A novel multiplex PCR assay for the detection of 18 bacterial, yeast and viral causes of meningitis

John R Melki, Shoo Peng Siah, Kiran Kaur, Jiny Nair, William Rawlinson* and Douglas S Millar

Genetic Signatures, Sydney, Australia, *Virology Division, SEALS Microbiology, Prince of Wales Hospital, Randwick, NSW, Australia

Abstract

Meningitis is a central nervous system (CNS) infection that can have serious clinical manifestations. Viral meningitis as a result of infection with Enterovirus is very common, usually self-limiting and does not require antibiotic treatment but is often treated empirically due to the length of time taken for conventional diagnosis. Bacterial meningitis can be life threatening and patients should be treated with the appropriate antibiotics as a matter of urgency. Our aim was to produce a real time PCR assay that could detect the presence of 18 bacterial, yeast and viral agents commonly associated with meningitis (see Table 1) in less than 2 hours. In addition, the assay utilises only 4 reaction tubes to minimise sample set up by employing dual labelled probes to increase the multiplexing capability of all common real time PCR instruments.

Background

We have developed a novel technology that has been clinically validated for the detection of HPV (1) and more recently multiplexed PCR panels for the detection of a wide range of Gastrointestinal pathogens (2).

The Genetic Signatures sample processing method converts the native nucleic acids from a 4 base code to a 3base™ form. The conversion process simplifies the design of multiplex PCR reactions by eliminating the large Tm differences that can be present when targeting multiple pathogens (see Figure 1).

Conventional Sequence	Tm	3base™ Sequence	Tm
Primer1 GTACACACCGCCCGTCGCTCCTACC	77ºC	GTATATATTGTTTGTTGTTTTTATTT	52 ºC
Primer2 GAAGGAGAAGTCGTAACAAG	56 ºC	GAAGGAGAAGTTGTAATAAG	50ºC
Probe1 TGAATAAAGAGGTGAAATTCTAGG	59 ºC	TGAATAAAGAGGTGAAATTTTAGG	59 ºC
Probe2 GAAGGGCCGCGAGCCCCCGCGC	87 ºC	GAAGGGTTGTGAGTTTTTGTGT	62 ºC

Figure 1. The DNA sequence for 2 primers and probes before and after the 3base™ modification approach

In addition, due to the large number of microorganisms targeted in the assay and the fact that meningitis does not tend to commonly manifest as multiple infections it was decided to utilise dual labelled probes (3).

Materials & Methods

Table 1. Microorganism targeted in the *EasyScreen*™ assay

Viral Meningitis		Bacterial Meningitis	
Panel A	**Panel B**	**Panel A**	**Panel B**
Enterovirus	VZV	*M. pneumoniae*	*E. coli*
Internal Control	Internal Control	Internal Control	Internal Control
	HSV-1	*S. agalactiae*	*M. tuberculosis*
	EBV	*H. influenzae*	*C. neoformans*
	HSV-2	*S. pneumoniae*	*L. monocytogenes*
	HHV-6	*N. meningitidis*	16S rDNA
	CMV		

Table 2. Commercially available strains used for the validation of the meningitis panels.

Organism	Supplier
Enterovirus	Zeptometrix
• Coxsackie A9	• CATALOG# NATCXVA9-ST
• Coxsackie B3	• CATALOG# NATCXVB3-ST
• Coxsackie B4	• CATALOG# NATCXVB4-ST
• Coxsackie B5	• CATALOG# NATCXVB5-ST
• Echovirus 6	• CATALOG# NATECHO6-ST
• Echovirus 11	• CATALOG# NATECHO11-ST
EBV	• CATALOG# NATEBV-0006
HSV-1	• CATALOG# NATHSV1-0005
HSV-2	• CATALOG# NATHSV2-0004
Human Herpes Virus 6	• CATALOG# NATHHV6-ST
VZV	• CATALOG# NATVZV-0005
Cryptococcus neoformans	• CATALOG# 0801539
N.menigitidis	• CATALOG# 0801511
Cryptococcus gattii	ATCC® 66031D-5
Mycobacterium tuberculosis	ATCC® 25177D-5
DNA from VZV strain Ellen	ATCC® VR-1367D
DNA from HSV-1 strain McIntyre	ATCC® VR-539D
DNA from HSV-2 strain G	ATCC® VR-734D™
Listeria monocytogenes	ATCC 19115D
Escherichia coli	ATCC 10798D
Streptococcus pneumoniae	ATCC® BAA-255D-5
Haemophilus influenzae	ATCC® 51907D
Haemophilus ducreyi	ATCC® 700724D-5
S. agalactiae	ATCC® (+clinical isolate)
S. pneumoniae	ATCC®

The *EasyScreen*™ assays employ a common sample processing technique that is capable of the simultaneous lysis and conversion of viral, bacterial and fungal nucleic acids to a 3base™ form. Thus a single patient specimen can be screened for all targets types including RNA viruses thus eliminating the need for different sample preparation techniques.

A total of 2-150µl of sample was added to 250µl of *EasyScreen*™ lysis buffer and heated at 95ºC for 15 minute. Sample preparation can be completed on a wide range of automated extraction platforms including the KingFisher Flex (Thermo, Waltham, USA), MagNA™ Pure (Roche, Pleasanton, USA), bioMerieux EasyMag (Marcy l'Etoile, France), Qiagen M48, Qiasymphony and EZ1 workstations (Hilden, Germany). In addition real-time PCR can be carried on a number of platforms such as the LC480 (Roche, Pleasanton), 7500 fast (Applied Biosystems, Foster City), Rotorgene-Q (Qiagen, Hilden) and the Smartcycler II (Cepheid, Sunnyvale) and CFX96 (Bio-Rad, Hercules, USA).

Sensitivity & Specificity

To assess the sensitivity and specificity of the panels 110bp oligonucleotides were designed containing the identical target region of each of the organisms included in the panels. Sensitivity and specificity was further assessed using the intact bacterial/viral/fungal and genomic nucleic acids listed in Table 2.

Figure 3a. Sensitivity and linearity of Enteroviral detection.

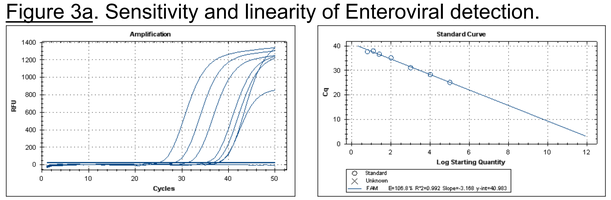


Figure 3b. Sensitivity of detection for bacterial targets.

N. meningitidis *S. pneumoniae* *H. influenzae*

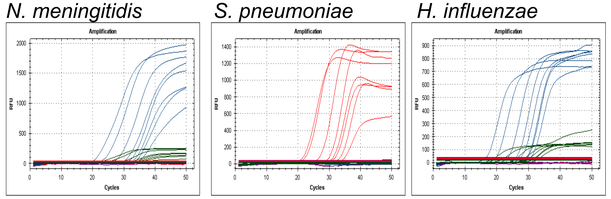


Figure 3c. Specificity of *N. meningitidis* using a wide range of non-target organisms (Blue traces *N. meningitidis*, Green traces 16S rDNA control).

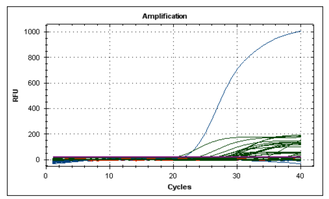
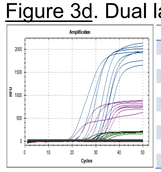
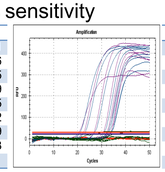


Figure 3d. Dual labeled probes sensitivity



Copies	Fam	Cy5
1,000,000	17.83	18.81
100,000	21.02	21.06
10,000	23.77	24.65
1,000	26.25	28.79
100	28.15	29.65
50	29.63	31.12
15	29.18	31.19
12.5	31.09	32.53
NTC	N/A	N/A

HSV-1



Copies	Fam	Cy5
1,000,000	21.44	21.01
100,000	23.5	22.94
10,000	27.56	27.12
1,000	31.51	31.08
100	32.95	32.44
50	33.36	32.93
15	34.37	34.06
12.5	34.53	34.06
NTC	N/A	N/A

C. neoformans

Summary

The sensitivity of the Enteroviral component was found to be approximately 6.25 copies when quantitated by real-time PCR (Fig 3a). The Enteroviral assay detects all major Enteroviral species associated with meningitis. Using commercially available strains the assay sensitively detected Coxsackie A9, B3, B4, B5, Echovirus 6 and 11 using whole viral particles (Zeptometrix). All other components of the assay were detected at a level of 10 or fewer genomic copies of target organism (Fig 3b). No inter-panel cross reactivity was observed with any components or any cross reactivity detected using a wide range of non-target organisms at an input level of 10^5 copies/PCR reaction (Fig 3c).

The use of dual labeled probes did not reduce assay sensitivity in the fully multiplexed reaction (Fig 3d). In addition, the Ct value of both fluorescent probes was near identical enabling mixed infections, if present, to be identified by differences in the Ct of the various components.

Discussion

We have developed a sensitive and specific assay for the detection of 18 causes of meningitis that is able to ultilise dual label probe technology to reduce the number of individual assays required to perform a complete meningitis screen. A simple sample processing method has been worked up which consists of taking 150µl of CSF, mixing with 250µl of *EasyScreen*™ lysis buffer and heating at 95ºC for 15 minutes. Sample purification can then be carried out on a wide range of automated platforms commonly found in pathology and hospital setting. We are in the process of collecting a large number of CSF samples to determine the advantages of using a complete meningitis screening panel to improve patient diagnosis and management.

References

1. Comparison of a novel HPV test with the Hybrid Capture II (hcII) and a reference PCR method shows high specificity and positive predictive value for 13 high-risk human papillomavirus infections. Baleriola C, Millar D, Melki J, Coulston N, Altman P, Rismanto N, Rawlinson W. *J Clin Virol* 2008;42:22-26.
2. Improved Detection Of Gastrointestinal Pathogens Using Generalised Sample Processing and Amplification Panels. Shoo Peng Siah*, Kiran Kaur*, Jiny Nair*, Peter G Huntington§, Thomas Karagiannis§, Damien Stark¶, Juan Merif¶, William Rawlinson¥, Tom Olma‡, Lee Thomas‡, John R Melki* and Douglas S Millar. Pathology (Accepted).
3. Multicolor combinatorial probe coding for real-time PCR. (2011) Huang Q, Zheng L, Zhu Y, Zhang J, Wen H, Huang J, Niu J, Zhao X, Li Q. PLoS One. 2011 Jan 14;6(1):e16033