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BRIEF REPORT

Detection of *Streptobacillus moniliformis* in whole blood by real-time PCR and review of clinical cases 2004-2015 in New York State

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ABSTRACT

Objectives: Early identification of *Streptobacillus moniliformis* infections that cause rat bite fever (RBF) can be challenging and treatment of these infections is important for the reduction of morbidity and mortality. We report a rapid and sensitive real-time PCR assay for use on whole blood (WB) specimens in less than 3 hrs.

Materials and Methods: We developed a novel two-target real-time PCR assay and assessed sensitivity, specificity, and compared assay performance to 16S rDNA analysis. A retrospective study of 9 clinical specimens received from 2004-2015 was performed and available epidemiological data was compiled.

Results: This assay was found to have a limit of detection of <1 colony forming unit in WB and to be specific for *S. moniliformis* as well as provide detection *of S. felis, S. notomytis*, and *S. ratti*. Analysis of retrospective specimens determined this assay to be 100% concordant with 16S rDNA analysis and epidemiological data review provided insight into exposures, symptoms and treatment.

Conclusion: This real-time PCR assay represents a valuable tool as it can be performed with instrumentation available in many public health and clinical laboratories, providing results within 3 hours from receiving a WB specimen. Its utilization may improve the detection of RBF and further the understanding of the burden of this disease. *J Microbiol Infect Dis 2017; 7(2): 88-92*

Keywords: *Streptobacillus moniliformis,* Rat Bite Fever, blood, real-time PCR, clinical

INTRODUCTION

Streptobacillus moniliformis is a causative agent of rat bite fever (RBF), a rare zoonotic disease. It is a gram-negative, facultative, anaerobic bacillus that can be fastidious and difficult to isolate. Rats are considered the predominant reservoir for *S. moniliformis*, however animals that consume rats may also become infected or colonized [1,2]. It is estimated that 50-100% of wild rats and 10-100% of domesticated rats are asymptomatically colonized with *S. moniliformis* [3], and approximately 10% of rat bites result in infection [4]. Therefore, RBF and rat colonization with *S. moniliformis* represent important public health issues.

Over 200 cases of RBF have been documented in the United States [1], however this is likely a significant under representation of disease burden since RBF is not a mandated nationally reportable disease. Over the past several decades, the likelihood of rodent-human interaction and chances of infection with RBF have increased as rats have gained popularity as pets, as use for reptile food, and wild rats have encroached into populated areas [5,6]. More than 50% of infections occur in children; however laboratory personnel and pet shop employees are other high risk populations [1,7]. Infections often result from animal bites, exposure to rat waste, or consumption of contaminated food and drink [8-10]. RBF often has a non-specific clinical presentation with a broad differential diagnosis, and identification and isolation of *S. moniliformis* is not always straightforward [1,5,11] and several new spcies have recently been described and compared to

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S. moniliformis [12]. Complications from RBF can include endocarditis, myocarditis, meningitis, pneumonia, or sepsis, and when left untreated the mortality rate is approximately 13% [13, 14]. Typically patients with RBF are treated with penicillin or tetracyclines [1,5,15]. Early identification of *S. moniliformis* is important for implementing immediate patient treatment to prevent severe disease and death [16,17], however few clinical laboratories have the ability to rapidly perform this testing on clinical specimens.

Due to the fastidious nature of this organism, culture from a primary specimen is not always feasible and 16S rDNA analysis (16S) has been the only method suitable for identification at the New York State (NYS) Department of Health Wadsworth Center laboratory. While accurate, 16S is labor intensive and has a long turnaround time, often greater than one week. PCR assays have been utilized [3,18,19] but these require running agarose gels which pose the potential for amplification contamination, and are not permitted as a stand-alone test for laboratory developed tests in New York State [20]. Therefore, we developed a TaqMan® real-time PCR assay that utilizes two fluorescently labeled target-specific probes that detect the 16S rRNA and *gyrB* genes and allows for rapid detection of S. moniliformis from whole blood (WB) in less than 3 hrs.

METHODS

Whole blood (WB) extraction was performed from 180 µl of specimen using the automated bioMerieux NucliSens easyMAG specific B D2 protocol. Prior to extraction, WB samples were seeded with 20ul of an assay specific inhibition control to assess extraction efficiency and inhibition [21]. Primer and probe sequences were designed to amplify unique sequences within the 16S and *gyrB* genes of the *S. moniliformis* genome (Table 1). Real-time PCR utilized Quanta Multiplex SuperMix with the following thermal cycling conditions: 1 cycle at 95ºC for 3 min, 40 cycles at 95ºC for 15s and 60ºC for 45s in an ABI 7500 Real-time PCR instrument (Applied Biosystems). Real-time PCR results were interpreted with a reporting algorithm shown in Table 2, and DNA sequencing was performed on all specimens to confirm the results using 16S rDNA sequence analysis as part of the assay validation.

The specificity of the assay was tested using a panel of 96 organisms including 3 strains of *S. moniliformis* (DSM 12112-type strain, CCUG 14554, and CCUG 31537), 4 other *Streptobacillus* species (*S. felis*, *S. honkongensis*, *S. notomytis*, and *S. ratti*), other organisms which are normal flora as well as those that are found in animal bite wounds and bloodborne infections that can cause disease with similar symptoms to *S. moniliformis* infection. Briefly the panel included *Actinomyces neuii*, 2 *Aeromonas* species, *Anaplasma phagocytophilum*, *Babesia microtii*, *Bacillus cereus*, *Bartonella vinsonii*, 6 *Borrelia* species, 2 *Campylobacter* species, *Citrobacter freundii*, 2 *Clostridium* species, 2 *Corynebacterium* species, *Eikenella corrodens*, *Enterobacter cloacae*, 2 *Enterococcus* species, *Escherichia coli*, *Flavobacterium ceti*, 5 *Fusobacterium* species, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Legionella pneumophila*, 3 *Leptotrichia* species, *Listeria monocytogenes*, *Micrococcus luteus*, *Moraxella canis*, 4 *Mycobacterium* species, 4 *Neisseria* species, 3 *Nocardia* species, *Pasteurella multocida*, *Peptostreptococcus anaerobius*, 3 *Prevotella* species, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Rickettsia rickettsii*, *Salmonella* Enteritidis, *Serratia marcescens*, 3 *Staphylococcus* species, *Stenotrophomonas maltophilia*, 8 *Streptococcus* species, 3 *Treponema* species, 3 *Vibrio* species, and *Yersinia enterocolitica*.

RESULTS

As expected in the specificity study, the 3 *S. moniliformis* strains tested were positive for both 16S and *gyrB* targets. The other *Streptobacillus* species were detected with the 16S target, and three of the four*, S. felis, S. notomytis,* and *S. ratti* were also detected with the *gyrB* target. However, the Ct value of the *gyrB* target was several Cts greater than that seen with the 16S target, indicating less specific binding of the primers and probe. The remaining 89 strains all tested negative for both targets demonstrating that the assay has no cross-reactivity with other bacterial species. The limit of detection for the real-time PCR assay was determined to be less than 1 CFU per PCR reaction from WB using a 10-fold dilution series of the DSM 12112 *S.*

moniliformis type strain. Our testing algorithm, shown in Table 2, indicates how each real-time PCR result is reported and when further testing is performed. Real-time PCR reporting for either *S. moniliformis* or *Streptobacillus* species are reported at the time of testing.

A retrospective analysis of 9 different patient specimens received between 2004 and 2015 was performed. All patients were NYS residents who resided throughout the state, with most of the cases located in the western region of the state (n=5) or within New York City (n=3). Age of patients ranged from 1 year to 74 years, with a median of 28 years. Six of nine (67%) patients were male. Detailed epidemiological data was available for six of the nine patients, as shown in Table 3.

Table 1. *S. monoliformis* real-time PCR primer and probe sequences.

Target	Name	Sequence (5'-3')
16S[a]	Smoni-16S-F	ggttatcccagtctaagaggtaagttct
	Smoni-16S-R	agaatgcttaacacatgcaaatctatg
	Smoni-16S-P (Texas Red)	cacgttactcaccagtccaccatgtctcttatct
gyrB[b]	Smoni-*gyrB*-F	agtttaaaattccctgaaccacaatt
	Smoni-*gyrB*-R	acttccaaacactcctgaaactatacttg
	Smoni-*gyrB*-P (FAM)	tcacaaactaaggcaaaacttggttcatctgag
	Smoni-Bicoid-P (Cy5)	tcgctctgtttcatacccggcga

Primer Express™ version 2 software (Applied Biosystems, Foster City, CA) was utilized for the design of all primer and probes.
a A primer pair (Smoni-16S-F and Smoni-16S-R) and probe (Smoni-16S-P) were developed to amplify a 112 bp portion of the 1439 bp section of the 16S gene (accession number AB330760)2.
b A primer pair (Smoni-*gyrB*-F and Smoni-*gyrB*-R) and probe (Smoni-*gyrB*-P) were developed to amplify a 96 bp portion of the 529 bp *gyrB* gene (accession number JQ087403) and assess for inhibition with probe (Smoni-Biocoid-P).

Table 2: *S. monoliformis* real-time PCR reporting algorithm**.**

Result Reported	Real-time PCR result	Additional testing
Streptobacillus moniliformis DNA by real-time PCR: Detected	16S and *gyrB* <37.00 Ct and within 4.0 CT of each other	
Streptobacillus species DNA by real-time PCR: Detected	Either 16S or *gyrB* targets present at <37.00 Ct	Reflex testing is performed to determine the species present
Streptobacillus species DNA by real-time PCR: Detected	16S or *gyrB* targets both present at <37.00 CT but *gyrB* target is >4 Ct higher	Reflex testing is performed to determine the species present
Streptobacillus moniliformis DNA by real-time PCR: Not detected	Both targets >40.00 Ct	
Streptobacillus moniliformis DNA by real-time PCR: Inconclusive due to inhibition	Inhibition control has Ct value >4.0 from the seeded negative extraction control, if result repeats it should be reported as indicated.	Re-extract and retest sample

Briefly, patients presented for medical attention on average 5 days from the onset of symptoms (range 2-10 days) and remained hospitalized with an average length of stay of 13 days (range 5-31 days). Data collected from retrospective chart reviews found the most commonly reported symptoms included fever (100%), chills (67%), rash on extremities (67%), vomiting (50%), difficulty walking (50%), nausea (50%), joint effusions (50%), body/muscle aches (50%), and joint pain (33%). Overall, 83% of patients had confirmed or probable rat exposure in the days or weeks prior to becoming ill. Patients were treated with varied intravenous and/or oral antibiotic regimens, predominantly from the penicillin and tetracycline classes, as indicated in Table 3, and all six fully recovered. Testing performed on all patient samples demonstrated that the *S. moniliformis* real-time PCR assay can detect the organism from WB specimens and culture isolates as shown in Table 3, when compared to 16S rDNA sequence analysis the results were 100% concordant (data not shown).

Table 3. Epidemiologic data for RBF cases in NYS from 2004-2015

No	Country	Age, Gender	Specimen Source	Days from symptom onset to specimen collection	Symptoms	Treatment	Exposures of Interest	16S Average Ct	*gyrB* Average
1	Niagara	4, M	Abscess	*	*	*	*	18.17	18.96
2	Monroe	1, M	Pustule	*	*	*	*	18.16	17.11
3	Broome	10, F	Blood	*	*	*	*	16.21	17.27
4	Queens	23, F	Blood	2	Fever, Chills, Rash, Nausea, Vomiting, Myalgia, Headache, Sinus/ear ache	Ceftriaxone Amoxicillin	Pet rat owner	17.08	18.13
5	Bronx	72, M	Blood	10	Fever, Nausea, Vomiting, Body aches, Immobility, Numbness in extremities, Weak urine	Zosyn Tylenol	None	17.25	18.55
6	Queens	51, M	Blood	*	Fever, Chills, Body aches, Difficulty walking, No appetite	Benadryl Haldol Ativan, Simethicone Lopramide, Trimethobenzamide, Ibruprofen Thiamine, Famotidine Clonidine	Homeless with abrasions, found ill in NYC subway station	**	**
7	Monroe	74, M	Blood	2	Fever, Chills, Rash, Difficulty walking, Shortness of breath, Lethargy, Inflammation of extremities	Piperacillin-tazobactam, Ceftriaxone, Doxycycline Bromocriptine, Amoxicillin Rabies vaccine	Animal bite	35.14	37.04
8	Monroe	28, M	Serum	8	Fever, Chills, Rash, Nausea, Vomiting, Joint effusions, Fatigue, Diarrhea, Arthralgia	Penicillin, Amoxicillin	Pet rat owner	37.7	38.76
9	Monroe	52, F	Blood	5	Fever, Rash, Pain in extremities, Joint pain	Ceftriaxone, Doxycycline	Pet rat owner	36.22	38.58

*Epidemiologic data unavailable for these data points.
** Sample submitted as an isolate and was identified with MALDI-TOF Mass Spectrometry.

DISCUSSION

In conclusion, this study describes a highly sensitive and specific real-time PCR assay for detection of *S. moniliformis* that meets the regulatory requirements for diagnostic testing in NYS [20]. It offers a rapid, accurate, and cost effective alternative to 16S analysis and conventional culture methods for identification. Since culture of this organism from a primary specimen is not always successful this method also serves as a practical alternative to other methods of identification, such as MALDI-TOF MS. The six case studies presented here describe RBF cases from across NYS with detailed patient symptomology, exposure, and treatment information. This study highlights the

need for detection of *S. moniliformis* as it is believed that known cases in the US represent a significant underestimation as neither the disease nor the isolation of the bacteria as reportable to health departments [1].

Additionally, early identification of *S. moniliformis* is important for implementing timely patient treatment to prevent severe disease [1] and identification of other species of *Streptobacillus* may help to further our understanding of the contribution of these species as a public health threat [12]. This real-time PCR represents a valuable tool since it can be performed with instrumentation readily available in most public health and many clinical laboratories, providing results within 3 hours from receiving a WB specimen.

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