Available online at www.sciencedirect.com
ScienceDirect
Diagnostic Microbiology and Infectious Disease 60 (2008) 339–346
DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE
www.elsevier.com/locate/diagmicrobio

Real-time polymerase chain reaction detection of *Neisseria meningitidis* in formalin-fixed tissues from sudden deaths

Amparo Fernández-Rodríguez[a,*], Belén Alcalá[a], Roberto Álvarez-Lafuente[b]

[a]*Microbiology Laboratory, Instituto Nacional de Toxicología y Ciencias Forenses, c) Luis Cabrera no 9, 28002 Madrid, Spain*
[b]*Neurology Service, Hospital Clínico San Carlos, c) Profesor Martín Lagos s/n Madrid 28040, Spain*

Received 9 August 2007; accepted 15 October 2007

Abstract

Accurate identification of meningococcal sudden deaths is needed to avoid underestimation of the true incidence of the disease. This study analyzed the usefulness of a real-time polymerase chain reaction (PCR) protocol using MGB (3′-minor groove binder) probes to detect *Neisseria meningitidis* in formalin-fixed paraffin-embedded tissues from sudden deaths where a meningococcal fulminating infection was suspected. The protocol included detection of meningococcal DNA (*ctrA* gene), multiplex B/C PCR serogrouping (*siaD* gene), and rapid confirmation of PCR products by microcapillary electrophoresis. Sixty-nine tissues from 15 culture-confirmed meningococcal sudden deaths were analyzed (positive cases). Validation studies were performed. In each positive case, both the *ctrA* and the B/C *siaD* genes were detected. The *ctrA* was detected in 81.2% of the samples, whereas the serogroup (B or C) was identified in 44.9% of them. Therefore, this protocol may improve nonculture diagnosis and case ascertainment in meningococcal disease deaths, particularly when formalin-fixed tissues are the only available specimen.

Keywords: *Neisseria meningitidis*; Fixed tissues; Real-time PCR; Meningitis; Septic shock; Postmortem diagnosis; Sudden death

1. Introduction

Neisseria meningitidis is 1 of the main pathogens responsible for bacterial meningitis and septicemia in children and young adults. Most adult patients present with at least 2 of the 4 symptoms of headache, fever, neck stiffness, and altered mental status (Van de Beek et al., 2004). However, sometimes, symptoms are very subtle or nonspecific, making it difficult to start an early therapy (Van de Beek et al., 2004). To make matters worse, meningitis diagnosis in children is even more complicated; nonspecific features are commonly noted by parents, with fever, lethargy, and vomiting as the initial symptoms, and rash, headache, and neck stiffness as occasional manifestations (Riordan et al., 1996).

Moreover, the rapid onset of the disease may lead to a fatal evolution, causing sudden death before any clinical diagnosis has been established. In such cases, the rapid detection of *N. meningitidis* is a matter of urgency to procure the appropriate management of contacts. Case fatality rates of 3% to 13% and morbidity rates of 3% to 7% for meningococcal meningitis have been reported (Van de Beek et al., 2004, 2006). In Spain, a case fatality rate of 12.2% and an incidence of 1.61 confirmed cases per 100 000 inhabitants have been estimated in 2004 to 2005 (Jiménez et al., 2006). Reliable epidemiology studies should include forensic data from all sudden deaths due to meningococcal disease for a better evaluation of the mortality rate of this infection (Morentin and Fernández-Rodríguez, 2006). Most sudden deaths due to meningococcal disease occur at home, before the patient is admitted to hospital, or at arrival at the emergency room. Besides, in some of them, a malpractice is investigated (Fernández-Rodríguez et al., 2005; Fernández-Rodríguez and Morentin, 2005). When a sudden infectious death occurs, most frequently, antemortem cultures are not taken and the identification of the pathogen has to rely on postmortem analyses. Although in septic forms of meningococcal

* Corresponding author. Tel.: +34-91-768-98-26; fax: +34-91-563-69-24.
E-mail address: a.fernandez@mju.es (A. Fernández-Rodríguez).

0732-8893/$ – see front matter © 2008 Published by Elsevier Inc.
doi:10.1016/j.diagmicrobio.2007.10.014

disease postmortem histopathologic findings are often typical of the Waterhouse–Friderichsen syndrome (bilateral adrenal hemorrhage, disseminated intravascular coagulation, and shock liver), other organisms can also cause this syndrome. Moreover, in fulminating cases of meningitis, the histopathologic changes of the brain are difficult to detect, and a polymorphonuclear infiltrate can only be found microscopically. Furthermore, on other occasions, death can occur before meningitis develops, and the only present signs can be nonspecific, and thus, they may be overlooked (Fernández-Rodríguez et al., 2005; Fernández-Rodríguez and Morentin, 2005). For these reasons, differential diagnosis in those sudden deaths where an infection is suspected or the cause of death is unclear should include *N. meningitidis* detection. Due to the difficulty in recovering *N. meningitidis* from postmortem specimens (Ploy et al., 2005), postmortem diagnosis of meningococcal disease is mainly based on polymerase chain reaction (PCR) assays.

The most frequently analyzed specimens for *N. meningitidis* detection are blood and cerebrospinal fluid (CSF). However, they are not always available as postmortem samples, and sometimes, the only specimens collected at autopsy are formalin-fixed tissues (Fernández-Rodríguez et al., 2003). The aim of this study was to assess the usefulness of a real-time PCR protocol for the diagnosis of meningococcal infection in formalin-fixed paraffin-embedded tissues from sudden death cases where a meningococcal disease was suspected. In Spain, meningococcal disease is mainly caused by serogroups B and C (Vázquez, 2001), with a higher mortality of serogroup C than serogroup B (Cano et al., 2000; Jiménez et al., 2006). For this reason, our protocol included 2 real-time PCR assays: the 1st one, to detect meningococcal DNA, targeting the *ctrA* gene, common to all meningococcal serogroups, and the 2nd serogrouping assay, to identify serogroups B and C (*siaD* gene) in a multiplex format. The sensitivity and specificity of both assays were assessed. A rapid confirmation of the PCR products was done by using the microchip-based capillary electrophoresis (MCE) technology due to its reliability.

2. Materials and methods

2.1. Fixed tissue samples from meningococcal confirmed cases

Formalin-fixed paraffin-embedded tissues from 15 sudden deaths caused by a meningococcal fulminating disease were analyzed (positive cases) (9 females and 6 males; median age, 13 years; range, 2 months to 76 years). The median age of the specimens was 5 years (range, 1 to 11 years). All these cases fulfilled specific clinical criteria for meningococcal septicemia and/or meningitis, and in all of them, *N. meningitidis* had been previously detected in postmortem blood and/or CSF by conventional PCR (Manchester PHLS Laboratory, personal communications, and Borrow et al., 1997). A −80 °C frozen aliquot of these samples was reanalyzed with the real-time PCR presented here, obtaining a positive result (data not shown). Besides, in each case, an *N. meningitidis* strain had also been isolated in these samples (10 serogroup C and 5 serogroup B strains). These nonfixed samples had been collected according to a forensic protocol for fulminant septic shock and meningitis, based on our previous experience and consensual agreement with forensic pathologists (Fernández-Rodríguez and Morentin, 2005). Formalin-fixed paraffin-embedded tissues were selected according to the histopathologic findings: 69 specimens of tissues where a neutrophilic inflammatory infiltrate or thrombi had been detected were taken. The adrenal glands were also selected when hemorrhagic or necrotic. The samples analyzed were 12 lungs and livers, 10 hearts, 9 kidneys, 8 adrenals and brains, 7 spleens, 1 bone marrow, 1 intestine, and 1 skin.

2.2. Validation of the real-time PCR assays

2.2.1. Assays with bacteria, yeasts, and viruses

2.2.1.1. Sensitivity and lower limit of detection. A total of 34 *N. meningitidis* strains (7 reference strains and 27 strains from different sources) (Table 1) were used in the

Table 1
Microbial strains

Species	No. of strains tested
N. meningitidis	7 reference strains: 13900 ATCC (serogroup B), 700532 ATCC (serogroup C), and 5 reference strains (serogroups A, B, C, W135 and Y) from the Unité Meningocoque, WHO Collaborating Center, Marseille, France
	15 strains (3 serogroup B, 4 serogroup C, 4 serogroup W135, 2 serogroup Y, 1 serogroup 29E, and 1 serogroup X) from the reference laboratory for *Neisseria*[a]
	and 12 strains (3 serogroup B and 9 serogroup C) isolated from forensic cases in the INTCF
Related species	6 strains (*Neisseria cinerea*, *Neisseria lactamica*, *Neisseria sicca*, *Neisseria polysaccharea*, *Neisseria gonorrhoeae*, and *Moraxella catharralis*) from the Unité Meningocoque, WHO Collaborating Center, Marseille, France
Other[b]	14 reference bacterial strains, 5 reference yeasts strains, 5 viral strains

INTCF = Instituto Nacional de Toxicología y Ciencias Forenses.

[a] National Centre of Microbiology, Instituto de Salud Carlos III.

[b] The other reference bacterial species tested were *Haemophilus influenzae* (1 strain from serotype b and 1 strain from serotype f), *Listeria monocytogenes*, *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Citrobacter freundii*, *Morganella morganii*, and *Halomonas halodenitrificans*. The yeasts tested were *Candida albicans*, *Candida parapsilosis*, and 3 strains of *Rhodotorula glutinis*. The viruses tested were human herpesvirus-6 (Z-29), Epstein–Barr virus (B95-8), cytomegalovirus (AD-169), herpes simplex virus (MacIntyre strain), and varicella–zoster virus (clinical strain).