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Report on a Suspected Case of Meningococcal Meningitis

Abstract

Clinically, Meningococcal meningitis is diagnosed clinically by symptoms such as fever, nausea, vomiting, stiff neck and a petechial rash with pink macules. Pathologically, it is diagnosed and confirmed based on the isolation of *Neisseria meningitidis* from cultures. However, in cases treated with antibiotics prior to specimen collection or when specimens are incorrectly collected, bacteria may be dead before the specimens arrive at the laboratory, and the culture could be negative. It is thus important to detect pathogenic agents rapidly and accurately with molecular diagnostic methods. The Southern Region Branch Bureau of the Center for Disease Control (CDC) received a report from a military hospital of a suspected case of Meningococcal meningitis. Though cultures of the blood and CSF of the patient were negative, Gram stain of the CSF revealed the existence of Gram-negative diplococci. To investigate the sources of infection, the Laboratory of the CDC used PCR testing to detect the DNA of N. meningitides in the CSF, and proved, though indirectly, the possibility of Meningococcal meningitis infection. Soon after the report by the military hospital, the CDC also took some preventive measures such as health education of the contacts and disinfection of the environment.

Introduction

Meningococcal meningitis is an acute bacterial disease caused by *Neisseria meningitidis*. 80% of the infected persons will develop meningitis. Children and adolescents have a higher morbidity and mortality. *N. meningitidis* is an aerobic Gram-negative diplococcus, positive for catalase and oxidase, non-motile, and with a polysaccaride capsule as its main antigen. Serotypes are determined by the antigen[(1)]. Thus far, 13 serotypes have been identified; and of the 13, A, B, C, Y, and W135 are major pathogenic serotypes. Although serotypes causing outbreaks differ according to time and location, almost 90% of the infections are caused by serotypes A, B, or C, with B serotype accounting for more than a half[(2)]. Primarily nonpathogenic types of *N. meningitidis* are found in the nasopharynx of carriers. Most carriers will develop natural immunity; only a few will, due to deficiency in complement pathways C3 and C5-C9, develop septicemia and meningitis[(2)]. Infection is through direct contact, and respiratory droplets from the nose and throat of infected individuals. The incubation period is 3-4 days. Symptoms are fever, intense headache, nausea, vomiting, stiff neck, vesicles and pink macules. In some cases, delirium and coma may occur[(3)].

According to reports, 15-25% of the cultured blood and CSF specimens of patients of bacterial meningitis are negative[(1)]. Likely reasons are that patients have been given antibiotic treatment before specimen collection; or the collection of specimens is faulty; and/or the conditions under which transportation of specimens occurs is inadequate. Bacteria are dead by the time the specimens reach the laboratory. However this is to be expected, since it takes days to culture microbes that are fastidious or slow-growing by conventional methods; they cannot be read and diagnosed rapidly. It is, therefore, important and necessary to conduct non-culture-based confirmation by using for instance, rapid and accurate molecular diagnostic methods as laboratory testing tools[(4)]. When

results of the microbial cultures are negative or patients have been pre-treated with antibiotics, the following methods are used to rapidly detect pathogenic agents: (1) for antigen detection, counter immuno-electrophoresis (CIE), latex agglutination (LA), coagglutination (COA), ELISA, and radio-immunoassay (RIA); (2) for the detection of *N. meningitidis* DNA, PCR testing; (3) for cytokine assays, TNF-alpha (tumor necrosis factor-alpha) and IL-10 (interleukin-10) concentrations in CSF, a confirmatory test that can be matched with PCR findings[5,6]. PCR is a rapid, highly accurate, specific, and easy to perform test, and is one of the testing methods used by the Laboratory.

The Background

On December 15, 2003, the Southern Region Branch Bureau of the CDC received a report from a military hospital of a patient with suspected Meningoccal meningitis. The case, a 21-year soldier, was admitted with a fever of 39℃ to the hospital for evaluation and treatment at 14:15 on December 12. The initial diagnosis was a common cold. At 17:45 on December 13, the soldier had vomiting and headache. Stiff neck and delirium developed at 08:00 the following day, and the patient was intubated and sent to ICU. Meningitis was initially suspected, and antibiotics were given. On December 15, the diagnosis was changed to suspected Meningococcal meningitis. The soldier died on December 19. Cultures of the blood and CSF of the patient were negative. To understand the cause of the infection, the Laboratory of the CDC used the PCR method to confirm the existence of *N. meningitidis* DNA in CSF specimens, proving indirectly the possibility of Meningococcal meningitis infection. The conscript was originally doing his military service in the Neichiao

camp of Paiho, Tainan County, but was transferred on December 5 to the Army Infantry Academy in Fengshan of Kaohsiung County for training. Further investigation, found that the state of health of his fellow conscripts in the two camps was normal. It appeared initially that the case was an isolated one. In order to prevent further cases, nasopharyngeal specimens of the 24 contacts of the case were collected for testing; and caregivers were given prophylactic treatment. The environment was disinfected.

Targets of the Epidemiological Investigation

The suspected case of Meningococcal meningitis and his 24 contacts.

Specimen Collection

1.blood and CSF specimens from the suspected case of Meningococcal meningitis;
2.nasopharyngeal and blood specimens (should contain Heparin or EDTA anticoagulant);
3.all specimens were transported at room temperature[7].

Processing and Culturing of Specimens

Blood specimens were inoculated at a ratio of 1:5 – 1:10 on TSB for amplification in a 5-10% CO_2 culture box at 35-37℃ for daily observation after 14-17 hours. If there was turbidity or RBC lysis in the TSB, the fluid was evenly mixed and 0.5mL was removed and sub-cultured in chocolate agar for amplification in a 5-10% CO_2 culture box at 35-37℃ for 48 hours. Otherwise, the fluid was sub-cultured in chocolate agar for 14-17 hours, 48 hours and on the 7th day. Even though there was no turbidity, a small amount of the fluid should be collected for sub-culture 7 days later. The TSB should be sterilized and

destroyed by the usual procedures[7].

The volume of the CSF specimen exceeded 1mL, and therefore was centrifuged at 2000xg for 20 minutes, the supernatant was removed, and the remaining 0.5mL of the fluid, shaken for 30 seconds for sedimentation; one drop was placed on a glass plate. After drying, the plate was Gram-stained. 1-2 more drops were taken and inoculated onto the chocolate agar and BHI liquid agar under the same conditions mentioned above.

Nasopharyngeal swabs were placed on chocolate agar and underwent the same process mentioned above[7].

Assessment

On the chocolate agar, colonies of *N. meningitidis* grew, 1mm in diameter, protruding, shining, round, and colorless or milky grey. The bacteria were Gram-negative, shaped like coffee beans and in two lines with a diameter of 0.6-0.8μm. The bacteria could also be seen in and outside the polynuclear neutrophils[7].

PCR

MK Taha's procedure of 2000 was followed[8]. For DNA extraction, the commercialized DNA purification kit was used to extract DNA from *N. meningitidis* in the CSF for use as a template of PCR. Primers ctrA, orf-2(A), saiD(B), saiD(C), saiD(W135), and saiD (Y) were used (for details of the primer sequences, see Table 1). 15 μL was taken from the template and mixed with the reaction solution to a final volume of 50 μL. The reaction solution contained 60mM Tris-HCl (pH 8.8), 17mM $(NH4)_2SO_4$, 5mM $MgCl_2$, 0.5mM each of deoxynucleoside triphosphate, 0.3 μM each primer, 1U Taq polymerase. Testing conditions were: (1) for denaturation, heating at 94℃ for 3 minutes, (2) for annealing, reaction at 55℃ for 30 seconds, (3) for polymerization, reaction at

72℃ for 20 seconds for one cycle, (4) 92℃ for 40 seconds, 55℃ for 30 seconds, 72℃ for 20 seconds for 35 cycles, and (5) amplification at 72℃ for 10 minutes. The final product of PCR (amplicons) was electrophoresed with 2% agar, using 100 bp DNA Ladder as the molecular weight marker.

Results of Laboratory Testing

No bacteria grew from either the blood or CSF cultures. The nasopharyngeal and blood specimens of the case and his 24 contacts were all negative. A DNA section of 176bp, however, was detected by PCR in the CSF specimen of the case, indicating that the *N. meningitidis* DNA testing was positive (Figure 1).

Summary of the Medical Record of Case

General examination:

Blood testing on December 13 revealed: WBC=13,700/μL, N/L=79.5/14.2 (N: neutrophils, L:lymphocytes), platelet=259,000/μL, arterial blood gas (ABG): pH=7.405, $PaCO_2$/PaO=27.0/453.8 mmHg, HCO_3=16.5 mmol/L, O_2Sat=100% (PCV, FiO_2 80%). CSF testing: Glu=5mg/dL, Cl=115.4meq/L, TP (total protein)=704mg/dL, pH=7. WBC=21,888/μL, N/L=96/4, RBC=400 μL.

Blood testing on December 15 revealed: WBC=19,300/μL, N/L=88.8/3.2, platelet=195,000/μL, Anti HCV=negative, HIV=negative, HBsAg=negative; CSF testing: Cryptococcus Ag=negative, meningitidis-5=all negative, Gram-stain: Gram-negative cocci

December 17, negative on both bacterial and microbial testing of CSF

December 18, negative on both aerobic and anaerobic bacterial culturing of blood

December 19, arterial blood gas (AGS), pH=7.012, PaCO2/PaO_2=97.4/40.0 mmHg, HCO_3=24.1 mmol/L, O_2Sat=49.7% (PCV, FiO_2 100%) Records of special examinations

December 13, brainscan showed edema

December 17, chest x-ray showed a cavity in the lower lobe of right lung; December 18, pneumonia of the lower lobe of right lung; December 19, pneumonia of both lungs. The patient was unconscious throughout the entire course of treatment, and died on December 19. The causes of death were (1) acute meningitis complicated by central nervous system failure, (2) pneumonia complicated by respiratory failure, (3) bleeding of the upper gastric-intestinal tract, and pneumomediastinum complicated by subcutaneous emphysema of the neck.

Discussion

The following lab data was noted from the medical records of the case: (1) The WBC in CSF was 21,888/cumm, which was higher than the 13,700/μl of WBC in blood, suggesting suppurative meningitis due to bacterial infection. (2) an ABG is important to assess the oxygenation and effectiveness of respiration of patients, and is an important test in patients with respiratory failure. In this case, his $PaCO_2$ ($PaCO_2/PaO_2$=97.4/40.0 mmHg, HCO_3=24.1 mmol/L) was higher than 50 mmHg, PaO_2 lower than 60 mmHg, and pH lower than 7.35, suggesting respiratory failure and acidosis. (3) the Coma index dropped from 7 to 3. (4) On the testing of CSF with latex agglutination, no trace of C. neoformans, H. influenzae, S. pneumoniae or N. meningtidis gr.A/B/C (menigitidis-5) antigens was detected. Testing using acid-Fast stain, Fugus stain and India Ink was negative. Only the Gram-stain detected polymorphonuclear cells harboring paired Gram-negative cocci. This important evidence and that fact that the condition of the patient was deteriorating rapidly strongly suggested N. meningitides infection. A comprehensive review by the disease control members of the Southern Region Branch Bureau came to the conclusion that the patient suffered from acute Gram-negative bacterial meningitis caused by

Gram-negative diplococci, most probably Meningococcal meningitis. This deduction was further supported by the detection of *N. meningitidis* DNA with PCR testing of the CSF.

Bacterial culturing of the blood and CSF of the patient was negative, possibly because the case had been pre-treated with antibiotics of Lopilexin and Ceftriaxone, and also because the specimens were transported at inappropriately low temperatures. The carrier rate of *N. meningitidis* is 5-15%, and that rate is higher in the military [(3)]. That the testing of all nasopharyngeal specimens of the 24 patient contacts was negative was inconsistent. Possible reasons were that in collecting the nasopharyngeal specimens, a general type collector was used; the cotton swab was too thick for the collection of nasopharyngeal specimens from deep inside the nasal or pharyngeal cavities, resulting in a negative test. The collection of blood specimens was not adequate, either. Due to the lack of anticoagulant, the blood specimens were already coagulated when they reached the Laboratory; they were not fit for bacterial culturing. Generally, no pathogenic agents can be detected in the blood of asymptomatic cases; except for research purposes, collection of blood specimens from these cases is not recommended. Therefore, the Branch Bureau sent a notice regarding "how to correctly and accurately collect and transport specimens in suspected Meningococcal meningitis" to the eight county/city health bureaus in the southern part of Taiwan on December 23 with copies to health stations and medical care facilities, to avoid making similar collection mistakes. Control measures suggested by the Branch Bureau are: upon receipt of a report of a suspected case of Meningococcal meningitis, prophylactic Rifampin (600mg b.i.d. for two days) should be given immediately to close contacts; the contacts should be collectively managed and body temperatures monitored to screen for infection. All articles touched by the case should be disinfected with a disinfectant solution diluted to

10% with distilled water. According to the regulations of Article 39 of the Communicable Disease Control Act, upon consent of the family, the remains of the deceased were returned to Hsinchu for cremation. A further investigation on December 25 revealed no unusual symptoms in the contacts and the family members, thus the case was considered closed.

Six pairs of primers were used in the present study, ctrA for the detection of *N. meningitidis* species, and orf-2(A), siaD(B), siaD(C), siaD(W135), and siaD(Y) for serogroups. The ctrA is a very specific piece of gene of N. meningitidis, with very little variability, and is found in all serotypes. The conserved regulatory gene (crgA) can also be used for screening. This section of gene is primarily for the adjustment of bacteria adherence to target cells. Serotyping uses multiplex PCR techniques to type *N. meningitidis* serogroups A, B, C, Y and W135, orf-2 for serogroup A, and siaD for serogroups B, C, Y and W135. Gene orf-2 (A) is associated with the biosynthesis of the capsules of serogroup A. Though the present study detected the ctrA gene of *N. meningitidis* in the CSF, it did not detect orf-2 or siaD genes, probably because the DNA concentration was low (insufficient amount of specimens), or the primer concentration was low, resulting in insufficient amounts of DNA products able to be observed on the agarose gel. According to the study of MK Taha (2000), the specificity (96%) and sensitivity (93%) of these primers were high, and no cross-reactivity could be noted among different types of *N. meningitidis* or with other bacteria such as N. gonorrhoeae, L. monocytogenes, Streptococcus pneumoniae, N. lactamica[8].

The PCR method is used to detect *N. meningitidis* in the CSF by analyzing the existence of the following genes: (1) IS1106 insertion sequence genes: Newcombe J. (1996) et al.[9] reported the detection of *N. meningitidis* using IS1106 as a routine screening procedure in British meningitis reference laboratories. (2) 16S and/or 23S rRNA genes: Kotilainen P. (1998) et al.[10]

reported the use of a broad-range bacterial PCR method with 16S and/or 23S rRNA to detect N. meningitidis(4). Atobe JH (2000) also used one step heminested-PCR to analyze 16S rRNA[11]. (3) ctrA and said genes: Stephen JG (1999) et al. used ultrasound-enhanced latex immunoagglutination in combination with ctrA and said PCR as a complementary method for the non-culture-based confirmation of Meningococcal meningitis[12]. Porritt RJ (2000) et al. used the existence of IS1106 and ctrA gene as a screening method. Corless CE (2001) et al.[13] in their study of bacterial meningitis and septicemia, used real-time PCR to diagnose simultaneously three pathogenic agents causing meningitis: ctrA gene (capsular transport) for N. meningitidis, bexA gene (capsulation), which appears in all capsular Haemophilus influenzae, and ply gene (pneumolysin), since all Streptococcus pneumoniae will produce hemolysin clinically. This is a convenient and highly accurate diagnostic model. (4) porA and porB genes: Stefanelli P et al. (2001), used PCR-RFLP method to analyze porA and porB genes[14] when serological methods could not serotype invasive N. Meningitidis. (5) 16S rDNA genes: Baethgen LF et al. used direct-PCR (DT-PCR) to detect 16S rDNA, primarily an amplified 600bp DNA section[15]. (6) Analysis of the polymorphism of pilA genes[16].

From findings of the present study and the previous studies mentioned above, it is obvious that, when bacterial cultures are negative, the use of non-culture-based molecular diagnostic methods is most important for the confirmation of pathogenic agents. Though these methods cannot replace the conventional methods of microbial culturing, they are helpful in identifying pathogenic agents, in confirming and establishing a more accurate diagnosis, in avoiding false-negatives, and in providing patients with appropriate treatment at an earlier stage; these methods are also useful in the monitoring of diseases.

N. meningitidis resides primarily in the nasopharynx, and the carrier rate is

about 5-15%. Under normal circumstances, the infection is asymptomatic, or presents with symptoms of a typical upper respiratory tract infection like a common cold. Under other circumstances, pneumonia, septicemia, and meningitis, and even fulminant meningococcal sepsis (FMS) may develop. The development of these symptoms is associated with the transformation of the agents from non-pathogenic to invasive ones. Deuren M et al. (2000) suggested the following stepwise conditions in which *N. Meningitidis* may cause invasive infection[17] : (1) exposure to pathogenic agents, (2) the agents adhere to the nasopharyngeal mucosa and colonize there, (3) they penetrate the mucous membranes, and (4) enter the blood stream. Why some agents can colonize the nasopharynx and some cannot is an issue requiring further study. Bacteria grow on the surface of the mucous membrane cells and epithelial tissue. When the epithelial tissue is damaged, bacteria will colonize. Other physical damages such as smoking and inhalation of second-hand smoke will also increase the chances of carriers becoming patients with invasive disease. Stress and viral infections will alter the surface of the mucous membrane affecting local or general immunity. Pili are important substances that adhere to the mucous membrane cells. These thread-like glycolated proteins pass from the surface of bacteria through capsular polysaccharide to join the membrane cofactor protein or CD66 of the nasopharyngeal cells. Signals may then be transmitted to host cells, and phagocytosis and cell hormone production controlled by macrophage and endothelial cells will occur. The host destroys the bacterial invaders through this series of immunological reactions. N. meningitidis, however, produces capsules and LOS (lipo-oligosaccharide) to inhibit these reactions. These substances can also prevent the phagocytosis and the bacteriolyis of neutrophils controlled by the complement systems, and also prevent the bacteria from being attacked by Kupffer cells and macrophages produced by the spleen. These are some of the

reasons that *N. meningitidis* can survive in the blood[17].

The pathogenicity factors of *N. meningitidis* are[1,2]: (1) capsular polysaccharide, (2) outer membrane lipo-oligosaccharide functioning like an endotoxin. The outer membrane contains more than 50% of lipo-oligosaccharide (LOS), which is similar to the polysaccharide produced by Gram-negative bacteria, and contains lipid A subcomponent. This antigen can activate macrophages and release tumor necrosis factor (TNF). TNF will induce toxic shock and hemorrhage. This is an important control factor of shock caused by serious meningitis septicemia. LOS can also, by means of changing the permeability of the blood-brain barrier, allow bacteria to invade the central nervous system. (3) outer membrane protein (OMP) such as porin, whose major function is to control the water soluble molecule via hydrophil and hydrophobic membrane. Serotypes of *N. meningitidis* can be further subtyped by the different compositions of OMP. Different types of OMP have different infection powers on hosts. (4) Pili, the function being to adhere on the epithelium of the host, and is highly associated with bacteria becoming from non-pathogenic to pathogenic. (5) immunoglobulin A1 protease, *N. meningitidis* can produce IgA1 protease to dissolve the A(IgA) of the host. This process will induce tolerance in bacteria to the phagocytosis of macrophages, avoiding the complement pathway and remaining in the host.

No information was available to indicate whether the immune system of the case was impaired or whether he smoked. Nasopharyngeal and blood cultures of the 24 contacts of the case were all negative. Therefore, since the present study was unable to identify the source of infection, it was suspected that the incident was an isolated one. In order to reduce the incidence of similar infections, if an outbreak occurs in crowded living spaces such as military barracks, nursing homes, jails, or nurseries, they should be reported immediately and treated in isolation; and the physical health and symptomatology of all contacts should be

strictly monitored. Ventilation of the areas should at the same time be improved. Prophylactic antibiotics should not be used unless absolutely necessary to avoid the overuse of antibiotics resulting in drug resistance and other unnecessary obstacles to disease control. Reporting institutions should be instructed to adhere to strict procedures in the collection and transportation of specimens of Meningococcal meningitis. These measures are important to the early identification of the pathogenic agent, and also to the control of disease.

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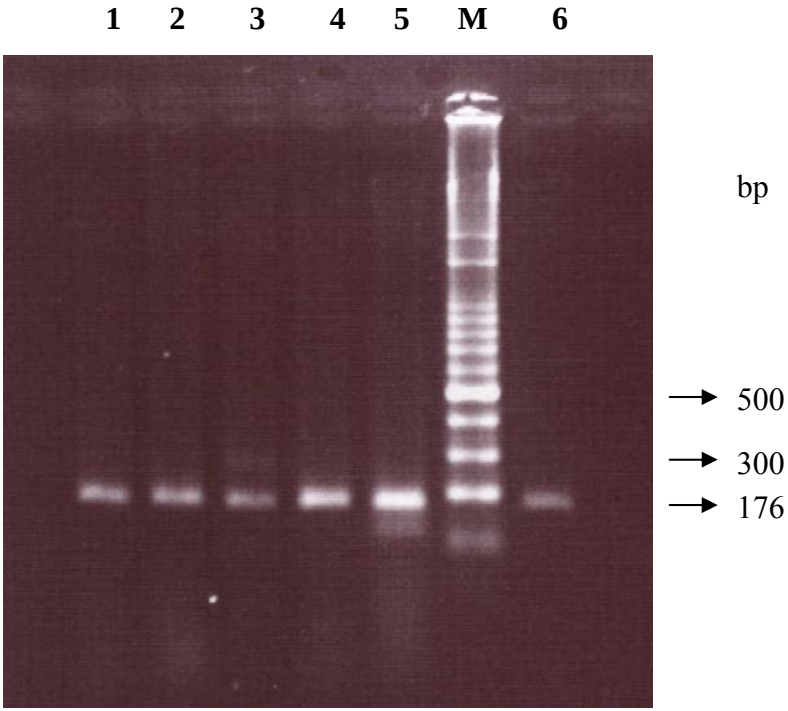
Table 1. Sequences of Primers Used

Oligonucleotide	Sequence	Gene amplified (serogroup)	Amplicon length (bp)
98-6	5'-ccagcggtattgtttgggtgg-3'	*ctrA*	176
98-10	5'-caggcggcctttaataatttc-3'		
98-28	5'cgcaataggtgtatatattcttcc-3'	*orf-2* (A)	400
98-29	5'-cgtaatagtttcgtatgccttctt-3'		
98-19	5'-ggatcatttcagtgttttccacca-3'	*siaD* (B)	450
98-20	5'-gcatgctggaggaataagcattaa-3'		
98-17	5'-tcaaatgagtttgcgaatagaaggt-3'	*siaD* (C)	250
98-18	5'-caatcacgatttgcccaattgac-3'		
98-32	5'-cagaaagtgagggatttccata-3'	*siaD* (W135)	120
98-33	5'-cacaaccattttcattatagttactgt-3'		
98-34	5'-ctcaaagcgaaggctttggtta-3'	*siaD* (Y)	120
98-35	5'-ctgaagcgttttcattataattgctaa-3'		

Figure 1. PCR Profile M: 100bp DNA Ladder



Lane 1: *Neisseria meingitidis* A

Lane 2: *Neisseria meningitidis* B

Lane 3: *Neisseria meningitidis* C

Lane 4: *Neisseria meningitidis* W135

Lane 5: *Neisseria meningitidis* Y

Lane 6: CSF of case