JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2010, p. 3016–3018 Vol. 48, No. 8
0095-1137/10/$12.00 doi:10.1128/JCM.00103-10

Multiple Nucleotide Substitutions in the *Neisseria meningitidis* Serogroup C *ctrA* Gene Cause False-Negative Detection by Real-Time PCR[▽]

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Received 18 January 2010/Returned for modification 20 April 2010/Accepted 21 May 2010

Two strains of *Neisseria meningitidis* serogroup C with disparate sequences of *ctrA* were isolated. The nucleotide substitutions did not alter the corresponding protein sequences, but they impeded the detection of these meningococcal isolates by real-time PCR.

CASE REPORT

In May 2008, a 27-year-old female was admitted to S. Orsola Hospital, Bologna, Italy, due to fever (39°C), myalgia, pharyngitis, and disseminated erythematous lesions on the legs. Her conscious status was conserved without signs of meningitis or neurological deficits, but she had an increased leukocyte count (29,000/ml) and elevated C-reactive protein (4.75 mg/dl). Her chest X ray was negative. Three blood culture samples were collected, and empirical antibiotic therapy, based on intravenous (i.v.) ceftriaxone (2 g/day), was initiated.

A *Neisseria meningitidis* (serogroup C) strain was isolated from individual blood cultures. The isolate, as tested by automated antimicrobial susceptibility test (AST) with the NH 214346 card (Vitek system; bioMérieux, Marcy l'Etoile, France), was sensitive to amoxicillin with clavulanic acid, ampicillin, chloramphenicol, azithromycin, sulfamethoxazole-trimethoprim, ceftriaxone, cefotaxime, and levofloxacin. Meningococcal sepsis was diagnosed, and the patient was discharged in good condition after 6 days of hospitalization. Home therapy with amoxicillin-clavulanic acid (2 g daily, orally) was recommended for the following 5 days.

In October 2008, a 21-year-old male was admitted to S. Orsola Hospital with hyperpyrexia (temperature of >38°C), mental confusion, and hypotonia of the right arm. On the day of admittance, neck stiffness and petechial rash developed. Meningoencephalitis that was associated with meningococcal sepsis was hypothesized, and a cerebrospinal fluid (CSF) specimen was collected. By latex agglutination test, the CSF was positive for *N. meningitidis*, and Gram-negative diplococci were detected by Gram staining. Elevated leukocyte levels (22,600/ml), comprising 89.3% neutrophils, and hyperbilirubinemia (2.82 mg/dl) were observed. A standard computed tomography (CT) scan of the brain was negative.

N. meningitidis (serogroup C) was isolated from the CSF. The AST of this isolate demonstrated that it was sensitive to ciprofloxacin, azithromycin, ceftriaxone, cefotaxime, and rifampin and only partially sensitive to sulfamethoxazole-trimethoprim. A therapy that was based on i.v. ceftriaxone, chloramphenicol, mannitol, and dexamethasone was begun. After 9 days of hospitalization, the patient was discharged in fairly good condition except for a mild ataxia. An identical oral home therapy was recommended.

The two bacterial strains from the blood culture and CSF of the two patients were identified as *N. meningitidis* by an API NH test (bioMérieux). The serogroup was identified using the Difco *Neisseria meningitidis* antiserum panel (Becton Dickinson, Sparks, MD). These isolates were also analyzed by real-time PCR (RT-PCR), as reported by Corless et al. (2).

This technique was tested in our laboratory by analyzing samples of DNA that were extracted from 12 meningococcal strains grown from CSF or blood culture specimens from patients with septic meningitis (10 isolates of serogroup B and 2 strains of serogroup C), and all were found to be positive. This assay is performed routinely in our laboratory to detect *N. meningitidis* in CSF specimens from patients who are suspected of having bacterial meningitis but yield negative cultures. In addition, each newly isolated *N. meningitidis* strain is evaluated by this real-time PCR method to determine its analytical performance.

Real-time PCR was performed as reported previously (2), with slight modifications. A partial region of *ctrA* (110 bp) was amplified using 300 nM (each) specific primers and detected with 50 nM 5′-6-carboxyfluorescein (FAM)-labeled probe using the LightCycler 480 Probes Master kit on a LightCycler 480 (Roche Molecular Diagnostics, Pleasanton, CA). The DNA was extracted from 200 μl of each bacterial suspension in tryptic soy broth (at a concentration of a 0.5 McFarland standard [turbidity]) after an incubation step of 15 min at 95°C using the automated NucliSens EasyMag nucleic acid extractor (bioMérieux) according to the manufacturer's protocol. The extracted DNA was eluted in a final volume of 55 μl.

Notably, the two bacterial strains, isolated from the patients in this report, were negative by real-time PCR. The test was repeated twice, yielding negative results.

To exclude the presence of PCR inhibitors, we amplified these meningococcal strains using a different target gene—an *N. meningitidis* serogroup-specific gene. The *siaD* gene was

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▽ Published ahead of print on 2 June 2010.

amplified using a set of primers that were specific for serogroup C, as reported by Tzanakaki et al. (9). Briefly, DNA was amplified in a PCR mix (50 μl of total volume), containing 1 μM (each) primer, 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates (dNTPs), and 1 unit of *Taq* DNA polymerase (Fermentas Life Sciences, Burlington, Ontario, Canada). The reaction was performed with the originally described amplification conditions (9).

By agarose gel electrophoresis, a single band that had the expected size (250 bp) was amplified in both samples. These results confirmed that the bacterial isolates were meningococci that belonged to the serogroup C strains, as detected by the serological test. Yet, these results contrasted with those of real-time PCR of *ctrA*.

To examine the presence of a target variation that could explain these negative results, *ctrA*, from nucleotide (nt) 109 to the stop codon (bp 1068)—containing the real-time PCR primers and probe annealing sequences—was sequenced completely in the two *N. meningitidis* strains. The primers L1289 (5′-GTCTCTTTAGGGCAACAATCTGA-3′, nt 251 to 273) and U157 (5′-AACTTTTCTTTTTCGGCTTTTTA-3′, nt 1405 to 1383), designed by Mothershed et al. (7), were used. These primers are positioned in the *N. meningitidis* capsular transport gene sequence (GenBank accession number M57677).

The amplification reaction was performed using Ampli *Taq* Gold (Applied Biosystems) in a 50-μl mix, containing 1.5 mM $MgCl_2$, 0.4 μM (each) primer, 200 μM dNTPs, 1.25 U *Taq* Gold DNA polymerase, and 5 μl of extracted DNA. The PCR mixtures were first incubated for 7 min at 95°C, and then 35 cycles were performed as follows: 35 s at 95°C, 55 s at 55°C, and 1 min at 72°C. Finally, the PCR mixtures were incubated at 72°C for 10 min.

By agarose gel electrophoresis analysis, a single band that had the expected size (1,154 bp) developed in both strains. For each sample, both strands of the amplicon were sequenced using the PCR and sequencing primers F623, R793, and F1097 (3). Our BLAST analysis (BLAST software is available at http://www.ncbi.nlm.nih.gov/blast) of the sequenced region revealed 97% identity (over 1,154 bp) with the *N. meningitidis* genome and no higher homology with any published DNA sequence. The partial *ctrA* sequences (1,068 bp) of the two strains were 100% identical. None of the previously published *N. meningitidis ctrA* gene sequences had such high homology with them; consequently, these sequences were submitted to GenBank under accession numbers GU391295 and GU391296.

The real-time reverse primer annealing sequence (2) has four different nucleotides (A594G, T597G, A600C, and A609G) in GU391295 and GU391296, and the probe annealing sequence (2) has 1 different nucleotide (G552A); the forward primer (2) is conserved.

To confirm these results, *ctrA* amplification and sequencing were performed using an additional *N. meningitidis* serogroup C isolate, which resulted in a positive reaction in our RT-PCR assay. By sequence analysis, the *ctrA* amplicon of this strain showed 100% identity and lacked nucleotide substitutions compared with the *ctrA* gene sequences in GenBank.

These findings clearly suggest that the presence of different nucleotides in the reverse primer and probe annealing sequences caused the negative results that were obtained when our two *N. meningitidis* isolates were evaluated by RT-PCR of *ctrA*.

After the introduction of the conjugate meningococcal serogroup C vaccine by the National Vaccine Plan 2005-2007 in Italy, the need for accurate diagnosis of *N. meningitidis* infections to monitor the effect of vaccine efficacy has grown. Consequently, the surveillance of invasive meningococcal disease that is caused by serogroup C isolates is required, and the laboratory-based identification of *N. meningitidis* serogroup C strains is an integral component of this process.

Culture-confirmed diagnosis of invasive meningococcal infections is often hampered by early antibiotic treatment, and the discrepancy between the numbers of clinically suspected and culture-confirmed cases of bacterial meningitis has prompted the development and routine application of nonculture-based methods, such as PCR, in the laboratory-based diagnosis of meningococcal infection (4, 8). One of the most frequently used PCR targets for *N. meningitidis* detection is *ctrA* (1, 3, 6), which encodes an outer membrane protein that regulates capsule transport. The *ctrA* gene is unique to *N. meningitidis*, and parts of the gene are highly conserved in all meningococcal serogroups (5).

In this report, we have described two strains of *N. meningitidis* serogroup C that were isolated within less than 6 months of each other from the CSF and blood, respectively, of two patients who resided in the same area (Emilia Romagna, Italy). These isolates have alternate *ctrA* sequences compared with published *N. meningitidis* sequences. The nucleotide substitutions in the *ctrA* gene do not modify the corresponding protein sequence, and these data are consistent with the normal expression of bacterial capsules. In both cases, *ctrA* mutant isolates caused typical meningococcal invasive infections, which resolved following specific antibiotic treatment.

Yet, the nucleotide substitutions in *ctrA* impeded the detection of these meningococcal isolates by the real-time PCR assay of Corless et al. (2). Specifically, nucleotide substitutions were identified in the primer and probe sequences. Corless et al. have recommended the use of this assay to detect *N. meningitidis* in clinical samples, due to its high specificity and sensitivity.

Based on our evidence, this RT-PCR method can generate false-negative results when *ctrA* has sequence variations. Consequently, we suggest that this assay should not be used as the sole molecular method for diagnosing *N. meningitidis* infections in the laboratory, unless a new set of primers that is not affected by the nucleotide substitution here described is used.

Nucleotide sequence accession numbers. The partial *ctrA* sequences from this study were submitted to GenBank under accession numbers GU391295 and GU391296.

REFERENCES

1. **Bennett, D. E., R. M. Mulhall, and M. T. Cafferkey.** 2004. PCR-based assay for detection of *Neisseria meningitidis* capsular serogroups 29E, X, and Z. J. Clin. Microbiol. **42:**1764–1765.
2. **Corless, C. E., M. Guiver, R. Borrow, V. Edwards-Jones, A. J. Fox, and E. B. Kaczmarski.** 2001. Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. J. Clin. Microbiol. **39:**1553–1558.
3. **Fernandez-Rodriguez, A., B. Alcala, and R. Alvarez-Lafuente.** 2008. Real-time polymerase chain reaction detection of *Neisseria meningitidis* in formalin-fixed tissues from sudden deaths. Microbiol. Infect. Dis. **60:**339–346.

4. **Fox, A. J., M. K. Taha, and U. Vogel.** 2007. Standardized nonculture techniques recommended for European reference laboratories. FEMS Microbiol. Rev. **31:**84–88.
5. **Frosch, M., D. Müller, K. Bousset, and A. Müller.** 1992. Conserved outer membrane protein of *Neisseria meningitidis* involved in capsule expression. Infect. Immun. **60:**798–803.
6. **Guiver, M., R. Borrow, J. Marsh, S. J. Gray, E. B. Kaczmarski, D. Howells, P. Bosely, and A. J. Fox.** 2000. Evaluation of the Applied Biosystems automated TaqMan PCR system for the detection of meningococcal DNA. FEMS Immunol. Med. Microbiol. **28:**173–179.
7. **Mothershed, E. A., C. T. Sacchi, A. M. Whitney, G. A. Barnett, G. W. Ajello, and S. Schmink.** 2004. Use of real-time PCR to resolve slide agglutination discrepancies in serogroup identification of *Neisseria meningitidis*. J. Clin. Microbiol. **42:**320–328.
8. **Taha, M. K., J. M. Alonso, M. Cafferkey, D. A. Caugant, S. C. Clarke, M. A. Diggle, A. Fox, M. Frosch, S. J. Gray, M. Guiver, S. Heuberger, J. Kalmusova, K. Kesanopoulos, A. M. Klem, P. Kriz, J. Marsh, P. Mölling, K. Murphy, P. Olcén, O. Sanou, G. Tzanakaki, and U. Vogel.** 2005. Interlaboratory comparison of PCR-based identification and genogrouping of *Neisseria meningitidis*. J. Clin. Microbiol. **43:**144–149.
9. **Tzanakaki, G., M. Tsolia, V. Vlachou, M. Theodoridou, A. Pangalis, M. Foustoukou, T. Karpathios, C. C. Blackwell, and J. Kremastinou.** 2003. Evaluation of non-culture diagnosis of invasive meningococcal disease by polymerase chain reaction (PCR). FEMS Immunol. Med. Microbiol. **39:**31–36.