
6	*L. ivanovii*	L1541F	L2038R
7	*L. ivanovii*	L1993F	L2534R
8	*L. innocua*	L318F	L559R
9	*L. innocua*	L1541F	L2038R
10	*L. innocua*	L1993F	L2534R
11	*L.seeligeri*	L318F	L559R

Lane	Sample	Primer1	Primer2
12	*L.seeligeri*	L1541F	L2038R
13	*L.seeligeri*	L1993F	L2534R
14	*B. subtilis*	L318F	L559R
15	*B. subtilis*	L1541F	L2038R
16	*B. subtilis*	L1993F	L2534R
17	*S. aureus*	L318F	L559R
18	*S. aureus*	L1541F	L2038R
19	*S. aureus*	L1993F	L2534R
20	No DNA	L1541F	L2534R
21	ladder	123 bp	
22			

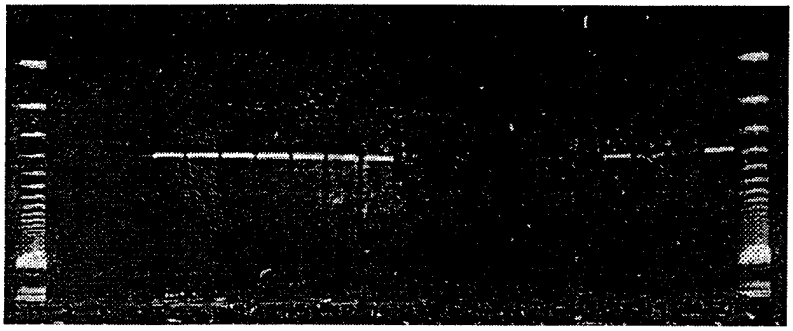
FIGURE 31



Lane	Sample	Primer1	Primer2
1	ladder	123 bp	
2	*L. monocytogenes*	L318F	L559R
3	*L. monocytogenes*	L1541F	L2038R
4	*L. monocytogenes*	L1993F	L2534R
5	*L. ivanovii*	L318F	L559R
6	*L. ivanovii*	L1541F	L2038R
7	*L. ivanovii*	L1993F	L2534R
8	*L. innocua*	L318F	L559R
9	*L. innocua*	L1541F	L2038R
10	*L. innocua*	L1993F	L2534R
11	*L.seeligeri*	L318F	L559R

Lane	Sample	Primer1	Primer2
12	*L.seeligeri*	L1541F	L2038R
13	*L.seeligeri*	L1993F	L2534R
14	*B. subtilis*	L318F	L559R
15	*B. subtilis*	L1541F	L2038R
16	*B. subtilis*	L1993F	L2534R
17	*S. aureus*	L318F	L559R
18	*S. aureus*	L1541F	L2038R
19	*S. aureus*	L1993F	L2534R
20	No DNA	L1541F	L2534R
21	ladder	123 bp	
22			

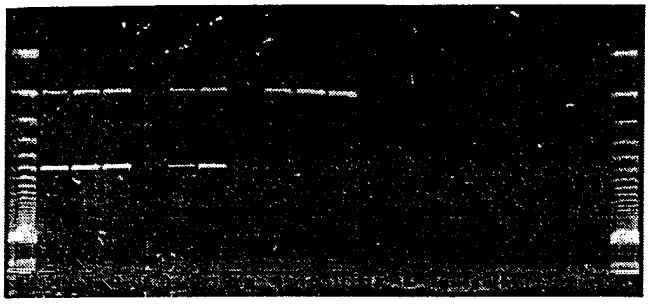
FIGURE 32



Lane	Sample	[$MgCl_2$]
1	ladder 123 bp	
2	*L. monocytogenes*	0.5 mM
3	*L. monocytogenes*	1.0 mM
4	*L. monocytogenes*	1.5 mM
5	*L. monocytogenes*	2.0 mM
6	*L. monocytogenes*	2.5 mM
7	*L. monocytogenes*	3.0 mM
8	*L. monocytogenes*	3.5 mM
9	*L. monocytogenes*	4.0 mM
10	*L. monocytogenes*	4.5 mM
11	*L. monocytogenes*	5.0 mM

Lane	Sample	[$MgCl_2$]
12	*B. subtilis*	0.5 mM
13	*B. subtilis*	1.0 mM
14	*B. subtilis*	1.5 mM
15	*B. subtilis*	2.0 mM
16	*B. subtilis*	2.5 mM
17	*B. subtilis*	3.0 mM
18	*B. subtilis*	3.5 mM
19	*B. subtilis*	4.0 mM
20	*B. subtilis*	4.5 mM
21	*B. subtilis*	5.0 mM
22	ladder 123bp	

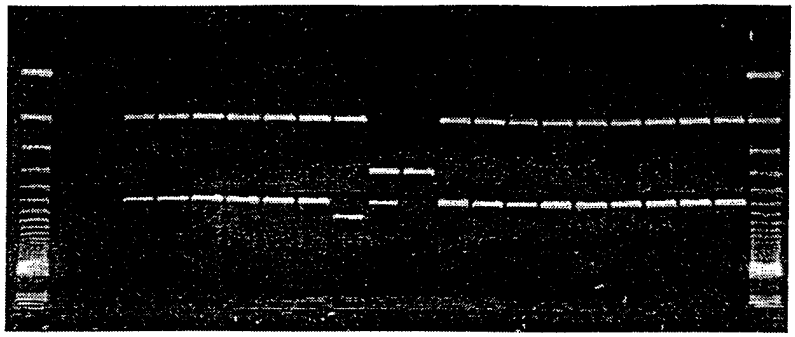
FIGURE 33



Lane	Sample
1	ladder 123 bp
2	*L. monocytogenes*
3	300 Sliced Ham
4	307 Sliced Ham
5	298 Sliced Ham
6	297 Sliced Ham
7	382
8	385
9	*L. ivanovii*
10	*L. innocua*

Lane	Sample
11	*L. seeligeri*
12	*S. flexneri*
13	*S. menston*
14	*Y. enterolitica*
15	*C. jejuni*
16	*B. cereus*
17	*B. subtilis*
18	*S. aureus*
19	Blank

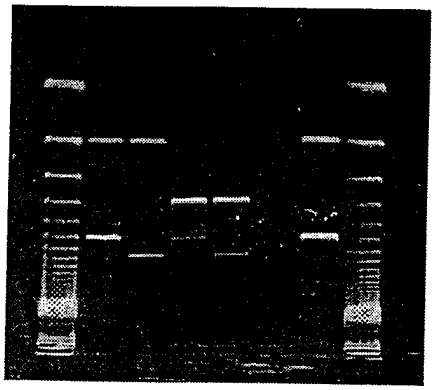
FIGURE 34



Lane	[MgCl]
1	123 bp ladder
2	0.5 mM
3	1.0 mM
4	1.5 mM
5	2.0 mM
6	2.5 mM
7	3.0 mM
8	3.5 mM
9	4.0 mM

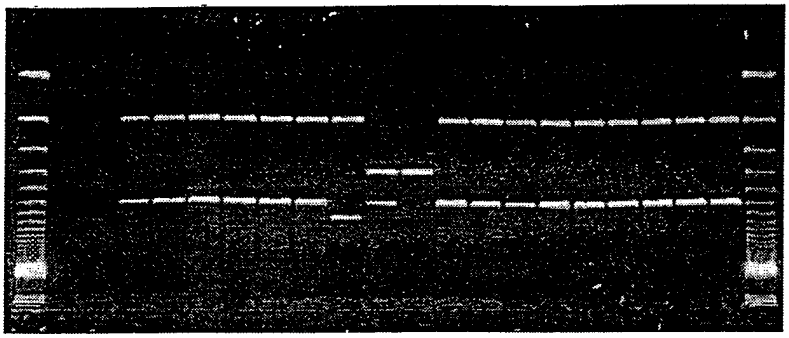
Lane	Primers
10	310F, 1183R, L318F and L559R
11	310F, 1016R, L1541F and L2038R
12	310F, 1183R, , L1541F and L2038R
22	123 bp ladder

FIGURE 35



Lane	Sample	Extraction procedure	*L. mono* Primer 1	*L. mono* Primer 2	*Listeria* Primer 1	*Listeria* Primer2	$[MgCl_2]$
1 & 8	ladder						
2	*L. mono*	Current	310F	1016R	L318F	L559R	2.5 mM
3	*L. mono*	Current	310F	1183R	L318F	L559R	2.5 mM
4	*L. mono*	Current	310F	1016R	L1541F	L2038R	2.0 mM
5	*L. mono*	Current	310F	1183R	L1541F	L2038R	2.0 mM
6	No DNA		310F	1016R	L318F	L559R	2.5 mM
7	*L. mono*	New	310F	1016R	L318F	L559R	2.5 mM

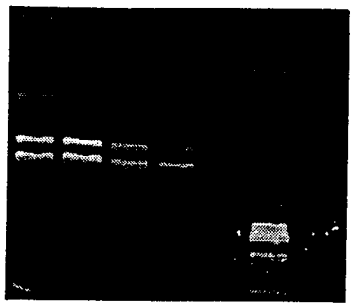
FIGURE 36



Sample No.	Lane	Sample
1	13	L.monocytogenes (A)
2	14	L.monocytogenes (B)
3	15	L.monocytogenes (C)
4	16	Ham 10^{-6} (A)
5	17	Ham 10^{-6}(B)

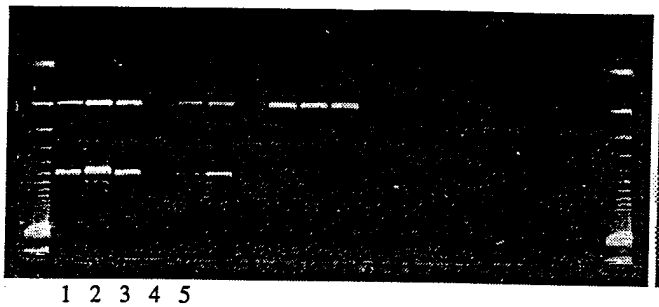
Sample No.	Lane	Sample
6	18	Ham 10^{-6}(C)
7	19	Ham 10^{-9}(A)
8	20	Ham 10^{-9}(B)
9	21	Ham 10^{-9}(C)

FIGURE 37



Sample No.	Lane	Sample
1, 2, 3	1	*L. monocytogenes*
	2	10^{-2}
4, 5, 6	3	10^{-6}
	4	10^{-8}
7, 8, 9	5	10^{-9}
	6	123 bp ladder
	7	Negative Ham sample

FIGURE 38



Sample No.	Sample.
1	*L. monocytogenes*
2	300 Sliced Ham
3	307 Sliced Ham
4	298 Sliced Ham
5	297 Sliced Ham

FIGURE 39

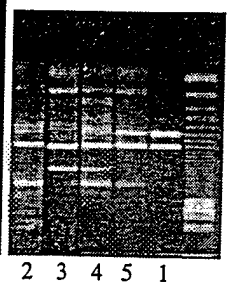


FIGURE 40

Figure 21. Food samples using the new PCR procedure

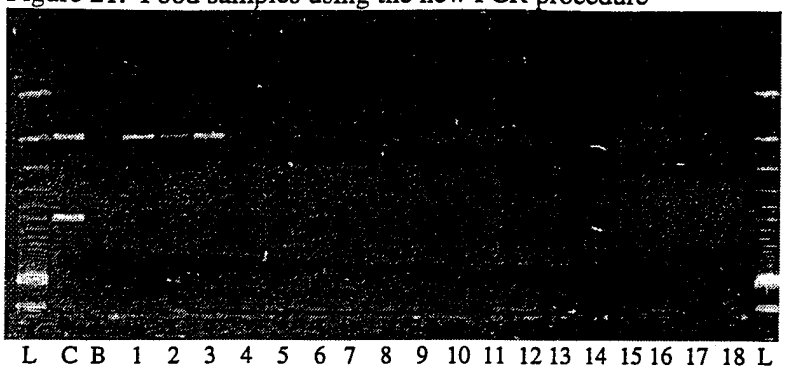
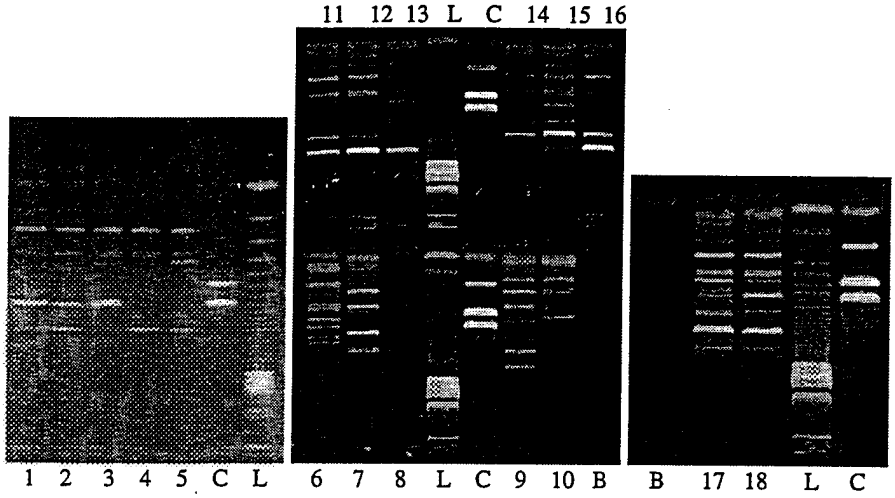


FIGURE 41



Sample No.	Sample
1	436 sandwich ham
2	437 sandwich ham
3	438 sandwich ham
4	439 sandwich ham
5	440 sandwich ham
6	011 ice cream
7	012 ice cream
8	013 ice cream
9	015 luncheon
L	123 bp ladder
B	Blank

Sample No.	Sample
10	016 luncheon
11	017 luncheon
12	018 luncheon
13	019 luncheon
14	020 luncheon
15	021 sandwich ham
16	022 sandwich ham
17	023 sandwich ham
18	024 sandwich ham
C	*L. monocytogenes* control

FIGURE 42

Figure 23. Food samples using the new PCR procedure

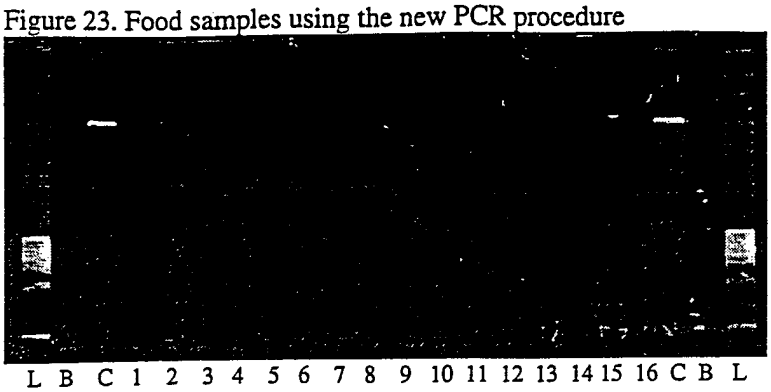
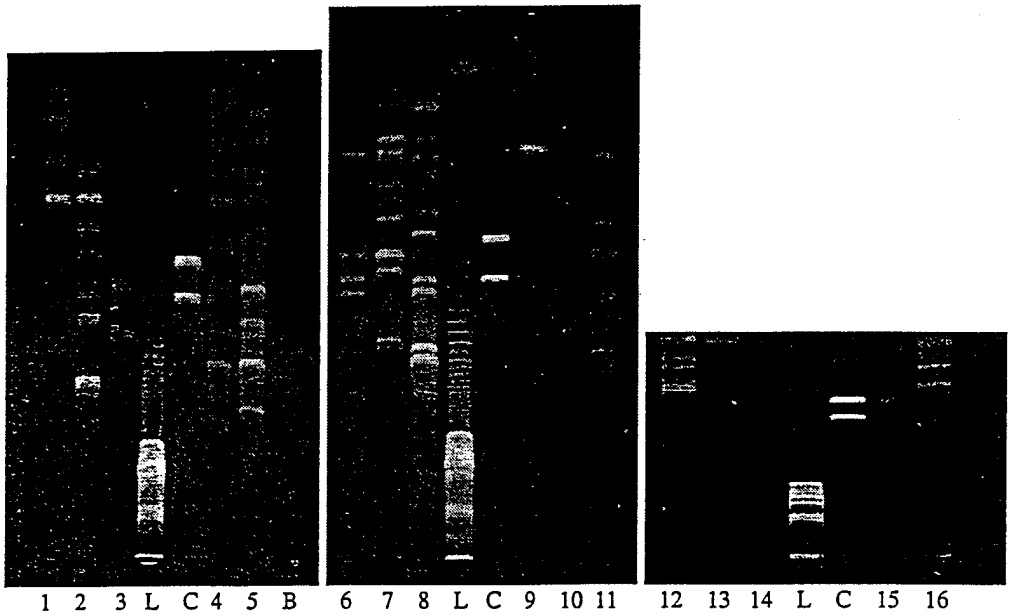


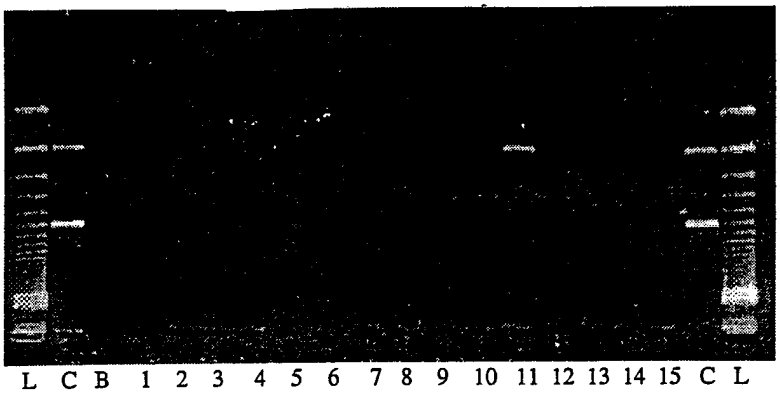
FIGURE 43



Sample No.	Sample
1	055 salami & salad sandwich
2	056 ham sandwich
3	057 ham & pickle sandwich
4	058 ham & egg sandwich
5	059 ham & egg sandwich
6	060 ham & salad sandwich
7	061 ham, salad, egg filled roll
8	062 ham sandwich
9	063 ham sandwich
10	064 ham sandwich

Sample No.	Sample
11	065 ham & egg sandwich
12	066 ham sandwich
13	067 ham,salad,cheese filled roll
14	068 ham, pickle,salad sandwich
15	069 ham,egg,tomato sandwich
16	070 ham & egg sandwich
L	123 bp ladder
C	*L. monocytogenes* control
B	Blank

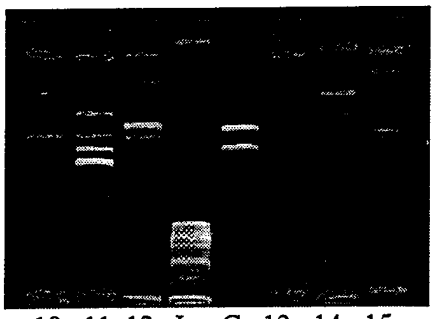
FIGURE 44



-Listeria

-L monocytogenes

FIGURE 45



- L.monocytogenes
- Listeria

Sample No.	Sample
1	*E. coli*
2	*E. faecalis*
3	*A. hydrophila*
4	*S. sonnei*
5	*S. epidermidis*
6	*P. aeruginosa*
7	*M. morganii*
8	*E. aeruginosa*
9	*R. coprophilus*

Sample No.	Sample
10	071 corned beef & lettuce sandwich
11	072 ham & mustard sandwich
12	073 ham sandwich
13	074 corned beef & pickle sandwich
14	075 ham sandwich
15	076 ham sandwich
L	123 bp ladder
B	Blank
C	*L.monocytogenes* control

FIGURE 46

Name:	l123srna2	Lactococcus lactis DSM 20481
Name:	l123srna1	Lactococcus lactis DSM 20069
Name:	su23srrn	Streptococcus uberis ATCC 19436
Name:	s60799	Streptococcus uberis ATCC 19436
Name:	sp23srrn	Streptococcus parauberis NCDO 2020
Name:	s60368	Streptococcus parauberis NCDO 2020
Name:	ld23srrn	Lactobacillus delbrueckii
Name:	lm23rrna	L. murrayi CCUG 4984
Name:	lg23srrna	L. grayii ATCC 19120
Name:	lm23srrna	L. monocytogenes ATCC 15313
Name:	lm23srrn	L. monocytogenes ATCC 19115
Name:	li23srrna	L. innocua ATCC 33090
Name:	ls23srrna	L. seeligeri CCUG 15530
Name:	lw23srrna	L. welshimeri CIP 8149
Name:	li23rrna	L. ivanovii CIP 7842
Name:	lm23srdna	L. monocytogenes LM149
Name:	bc16s23sd	Bacillus cereus DSM 31
Name:	sa23srrn	Staphylococcus aureus ATCC 12600
Name:	s60371	Leuconostoc carnosum
Name:	lc23srrnx	Leuconostoc carnosum
Name:	s60370	Leuconostoc mesenteroides
Name:	s60375	Lactobacillus confusus
Name:	lc23srrn	Lactobacillus confusus NCDO 1586
Name:	s60373	Leuconostoc paramesenteroides
Name:	s60377	Leuconostoc oenos
Name:	parrn23s	Pseudomonas aeruginosa ATCC 10145
Name:	ah23srrn	Aeromonas hydrophila ATCC 7960T

FIGURE 47

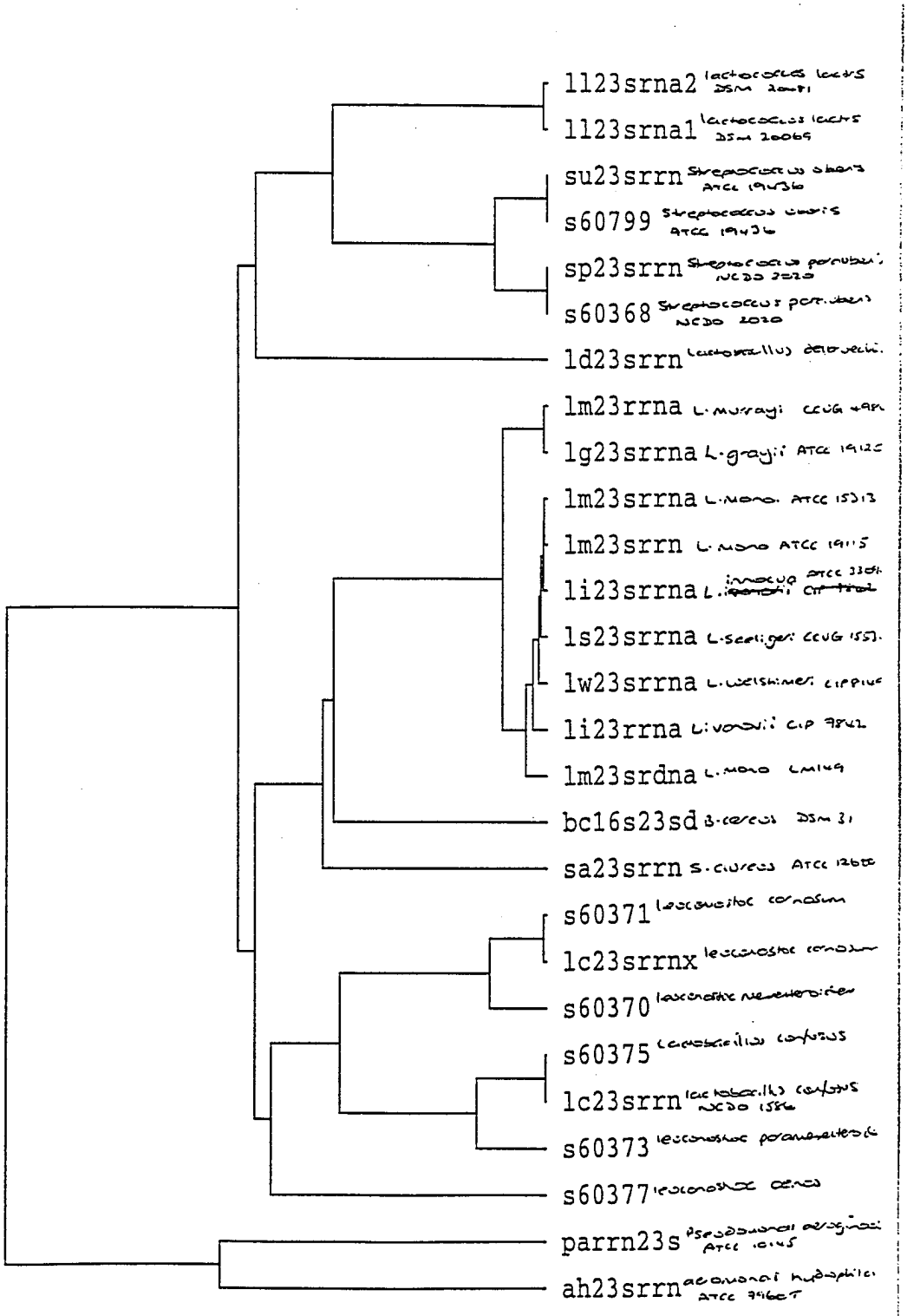
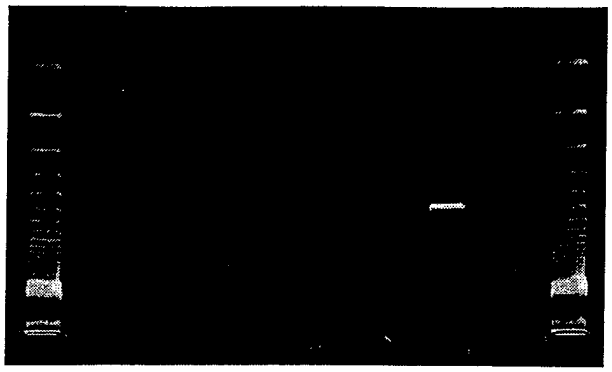

l123srna2
l123srna1
su23srrn
s60799
sp23srrn
s60368
ld23srrn
lm23rrna
lg23srrna
lm23srrna
lm23srrn
li23srrna
ls23srrna
lw23srrna
li23rrna
lm23srdna
bc16s23sd
sa23srrn
s60371
lc23srrnx
s60370
s60375
lc23srrn
s60373
s60377
parrn23s
ah23srrn

FIGURE 48

Lane	Sample	Primer1	Primer 2
1	123 bp	ladder	
2	*E. coli*	LF	1016R
3	*E. coli*	LF	1183R
4	*E. coli*	LF	LR
5	*E. coli*	715F	1016R
6	*E. coli*	715F	1183R
7	*E. coli*	715F	LR
8	*E. coli*	310F	1016R
9	*E. coli*	310F	1183R
10	*E. coli*	310F	LR
11	*B.subtilis*	LF	1016R

Lane	Sample	Primer1	Primer2
12	*B.subtilis*	LF	1183R
13	*B.subtilis*	LF	LR
14	*B.subtilis*	715F	1016R
15	*B.subtilis*	715F	1183R
16	*B.subtilis*	715F	LR
17	*B.subtilis*	310F	1016R
18	*B.subtilis*	310F	1183R
19	*B.subtilis*	310F	LR
20	*L.mono.*	310F	1016R
21	No DNA	310F	1016R

FIGURE 49



Lane	Sample	Primer1	Primer 2
1	123 bp	ladder	
2	*B.cereus*	LF	1016R
3	*B.cereus*	LF	1183R
4	*B.cereus*	LF	LR
5	*B.cereus*	715F	1016R
6	*B.cereus*	715F	1183R
7	*B.cereus*	715F	LR

Lane	Sample	Primer1	Primer2
8	*B.cereus*	310F	1016R
9	*B.cereus*	310F	1183R
10	*B.cereus*	310F	LR
11	*L.mono.*	310F	1016R
12	No DNA	310F	1016R
13	*B.subtilis*	310F	1016R
14	123 bp	ladder	

FIGURE 50

Lane	Sample	Primer1	Primer 2
1	123 bp	ladder	
2	*S.aureus*	LF	1016R
3	*S.aureus*	LF	1183R
4	*S.aureus*	LF	LR
5	*S.aureus*	715F	1016R
6	*S.aureus*	715F	1183R
7	*S.aureus*	715F	LR

Lane	Sample	Primer1	Primer2
8	*S.aureus*	310F	1016R
9	*S.aureus*	310F	1183R
10	*S.aureus*	310F	LR
11	*L.mono.*	310F	1016R
12	No DNA	310F	1016R
13	123 bp	ladder	

FIGURE 51

Lane	Sample	Primer1	Primer 2
1	123 bp	ladder	
2	*E. faecalis*	LF	1016R
3	*E. faecalis*	LF	1183R
4	*E. faecalis*	LF	LR
5	*E. faecalis*	715F	1016R
6	*E. faecalis*	715F	1183R
7	*E. faecalis*	715F	LR
8	*E. faecalis*	310F	1016R
9	*E. faecalis*	310F	1183R
10	*E. faecalis*	310F	LR
11	*A. hydrophila*	LF	1016R

Lane	Sample	Primer1	Primer2
12	*A. hydrophila*	LF	1183R
13	*A. hydrophila*	LF	LR
14	*A. hydrophila*	715F	1016R
15	*A. hydrophila*	715F	1183R
16	*A. hydrophila*	715F	LR
17	*A. hydrophila*	310F	1016R
18	*A. hydrophila*	310F	1183R
19	*A. hydrophila*	310F	LR
20	*L.mono.*	310F	1016R
21	No DNA	310F	1016R

FIGURE 52

FIGURE 53

Bacterial Strain	Source
Listeria Species	
Listeria monocytogenes Serotype 1a	NZRM 2591, ATCC 19111
Listeria monocytogenes Serotype 2	NZRM 2592, ATCC 19112, NCTC 5348
Listeria monocytogenes Serotype 3a	NZRM 2594, ATCC 19113, NCTC 5105
Listeria monocytogenes Serotype 4a	NZRM 2595, ATCC 19114, NCTC 5214
Listeria monocytogenes Serotype 4c	NZRM 2596, ATCC 19116
Listeria monocytogenes Serotype 4d	NZRM 2597, ATCC 19117
Listeria monocytogenes	NZRM 3312, ATCC 7646
Listeria monocytogenes	NZRM 3370, ATCC 49594
Listeria monocytogenes	NZRM 3384 Coleslaw, Halifax, Canada
Listeria monocytogenes	NZRM 3387 Cucumber, Halifax, Canada
Listeria monocytogenes	NZRM 3449 NZ Smoked mussels
Listeria monocytogenes	NZRM 3450 NZ Smoked mussels
Listeria grayii	NZRM 1088 T
Listeria innocua	NZRM 3024, ATCC 33090
Listeria ivanovii	NZRM 797
Listeria seeligeri	NZRM 3287, ATCC 35967
Listeria welshimeri	NZRM 3286, NCTC 11857 T
Listeria Related Species	NZRM 3583, ATCC 51268, NCTC 12142
Aerococcus urinae	NZRM 3204,ATCC 11563
Aerococcus viridans	NZRM 5 ATCC 10702, NCTC 8035,
Bacillus cereus	NZRM 143, ATCC 6051, NCTC 3610,
Bacillus subtilis	NZRM 2981
Bacillus thuringiensis subsp israelensis	NZRM 3320, ATCC 11509
Brochothrix thermosphacta	NZRM 3569, ATCC 43754
Brochothrix campestris	NZRM 3572, ATCC 35677, NCIMB 11952
Carnobacterium divergens	NZRM 3575, ATCC 49517
Carnobacterium gallinarum	NZRM 3576, ATCC 49516, DSM 4848,
Carnobacterium mobile	NZRM 12847
	NZRM 3571, ATCC 35586, NCIMB 2264
Carnobacterium piscicola	NZRM 3574, NCIMB 13013
Enterococcus colombae	NZRM 3570, ATCC 49903, NCIMB 13117
Enterococcus sulfureus	NZRM 917, ATCC 25923
Enterococcus aureus	NZRM 3573, ATCC 49515, NCIMB 13038
Vagococcus fluvialis	NZRM 3577, ATCC 51200, NCIMB 13133
Vagococcus salmonarium	

Bacterial Strain	Source
Other Bacteria	
Aeromonas hydrophila	NZRM 804, ATCC 7699, NCTC 8049
Campylobacter jejuni	NZRM 2397, NCTC 11351, ATCC 33560
Enterobacter aerogenes	NZRM 798, ATCC 13048, NCTC 10006
Enterococcus faecalis	NZRM 1106, ATCC 19433, NCTC 775
Escherichia coli	NZRM 916, ATCC 25922
Morganella morganii	NZRM 65, ATCC 25830, NCTC 235
Pseudomonas aeruginosa	NZRM 981, ATCC 25668, NCTC 10662
Rhodococcus coprohilus	ATCC 29080, NCTC 10994, DSM 43347T
Salmonella menston	NZRM 383
Shigella flexneri	NZRM 972
Shigella sonnei	NZRM 86
Staphylococcus epidermidis	NZRM 1210, ATCC 12228
Yersinia enterolitica	NZRM 2603, ATCC 9610

ATCC: American Type Culture Collection.
NCTC: National Collection of Type Cultures.
NCIMB: National Collection of Industiral and Marine Bacteria.
DSM: Deutsche Sammlung von Mikroorganismen.
NZRM: New Zealand Reference Culture Collection, Medical Section.

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/NZ 98/00044

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12Q 1/68
// C12R 1:01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q 1/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Medline & CAS: (1) Listeria & Polymerase Chain Reaction & amplification
(2) (Rhodococcus & Polymerase Chain Reaction) or (Rhodococcus & ribosomal)
Orbit: (1) Listeria & C12Q 1/68
(2) Rhodococcus & C12Q 1/68

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 523 205 (INSTITUT PASTEUR) 4 June 1996 & WO 89/06699 Whole Document	1-3, 8, 27, 29, 30
X	Blais, B; et al *Applied and Environmental Microbiology*, Vol 59(9) 1993 pages 2795-2800 "A Simple RNA Probe System for Analysis of Listeria Monocytogenes Polymerase Chain Reaction Product" Whole Document	1-3, 8, 27, 29, 30

[X] Further documents are listed in the continuation of Box C

[ ] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 June 1998	16 JUL 1998
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929	Authorized officer **MR LEIGH R. TRISTRAM** Telephone No.: (06) 283 2075

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/NZ 98/00044

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Thomas, E. J.; et al *Applied and Environmental Microbiology*, Vol 57(9) 1991, pages 2576-2580 "Sensitive and Specific Detection of Listeria monocytogenes in Milk and Ground Beef with the Polymerase Chain Reaction"	
X	Whole Document (See page 9 lines 27-35)	1-3, 8, 27, 29, 30
	WO 90/08841 (GENE-TRAK SYSTEMS) 9 August 1990	
X	Whole Document	1, 2, 9-11, 16, 27, 29, 30
	EP 0 314 294 (GENE-TRAK SYSTEMS) 3 May 1989	
X	Whole Document (See especially column 9 lines 40-42)	1, 2, 9-11, 16, 27, 29, 30
	Sallen, B; et al *International Journal of Systematic Bacteriology*, Vol 46(3) 1996, pages 669-674 "Comparative Analysis of 16s and 23s rRNA Sequences of Listeria Species"	
X	Whole Document	1, 2, 9-11, 16, 27, 29, 30
	Rainey, A. R.; et al *Microbiology*, Vol 141 1995, pages 532-528 "Phylogenetic analysis of the genera *Rhodococcus* and *Norcardia* from within the radiation of *Rhodococcus* species"	
X, Y	Whole Document	19-21, 26, 28-30
	Mordarski, M.; et al *Journal of General Microbiology*, Vol 118 1980, pages 313-319 "Ribosomal Ribonucleic Acid Similarities in the Classification of *Rhodococcus* and Related Taxa"	
X	Whole Document	19-20, 26, 28-30
Y	Whole Document	21

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/NZ 98/00044

Box 1 Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1, 2, 8, 9-10, 16, 19-20, 26
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The breadth of the claims was such that an economically viable search could not encompass the full scope of the claims.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplement Sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/NZ 98/00044

Box II Continuation

1. Claims 1-8 are directed to distinguishing *Listeria monocytogenes* from all other bacteria including all other *Listeria* species. The method described to achieve this uses primers against the Listeriolysin O gene. It is considered that a species specific test for *Listeria monocytogenes* comprises the first "special technical feature".
2. Claims 9-16 are directed to distinguishing *Listeria* species from other bacteria. The method described to achieve this uses primers against the 23s rRNA gene. It is considered that a genus specific test for *Listeria* comprises the second "special technical feature".
3. Claims 19-26 directed to distinguishing *Rhodococcus coprophilus* from all other bacteria including all other *Rhodococcus* species. The method described to achieve this uses primers against the 16s rRNA gene. It is considered that a species specific test for *Rhodococcus coprophilus* comprises the third "special technical feature".

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/NZ 98/00044

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	89/06699	DE	68922252	EP	0 355 147	JP	2502880
		US	5 389 513	US	5 523 205		
WO	90/08841	CA	2 025 236	EP	0 418 346	JP	3504677
		AU	51881/90	US	5 376 528		
EP	0 314 294	AT	110419	AU	22134/88	DE	3851200
		EP	0 314 294	JP	1304899	NZ	226074
		US	5 089 386				

END OF ANNEX