The Application of a Novel DNA Simplification Approach for the Universal Detection and Subtyping of Gram Positive Bacteria D.S. Millar, C.J. Vockler, J.R. Melki; Human Genetic Signatures, Australia

Abstract:

Background: The use of molecular techniques to detect groups of pathogens, such as Gram-negative species, has been hampered by the large number of members of each family and the very divergent nucleotide sequence differences between species. Methods: Treatment of DNA with sodium bisulfite results in the conversion of cytosine residues to uracil, which are subsequently amplified as thymine. This conversion essentially results in the simplification of the conventional 4 base genome to a 3 base genome to a 3 base genome to a 3 base genome making the design of probes and primers for the specific detection of families of pathogens easier. This results in less nucleotide differences between species and thus more reliable amplification. We have developed a robust PCR assay directed towards bisulfite simplified 23S rRNA DNA to differentiate between Gram positive and Gram negative bacteria. In addition, genus-specific primers for Streptococcus and Staphylococcus were designed to specific genus identification. Results: DNA was extracted from a wide range of medically important pathogens, chemically simplified and amplified with Gram-positive specific primers. All of the 12 Gram-positive bacteria tested amplified well, including Staphylococcus, Streptococcus, Enterococcus, Bacillus, Listeria and Clostridium. Conversely all 9 Gram-negative bacteria tested negative, including Proteus, Pseudomonas, Moraxella, Neisseria, Salmonella, Shigella, Bordetella and Escherichia. In addition, the Staphylococcus or Streptococcus specific primers correctly identified all strains tested, with no cross-reactivity with any other bacterium assayed. Conclusion: DNA simplification via sodium bisulfite is a novel approach for the reliable detection of pathogens consisting of a large number of sub-types such as Gram-positive organisms. In addition, the simplified DNA retains enough divergent sequence for the species-specific detection of groups of pathogen such as Streptococcus or Staphylococcus.

Introduction:

A rapid and sensitive molecular assay that can discriminate between Gram Positive and Gram Negative bacteria would be a valuable tool in clinical microbiology and the early detection of bacterial infection. In particular this could be useful in reducing the empirical use of broad-spectrum antibiotics for treating systemic infections. We have developed a broad-range assay that successfully detects all species tested that are in the Gram Positive classification. This Gram positive/negative discrimination is achieved through the conversion of the microbial DNA with sodium bisulfite, which converts all unmethylated cytosine bases within a DNA sample to thymine base (via a uracil intermediary) as indicated in Figure 1 below:

CGTAGCCTCACTTCCAGGACTGGC Add TGTAGTTTTATTTTAGGATTGGT

Figure 1: The effect of sodium bisulfite on cytosine

This simplification of the DNA serves to make nucleic acid sequences from different organisms or families more similar to each other enabling the following benefits:

- 1) The detection of multiple microbial strains in a single reaction without the need for multiplexing, which is complex and difficult to optimise.
- A single consensus primer detects the presence of any/all variants of species of interest (e.g. Gram Positive organisms), with high degree of specificity in a single reaction.
- 3) Genotyping or specific species detection can still be performed as there is sufficient heterogeneity remaining in the samples post simplification.

An example of how simplification of DNA can be applied to the detection of Gram Positive bacteria is shown in Figure 2. Our assay can effectively reduce the consensus primer heterogeneity from 1024 combinations to just 32 combinations and increase the sequence homology from 62% to 80%. This represents a 97% simplification of the original divergent sequences.

Unique Gram Positive sequence

	Non-Converted sequence			Simplifie	Simplified sequence	
Staphylococcus spp Leuconostoc spp Listeria spp Bacillus spp Clostridium spp	AGTTAC AGTTAC AGTTAC AGTTAC ACTTAC	CGTATTCAGA TGTATTCAGA CGTATTCAGA CGTATTCAGT CGTACCTTAT	CAAACGCCGA TAAACGGCGA TAAACGCCGA CAAACGCCGA CAAACGCCGA	AGTTAT AGTTAT AGTTAT AGTTAT ATTTAT	TGTATTTAGA TGTATTTAGA TGTATTTAGA TGTATTTAGT TGTATTTAGT	
Consensus sequence	ASTTAC YGTAYYYWRW YAAACGSCGA			AKTTAT	TGTATTTWRW	
	1024 Possible primer combinations 62% sequence similarity			32 Possible primer combina 80% sequence similarity		

Figure 2: Consensus sequence of Gram Positive micro-organisms before and after DNA simplification. The simplification has resulted in an increased homology from 62% to 80%.

Furthermore we have designed similar assays that are able to specifically detect any species from the Staphylococcus or Streptococcus genera with no cross reactivity with other genera. To demonstrate that the sequences still retain enough nucleic acid diversity after simplification we have also developed an assay that can discriminate S. Epidermidis from all other strains of Staphylococcus tested.

Nethods:

Microbial DNA was purchased from the ATCC and re-suspended in TE (pH 8.0). Two micrograms of microbial DNA was converted by using the MethyEasy™ DNA bisulphite conversion kit from Human Genetic Signatures (North Ryde, Australia) as per the manufacturer's instructions and resuspended to a final concentration of 20 ng/µL. One microliter of converted DNA was added to each PCR.

Gram Positive PCR: Twenty nanograms of simplified DNA was amplified in Promega 2x PCR mastermix (Madison USA) with 100 ng of each consensus primer in a final volume of 25 µL. The cycling conditions were: 95°C, 3 min x1; (95°C, 1 min; 55°C, 2 min; 65°C, 2 min) x 20 cycles. One microlitre of this PCR reaction was seeded into a 2nd round PCR and amplified according to the previous conditions with only 15 cycles. Ten microlitres of the 2nd round PCR reaction was electrophoresed on a 2% precast agarose gel (Invitrogen, USA).

Staphylococcus and Streptococcus specific PCR: Twenty nanograms of simplified DNA was amplified in Promega 2x PCR mastermix (Madison USA) with 100 ng of each consensus primer in a total volume of 20 µL. The cycling conditions were as indicated above. One microlitre of this PCR reaction was seeded into a 2nd round PCR and amplified as indicated above. Ten microlitres of the 2nd round PCR reaction was electrophoresed on a 2% precast agarose gel (Invitrogen, USA).

Gram Positive Real-time PCR and High-Resolution Melt: A total of 200 pg of simplified microbial DNA was amplified in Promega 2x PCR mastermix (Madison, USA) with 100ng of each consensus primer in a final volume of 25 µL. This reaction was supplemented with an additional 3 mM MgCl₂ (total of 4.5 mM MgCl₂). Positive reactions were detected via a Gram Positive specific MGB probe (Applied Biosystems, Foster City, USA). The cycling conditions were: 95°C 2 min x1; (95°C 15 sec; 55°C 2 min) x 45 cycles. The reactions were run in a Corbett Rotor Gene 6000 (Sydney, Australia) and data collected in the green channel. Once the reaction was completed Syto-9 (Invitrogen, USA) was added to a final concentration of 1 µM and a high resolution melt curve profile was performed, with data collected every 0.25°C from 50°C to 90°C.

Results:

A range of micro-organisms from both gram classifications were tested with our Gram Positive specific assay, an example of which is shown in Figure 3. A list of all micro-organisms tested and the results are listed in Table 1. Twenty Two Gram Positive micro-organisms were successfully amplified from a total pool of 48 different micro-organsims. There was no observed cross-reactivity as all non-gram positive microorganisms failed to generate an amplicon in our Gram Positive specific assay. Figure 3 and Table 1 also show the results generated with our Gram Negative specific assay that is designed to specifically amplify any/all Gram Negative micro-organisms. Once again good specificity is observed without any non-specific amplification of Gram Positive or other micro-organisms.

Gram negative specific PCR



TAAATGTTGA

TAAATGTTGA

TAAATGTTGA TAAATGTTGA

TAAATGKTGA

TAAATGGTGA



- Escherichia coli
- 2. Neisseria gonorrhea
- . Klebsiella pneumoniae
- 4. Moraxella catarrhalis 5. Pseudomonas aeruginosa
- 6. Proteus vulgaris
- 7. Enterococcus faecalis
- 8. Staphylococcus epidermidis Positive Staphylococcus aureus
- **10.** Staphylococcus xylosis **11.** *Streptococcus pneumoniae* Positive
- **12.** Streptococcus haemolyticus Positive

Negative Negative Negative Negative Positive Positive Positive

Gram Strain

Negative

Figure 3. Results of our Gram Positive specific assay and Gram Negative specific assay.

Table 1: Results of PCR amplification with assays specific for either Gram Positive or Gram Negative micro-organisms.

Micro-organism	Gram +ve	Gram –ve Result	Micro-organism	Gram +ve result	Gram – ve Result
Staphylococcus aureus subsp.	Positive	Negative	Proteus vulgaris	Negative	Positive
Staphylococcus epidermidis	Positive	Negative	Pseudomonas aeruginosa	Negative	Positive
Staphylococcus saprophyticus	Positive	Negative	Moxarella catarrhalis	Negative	Positive
Staphylococcus lugdunensis	Positive	Negative	Klebsiella pneumoniae	Negative	Positive
Staphylococcus xylosus	Positive	Negative	Neisseria flava	Negative	Positive
Staphylococcus hominis	Positive	Negative	Neisseria perflava	Negative	Positive
Staphylococcus schleiferi	Positive	Negative	Neisseria gonnorrhoeae	Negative	Positive
Streptococcus salivarius	Positive	Negative	Salmonella enterica subsp.	Negative	Positive
Streptococcus gallolyticus	Positive	Negative	Shigella flexneri	Negative	Positive
Streptococcus agalactiae	Positive	Negative	Escherichia coli	Negative	Positive
Streptococcus thermophilus	Positive	Negative	Bordetella pertussis	Negative	Positive
Streptococcus mutans	Positive	Negative	Mycobacterium sp.	Negative	Negative
Group B Streptococcus	Positive	Negative	Campylobacter jejuni	Negative	Negative
Streptococcus pneumoniae	Positive	Negative	Mycoplasma arginini	Negative	Negative
Streptococcus sanguinis	Positive	Negative	Mycoplasma arthritidis	Negative	Negative
Streptococcus mitis	Positive	Negative	Mycoplasma genitalium	Negative	Negative
Enterococcus faecalis	Positive	Negative	Mycoplasma hyorhinis	Negative	Negative
Bacillus cereus	Positive	Negative	Mycoplasma orale	Negative	Negative
Bacillis subtilis	Positive	Negative	Mycoplasma pirum	Negative	Negative
Listeria monocytogenes	Positive	Negative	Mycoplasma salivarium	Negative	Negative
Clostridium perfringens	Positive	Negative	Mycoplasma hominis	Negative	Negative
			Mycoplasma pneumoniae	Negative	Negative
			Candida albicans	Negative	Negative
			Candida kefyr	Negative	Negative
			Candida tropicalis	Negative	Negative
			Candida parapsilosis	Negative	Negative

In order to determine if our simplification technology will also allow pan-genus detection we designed assays for the detection of any species belonging to the Streptococcus or Staphylococcus genera. These assays also demonstrate the utility of using simplified DNA in order to simultaneously amplify DNA from a broad range of organisms. Table 2 indicates the excellent specificity of the Streptococcus specific and the Staphylococcus specific assays.

Table 2: Results of PCR amplification with assays specific for either Streptococcus (Strep) or Staphylococcus (Staph) micro-organisms.

Micro-organism	Strep	Staph	Micro-organism	Strep	Staph
Staphylococcus aureus subsp.	Negative	Positive	Proteus vulgaris	Negative	Negative
Staphylococcus epidermidis	Negative	Positive	Pseudomonas aeruginosa	Negative	Negative
Staphylococcus saprophyticus	Negative	Positive	Moxarella catarrhalis	Negative	Negative
Staphylococcus lugdunensis	Negative	Positive	Klebsiella pneumoniae	Negative	Negative
Staphylococcus xylosus	Negative	Positive	Neisseria flava	Negative	Negative
Staphylococcus hominis	Negative	Positive	Neisseria perflava	Negative	Negative
Staphylococcus schleiferi	Negative	Positive	Neisseria gonnorrhoeae	Negative	Negative
Streptococcus salivarius	Positive	Negative	Salmonella enterica subsp.	Negative	Negative
Streptococcus gallolyticus	Positive	Negative	Shigella flexneri	Negative	Negative
Streptococcus agalactiae	Positive	Negative	Escherichia coli	Negative	Negative
Streptococcus thermophilus	Positive	Negative	Bordetella pertussis	Negative	Negative
Streptococcus mutans	Positive	Negative	Mycobacterium sp.	Negative	Negative
Group B Streptococcus	Positive	Negative	Campylobacter jejuni	Negative	Negative
Streptococcus pneumoniae	Positive	Negative	Mycoplasma arginini	Negative	Negative
Streptococcus sanguinis	Positive	Negative	Mycoplasma arthritidis	Negative	Negative
Streptococcus mitis	Positive	Negative	Mycoplasma genitalium	Negative	Negative
Enterococcus faecalis	Negative	Negative	Mycoplasma hyorhinis	Negative	Negative
Bacillus cereus	Negative	Negative	Mycoplasma orale	Negative	Negative
Bacillis subtilis	Negative	Negative	Mycoplasma pirum	Negative	Negative
Listeria monocytogenes	Negative	Negative	Mycoplasma salivarium	Negative	Negative
Clostridium perfringens	Negative	Negative	Mycoplasma hominis	Negative	Negative
			Mycoplasma pneumoniae	Negative	Negative
			Candida albicans	Negative	Negative
			Candida kefyr	Negative	Negative
			Candida tropicalis	Negative	Negative
			Candida parapsilosis	Negative	Negative

Finally, as a demonstration of the level of specifically we can achieve with DNA simplification, we have designed an assay that specifically detect Staphylococcus epidermidis. This did not detect any other organisms as shown in Figure 4.

Staphylococcus epidermidis specific PCR



- **1.** Escherichia coli 2. Neisseria gonorrheae
- **3.** *Klebsiella pneumoniae*
- 4. Moraxella catarrhalis
- 5. Pseudomonas aeruginos 6. Proteus vulgaris
- 7. Enterococcus faecalis
- 8. Staphylococcus epidern **9.** Staphylococcus aureus
- **10.** Staphylococcus xylosis
- **11.** Streptococcus pneumor
- **12.** Streptococcus haemolyt

Figure 4. Results of our Staphylococcus epidermidis specific assay against a panel of micro-organisms.

As assay time is of extreme importance in a clinical setting such as the diagnosis of sepsis, we have also adapted our Gram Positive specific assay for detection in a real-time PCR setting (Figure 5). The MGB probe based assay was able to rapidly determine the specific presence of gram-positive micro-organisms. This assay is a single round assay which takes less than 90 mins to perform



Figure 5. Real-time amplification plot using an MGB probe specific for Gram Positive product. There was good amplification observed for the grampositive organisms (indicated by '+' in the legend) and no cross reactivity for the remaining microorganisms listed.

The real-time detection of the Gram specific micro-organisms also raises the interesting possibility of using high resolution melt curve analysis to identify which organism was originally amplified via the Gram Positive specific assay. This is theoretically possible as the amplicon sequence internal to the primers is still quite divergent between the different Gram Positive micro-organisms. Initial results show relatively distinct high resolution melt curves can be generated from different Gram Positive bacteria (Figure 6). Such a method could potentially lead to an assay that is able to rapidly determine Gram classification and then further identify the micro-organism(s) present via a high resolution melt reflex assay.



Figure 6. High Resolution Melt plot of Gram Positive micro-organisms using Syto-9 as the intercalating dye. The distinct melt curves suggest that Gram Positive samples may be identified through their individual high resolution melt characteristics.

	Gram Sta
	Negative
	Negative
	Negative
	Negative
sa	Negative
	Negative
	Positive
nidis	Positive
	Positive
	Positive
niae	Positive
ticus	Positive

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Discussion and Conclusion:

The early detection of bacterial infections can greatly impact upon the treatment and the resulting outcome of patients with a systemic infection, particularly if the bacteria can be accurately classified. This study confirms that the simplification of the DNA using sodium bisulfite is a novel way to allow the detection of multiple species in one single assay. The multiple species maybe grouped very broadly (e.g Gram positive/ negative), or via genus or most other classifications. In addition, the simplified DNA retains enough divergent sequence for the unique speciesspecific detection of particular micro-organisms.

Another benefit of simplification is the ability to target regions within the genome that are not naturally homologous to each other. Creating an artificial homology means that the whole genome can now be interrogated for similarities between the microbial grouping of interest. Moreover, as the strains are no longer complementary after the simplification we can look for suitable regions within both top and bottom strands of the nucleic acid. For example we have designed an assay that can detect the presence of any high-risk HPV species that targets the E7 region of the HPV genome and moved away from the commonly used L1 region. This is of clinical importance as the L1 region can be deleted upon viral integration and therefore an infection may be misdiagnosed.

A common criticism of using sodium bisulfite is that the incubations have traditionally required 4 to 16 hours to complete which drastically limits the usefulness in a clinical setting. We have successfully modified the incubations required and reduced them to as little as 10 minutes. We therefore feel that DNA simplification can now be easily integrated into clinical settings becoming a valuable tool in the simple detection of microbial infections.