Title: Evaluation of a multiplex PCR for early diagnosis of ventriculostomy-related infections

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Abstract

Object

Diagnosis of ventriculostomy-related infections (VRIs) is challenging due to the lack of rapid, sensitive assays for pathogen detection. We report the development of a multiplex PCR assay for differential diagnosis of common VRI pathogens.

Methods

MassTag PCR was employed to develop a multiplex assay for detection of 11 VRI pathogens. The assay was established and optimized using cloned template standards and spiked samples, and then evaluated on cerebral spinal fluid (CSF) specimens from ventricular drains. Subjects were grouped into definite VRI, possible VRI, or no VRI based on conventional microbiology, CSF evaluation and clinical parameters.

Results

CSF specimens were obtained from 45 subjects (median age 49; interquartile range 32-63yrs; males 51%). The assay detected 10-100 genome copies. The assay detected a pathogen in 100% (6/6) of definite VRI cases in which a pathogen targeted by the assay was present; these represented 67% of all definite VRI (6/9). Among subjects with a possible VRI, the assay detected a pathogen in 29% (5/17). In subjects without overt infection the presence of a pathogen was detected in 32% (6/19) of subjects, albeit with lower signal compared to the VRI group.

Conclusion

MassTag PCR enabled parallel testing of CSF specimens for 11 pathogens of VRI. The high sensitivity of PCR combined with possible device colonization, specimen contamination and concurrent antibiotic treatments limit the clinical value of the assay, similar to other current diagnostic approaches. With further optimization, multiplex PCR may provide timely identification of multiple possible VRI pathogens and guide management, complementing classical culture approaches.

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Introduction

Ventriculostomy devices are used in the management of acute hydrocephalus by providing therapeutic drainage of cerebral spinal fluid (CSF) and intracranial pressure monitoring. However, ventriculostomy-related infections (VRIs) frequently occur causing significant morbidity and mortality [6]. Diagnosis of ventriculomeningitis is difficult because similar clinical and CSF parameters are often present after intraventricular hemorrhage and neurosurgery [5]. To reduce the risk of VRIs, many institutions use prolonged antibiotic "prophylaxis" for the duration of the ventriculostomy devices despite limited evidence of efficacy [7]. Definitive diagnostic culture results can take several days and are often negative because of antibiotic inhibition [8,10]. Given the inability to obtain a rapid, sensitive diagnosis and the potential for serious neurological sequelae associated with delayed treatment, most clinicians treat empirically with broad-spectrum antibiotics when there is clinical suspicion of a VRI [14]. However, these strategies promote the selection of resistant microorganisms [7], and expose the patient to the risks of potentially unnecessary antibiotics. PCR assays allow for rapid and specific amplification of microbial nucleic acids. We have developed a multiplex PCR primer panel for the early differential diagnosis of VRIs using bacterial and fungal primers. This panel was used to build a MassTag PCR assay wherein microbial gene targets are recognized by the presence of tags with different molecular weights attached to agent-specific primers [2].

Materials and Methods

Development of multiplex PCR assay for VRIs

A review of culture-positive CSF specimens obtained from ventriculostomies performed at New York-Presbyterian Hospital (NYP)/Columbia University Medical Center between 2001-2006 was performed to determine common VRI pathogens. Bacteriologic cultures of CSF specimens were performed in accordance with standard laboratory protocol. Specimens were inoculated onto three agar plates (Colistin-nalidixic acid with 5% sheep blood, Chocolate and MacConkey, BBL, Sparks, MD) and an aliquot was added to pre-reduced thioglycollate broth (BBL, Sparks MD). Media were incubated at 35^{o} C in an atmosphere of 5% CO_2. Plates were examined by a technologist daily for up to four days, and broths were examined daily for 14 days and

subcultured when cloudy. All bacterial growth was identified to species level using VITEK TWO (bioMérieux, Durham, NC) for gram-negative bacteria and MicroScan Walk-Away (Dade Behring Inc., West Sacramento, CA) for gram-positive cocci.

An 11-plex MassTag primer panel was assembled targeting the most common VRI pathogens (Table 1). PCR primers were designed as previously described [2] and optimized using targeted sequences cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA, USA) or samples with 10^8 live organisms per mL lysis buffer (NucliSENS, bioMerieux, France) obtained from the NYP Clinical Microbiology Service. Samples were subjected to PCR amplification with a multiplex PCR kit (Qiagen), primers at 0.5 μM each in a final reaction volume of 20 uL, and the following cycling protocol: an annealing step with a temperature reduction in 1°C increments from 65°C to 58°C during the first 8 cycles and then continuing with a cycling profile of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s for 34 cycles in an MJ PTC200 thermal cycler (MJ Research, Waltham, MA, USA). Results from the MassTag PCR were obtained within 9 hours (range 8-9 hours), requiring approximately 180 min (range 150-210 min) of hands-on time when processing a 96-well plate with up to 69 samples from extraction to result (the remaining wells are blanks and quantitation standards for each target). The limit of detection for the assay was 10-100 genome copies per reaction for all pathogens as determined by serial dilution of nucleic acid extracted from the samples containing 10^8 live organisms per mL. Assay limitations have been described previously [2]. Definitive identification of pathogen(s) detected by the multiplex PCR assay was achieved through subsequent specific single-plex PCR amplification and sequencing of the product.

Assessment of assay performance using clinical specimens

The primer panel was evaluated on 45 CSF specimens collected from ventricular drains sent to the NYP Clinical Microbiology Service for culture diagnosis of VRIs from January 2008 to June 2010. A technician in the Clinical Microbiology Service would aliquot 250uL of CSF into 750uL of lysis buffer (NucliSENS, bioMerieux, France) under sterile conditions for this study, if sufficient CSF was available. All specimens were stored at -70°C and batched for PCR assay testing. This study included all specimens that were collected from ventricular drains and saved by the Clinical Microbiology Service during this period.

Retrospective laboratory and chart review was performed to determine CSF and clinical parameters, and to categorize subjects into the following clinical diagnoses based upon accepted definitions [6]: (1) definite VRI, (2) possible VRI and (3) no VRI. Definite VRI was defined by progressively declining CSF glucose level, increasing CSF protein profiles, advancing CSF pleocytosis, one or more positive CSF culture or Gram stain and clinical features such as fever, clinical signs of meningitis, including nuchal rigidity, photophobia, decreased mental status, seizures, or moribund appearance. Possible VRI was defined by the progressively declining CSF glucose level, increasing CSF protein profiles and advancing CSF pleocytosis with clinical signs and symptoms, in the absence of positive CSF cultures or Gram stain. No VRI was defined by expected CSF glucose, protein and cell count, lack of clinical signs and symptoms of infection, and lack of a positive culture or gram stain. Absolute criteria for acceptable CSF glucose and protein levels or cell counts were not specified because these parameters vary in a predictable manner, depending on the given clinical situation [6]. In three instances two successive samples from the same subject were available; only the first sample was included in the main analysis. During the period of the study, both conventional and antibiotic-coated ventricular drains with rifampin and minocycline were utilized, however records for the use on individual subjects were not available. Practice in our institution often included prolonged antibiotic prophylaxis with intravenous cefazolin while the drain was *in situ* [15] and data on antibiotic use <24 hours prior to the collection of the CSF specimen was collected. Data on indication for, or duration of, ventricular drain placement were not collected on these subjects. However, from a prior study from our institution, the primary indications for ventricular drain placement were subarachnoid hemorrhage (46%), intracranial hypertension (30%), tumor (21%), trauma (3%), and the average duration was 8.2±6.9 days [15].

Results

The median age of the 45 subjects with CSF specimens analyzed in this study was 49 years; interquartile range [IQR] 32-63 years and 51% (23/45) were male (Table 2). Presence of signs and symptoms of VRI, antibiotic administration <24 hours prior to CSF sample collection and relevant CSF parameters for VRI for each subject are shown in Table 2. Among subjects with a definite VRI, the MassTag PCR assay detected a microbial sequence in 6/6 (100%) cases for

which a pathogen targeted by the assay was present, and 6/9 (67%) cases overall (Table 3). In three cases, *Morganella morganii*, *Streptococcus parasanguinis* and *Micrococcus* species were detected by culture but not by the MassTag PCR, since the assay did not include specific primers for these organisms. Among subjects with a possible VRI, we detected at least one potential pathogen in 5/17 (29%) cases (Table 3). In subjects with no VRI, a nucleic acid of an organism was detected in 6/19 (32%) cases.

For methicillin-sensitive *Staphylococcus aureus* (MSSA), one of the most commonly detected pathogen, the positive samples with highest signal relative to the quantitative positive controls, and thus with the highest pathogen load, all occurred in the definite VRI group; considerably lower signal relative to the quantitative positive controls was evident in the positive samples from the group without VRI (Table 2). Similarly, for all *Staphylococcus* species and methicillin-resistant *Staphylococcus aureus* (MRSA) highest signals were in the definite VRI group. Although there were no definite VRIs with *Enterococcus* species or *Klebsiella* species, low signal relative to the quantitative positive control signal was observed in the group without a VRI for two of three *Enterococcus* positives and one of two *Klebsiella* positives, suggesting possible device colonization.

We calculated the sensitivity and specificity of the assay by using the definite VRI subjects as the case definition and considered only pathogens detectable by the current MassTag PCR assay (i.e. 6/6 had true positive results), and the no VRI subjects as the controls (i.e. 13/19 had true negative results). Using these criteria, the sensitivity of the assay was 100% (95% confidence interval [CI] 54-100%) and specificity was 68% (95% CI 44-87%). The negative predictive value was 100% (95% CI 75-100%) and the positive predictive value was 50% (95% CI 21-79%).

Antibiotic exposure within the 24 hours before the CSF sample, either as part of prolonged prophylaxis for the EVD placement or as treatment for another site of infection, occurred only in 3/9 (33%) of the definite VRI group as compared to 15/17 (88%) of the possible VRI group and 14/19 (77%) of the no VRI group, ($p<0.05$, both comparisons). Among the subjects with positive PCR results, 1/6 (17%) received antibiotics that were active against at least one of the organisms

detected by PCR (and culture) in the definite VRI group as compared to 3/5 (60%) in the possible VRI group and 2/6 (33%) in the no VRI group ($p>0.5$, both comparison)(Table 2).

Of the three subjects who had more than one specimen available, two were from the possible VRI group and showed consistent positive PCR results for *S. aureus*. The first patient had MSSA detected in CSF collected four days apart, while the second patient had MRSA detected in CSF collected five days apart. The agent in both cases was not detected using traditional culture methods. The third subject was from the definite VRI group and had *C. albicans* detected in CSF collected four days apart by culture and PCR despite directed antifungal therapy.

Discussion

Several multiplex PCR assays have been designed to obtain an earlier diagnosis in acute viral and bacterial meningitis cases compared to conventional culture methods [4,9,13]. Fewer studies have been published on multiplex approaches for the early diagnosis of VRIs. Banks *et al.* [1], developed a strategy that utilized a broad range PCR followed by a nested PCR using primers for selected gram-positive bacteria (*Propionibacterium acnes, S. aureus, MRSA*) in patients with a clinical suspicion of VRI. Deutch *et al* [3] utilized a similar approach in patients with and without VRI with a broad-range real-time PCR assay followed by sequencing of the amplicon to identify the pathogen, including gram-positive and gram-negative bacteria. The MassTag assay reported here differs from these previous studies by limiting the inherent contamination risk of multistep PCRs through restricting the approach to just one round of PCR, and by parallel testing multiple agents through inclusion of specific primers for 11 different bacterial and fungal pathogens and a *S. aureus* antibiotic resistance gene.

The assay performed well with definitive VRI cases in which a primer for the pathogen was present. One of the strengths of the MassTag PCR platform is that it is relatively straightforward to add additional targets as desired since only two short specific primer sequences are required. Our assay also detected microbial nucleic acids in 32% of subjects without active VRI. Positive PCR results from CSF sampled from ventricular drains in the absence of VRI may indicate device colonization, potential contamination events, or the presence of non-viable bacteria as a consequence of antibiotic treatment. While colonization or contamination was described as a

problem encountered in other multiplex PCR VRI approaches, the rate was not reported [3]. To further differentiate between colonization or contamination we tested 123 CSF samples from the NYP Clinical Microbiology Service that were collected from subjects without a ventricular drain and without clinical or laboratory evidence of central nervous system infection using standard clinical definitions [11,12]. Twelve samples (9.8%) yielded positive signal for bacteria: coagulase-negative Staphylococci [CoNS] [n=4, 33%], CoNS or MRSA [n=1, 8%], MSSA [n=2, 17%], Enterococci [n=3, 25%], *Klebsiella* [n=1, 8%], or Enterococci & CoNS [n=1, 8%]. In nearly all cases detection was with low signal level close to the assay cut-off. These data suggest that approximately 10% of CSF specimens obtained from a clinical microbiology laboratory may potentially produce 'false' positive results, due to contamination during sample collection, microbiology laboratory handling and/or PCR processing.

Therefore, in our subjects with a ventricular drain low positives may be due to contamination events, in addition to the possibility of ventricular drain colonization. Another possible explanation for low positive PCR results with a negative culture is the presence of non-viable bacteria due to antibiotics, from either prolonged cefazolin or other antibiotic therapy, or rifampin and minocycline coated drains. More precise quantitation of nucleic acid load may be helpful to distinguish between these scenarios; however, this remains to be shown. More recently, Rath *et al*., described the use of a multiplex PCR including intrathecal biomarkers (interleukin-6 and lactate)[8]. This approach represents another potential method to distinguish true infection from causes for the presence of microbial nucleic acid at low level.

Our study has several limitations. It was performed at a single center, the number of patient samples is relatively small, and the utilization of prolonged antibiotic prophylaxis and/or antibiotic-impregnated catheters was not consistent throughout the study period. Also, CSF samples were frozen prior to nucleic acid extraction which may have affected genomic DNA integrity; however, samples were stored -70°C and were not thawed prior to nucleic acid extraction. A potential for sample contamination may be reduced by taking a dedicated CSF sample at the patients' bedside and running the assay in real time with minimal sample handling; however, our results reflect true specimen processing in a clinical setting, and thus may be more

generalizable. Another limitation was that the current assay did not differentiate between MRSA and CoNS, if the *mecA* gene was the only *Staphylococcus* target detected, because both MRSA and also some strains of CoNS possess the *mecA* gene. This can be addressed by adding specific primers to distinguish CoNS in aoptimized version of the assay. Additional testing of the assay with a broader range of clinical samples is required to evaluate detection of gram-negative and fungal pathogens. Lastly, our study and other studies of novel diagnostic methods for VRI, are hampered by the lack of a gold-standard definition for true VRI, since the combination of symptoms, CSF profile and microbial gram stain or culture appear insufficient.

Currently, there are no PCR platforms in general use for the microbial diagnosis of VRI. The value of our current assay is also limited given the high sensitivity of PCR and the difficulty of differentiating between true infection and colonization/contamination. Nevertheless, an optimized PCR-based assay may provide timely and economic identification of multiple possible VRI pathogens to help guide choice of antibiotics and duration of therapy, complementing the classical culture approaches.

Conclusion

The VRI MassTag PCR primer panel performed well in definite VRI, if culture served as gold standard. In addition, pathogens were identified in 29% of possible VRI cases. Pathogen nucleic acid was also detected with low signal strength in a third of cases without VRI. This possibly reflects ventricular drain colonization, sample contamination or non-viable organisms due to antibiotic treatment. The lack of clear differentiation between these possibilities renders the current assay of limited clinical value. Nevertheless, in conjunction with clinical and CSF parameters our multiplex PCR assay can provide timely, economic and specific identification of multiple common VRI pathogens, improving upon current culture-based diagnostics. Further development of the PCR approach, potentially paired with intrathecal biomarkers, can be envisioned to improve differentiation between infection, device colonization or contamination, and to monitor the efficacy of antibiotic treatment.

Disclosure

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The author's declare no conflicts of interest.

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Table 1 VRI pathogens and gene targets

Organism	*Target Gene*	*Forward primer*	*Reverse primer*
Staphylococcus (genus level)	16S rRNA	CCATCATTAAGTTGGGCACTCTAAGTT	GGTTTCGCTGCCCTTTGTATTGT
Staphylococcus aureus	femA	TTCACGCAAACTGTTGGCCACTA	GGTAACAGGTACAGCAGTAAGTAAGCAA
Methicillin-resistant *S. aureus*	mecA	CCTTGTCCGTAACCTGAATCAGCT	GCTAGAGTAGCACTCGAATTAGGCAGT
Propionibacterium acnes	recA	GCAGCAGATCGAGAAGCAGCA	GATCTCCACGATTCTGCCACGT
Enterococcus sp.	16SrRNA	GGGGATAACACTTGGAAACAGGT	TCCATCCATCAGCGACACCCGAA
Pseudomonas aeruginosa	ExoS	GTCAACTGGTGCTCGACGCAA	CGATACTCTGCTGACCTCGCTCTCT
Enterobacter sp.	ompA	GCGCWGACTCCAGCAACARCAT	RCCGTCGCCGATGTTGTTAA
Acinetobacter baumannii	OXA-51	GCTTCCGCTATTCCRGTTTATCA	CGACTTGGGTACCGATATCTGCATT
Serratia marcescens	ompA	GGCCGTACTGGTCAGCGTCT	GCCCAGGCTCAGCATGGT
Klebsiella sp.	ompA	GCTTCTIGGITTTCCCGTA	ACGGGTTGCGATGTCACGAGTAACA
Candida albicans	CaAGM1	ACCAGTAGGAGTACAACGAACAGGAA	ATTTCATTGAATATTGGTGTGGTTCA

Table 2: Clinical and laboratory characteristics of 45 subjects with CSF specimens analyzed by MassTag PCR assay

	Subject No.	Age	Sex	Antibiotics <24 hours prior	Signs and symptoms present*	CSF parameters				Organism target(s) detected by PCR	Quantitation of PCR result^ (copies per assay)
						Increasing leukocytosis	Decreasing glucose	Increasing protein	Organism cultured		
Definite VRI	1	0.5	F	-	+	+	+	+	*Staphylococcus auricularis*	CoNS	10^3-10^4
	2	3	M	-	+	+	-	+	*Streptococcus parasanguinis*	-	
	4	46	F	-	+	+	-	+	MRSA	MRSA	$\geq 10^4$
	5	46	M	+	+	+	-	+	*Candida albicans*	*Candida albicans*[#]	$\geq 10^4$
	3	48	M	-	+	+	-	-	*Staphylococcus epidermidis*	CoNS	$\geq 10^4$
	6	50	M	+	+	+	-	+	MSSA	MSSA[**]	10^3-10^4
	7	64	M	-	+	+	-	+	MSSA	MSSA	10^3-10^4
	8	65	F	+	+	+	+	+	*Morganella morganii*	-	
	9	33	F	-	+	+	-	-	*Micrococcus*	-	
Possible VRI	10	30	M	+	+	+	-	+	-	*Enterococcus*[**], CoNS or MRS[**]	$<10^3$, $<10^3$
	11	33	M	+	+	+	-	+	-	*Enterococcus*[**]	$<10^3$
	12	41	M	+	+	-	-	+	-		

	13	41	F	+	+	+	-	+	-	*Enterococcus*[#], MSSA[**]	$<10^3$, $<10^3$
	14	46	F	+	+	+	-	-	-		
	15	47	M	-	+	+	+	+	-		
	16	48	M	+	+	+	-	+	-		
	17	49	F	+	+	+	-	+	-		
	18	50	M	+	+	+	-	+	-		
	19	56	M	+	+	-	-	-	-		
	20	56	M	+	+	+	-	-	-		
	21	56	M	+	+	+	+	+	-		
	22	58	F	+	+	+	-	-	-		
	23	63	F	+	+	+	+	+	-		
	24	65	F	+	+	+	-	+	-		
	25	80	F	-	+	+	+	+	-	MRSA	$<10^3$
	26	93	F	+	+	+	-	+	-	MRSA[#]	$<10^3$
No VRI	27	1	M	+	-	-	-	+	-		
	28	20	F	-	-	-	-	-	-	*Enterococcus*	$<10^3$
	29	22	F	-	-	-	-	-	-		

	30	23	M	+	-	-	-	-	-	MSSA**	$<10^3$
	31	24	M	-	-	-	-	-	-	*Klebsiella*	$\geq 10^4$
	32	26	F	+	-	-	-	+	-		
	33	26	F	+	-	-	-	+	-		
	34	31	M	+	-	-	-	+	-		
	35	32	F	-	-	-	-	-	-	*Klebsiella*	10^3-10^4
	36	53	M	+	-	-	-	-	-		
	37	60	M	+	-	-	-	+	-		
	38	62	M	+	-	-	-	+	-		
	39	62	M	+	-	-	-	-	-		
	40	63	F	+	-	-	-	+	-		
	41	64	F	+	-	-	-	+	-	MSSA**	$<10^3$
	42	71	F	+	-	-	-	+	-		
	43	79	M	+	-	-	-	-	-		
	44	84	F	-	-	-	-	-	-	*Enterococcus*	10^3-10^4
	45	93	F	+	-	-	-	+	-	*Enterococcus*#	$<10^3$

*Clinical signs and symptoms included fever, clinical signs of meningitis, including nuchal rigidity, photophobia, decreased mental status, seizures, or moribund appearance

#Antibiotics were not directed against this pathogen

** Antibiotics were directed against this pathogen

^ If more than one gene target for an individual organism was detected the highest value is shown.

Legend: Male (M), female (F), coagulase-negative *Staphylococci* (CoNS), methicillin-susceptible *S. aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), ventriculostomy-related infection (VRI)

Table 3. Evaluation of VRI MassTag Panel

Ventricular device present (n=45)			
Infection category	*No. samples tested*	*Positive MassTag result*	*Organisms detected (No.)*
Definite VRI	9	6* (67%)	Coagulase-negative *Staphylococci* [CoNS][#](2), Methicillin-susceptible *S. aureus* [MSSA] (2), Methicillin-resistant *S. aureus* [MRSA] (1), *C. albicans* (1)
Possible VRI	17	5 (29%)	MRSA (2), *Enterococcus* [ENT] (1), ENT & MSSA (1), ENT & MRSA or CoNS (1)^
No VRI	19	6 (32%)	MSSA (2), ENT (3), *Klebsiella* [KLEB] (2)

* Primers for the additional 3 organisms were not present on the MassTag panel. These included *Morganella morganii*, *Streptococcus parasanguinis* and *Micrococcus* sp.

[#] Coagulase-negative Staphylococci refers to all Staphylococci except *S. aureus.*

^ MecA gene target does not differentiate between methicillin-resistant CoNS or MRSA.