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ORIGINAL ARTICLE

Carriage rates of *Neisseria meningitidis* serogroups: determination among freshmen conscripts before vaccination

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ABSTRACT

Background and Objectives: *Neisseria meningitidis* is transmitted from person-to-person. Thus, close contact with a healthy carrier can facilitate the spread of the bacteria and lead to life-threatening meningococcal disease. The aim of this study was to identify oropharyngeal carriers of *N. meningitidis* in volunteers preparing for military service before vaccination.
Materials and Methods: In a cross-sectional study, 226 volunteers entering military service were referred to the Shemiranat Health Center for meningococcal vaccination and assayed. Before vaccination, the participants underwent sampling of the throat using separate swabs. Thayer-Martin Agar medium and microbiological standard methods were used for culture and isolation of the organisms. The bacterial isolates were subjected to DNA extraction and polymerase chain reaction. The obtained data were descriptively analyzed.
Results: Out of the 226 (100%) young volunteers, only 18 (8%) yielded Gram-negative diplococci. The results showed the presence of *N. meningitidis* (carriage rate: 8%) in their oropharyngeal regions. The isolated serogroups were C, A, Y, W-135, and X with frequencies of 50, 22.2, 16.6, 5.5, and 5.5, respectively.
Discussion: This study showed that the carriage rate in young volunteers for military service is around 8% before vaccination. Although the rates for serogroups A and C were dominant, the existence of serogroups Y and W indicate the necessary revision of the A/C vaccine. More research is needed to determine serogroup diversity and decrease the risk of meningococcal disease in individual groups.

Keywords: Conscripts, Healthy carrier, PCR, *Neisseria meningitidis*

INTRODUCTION

The human pathogen *Neisseria meningitidis* (a Gram-negative diplococci) has 13 serotypes and is capable of colonizing the nasopharyngeal surface membrane (1). These bacteria are responsible for meningococcal diseases such as epidemic cerebrospinal fever (2), clusters and sporadic cases of acute bacterial meningitis, mild bacteremia to devastating septicemia, pneumonia (3), septic arthritis, pericarditis, chronic bacteremia, conjunctivitis, epiglottitis, otitis, sinusitis (4, 5), urethritis, and proctitis (6, 7).

Globally, half a million cases of infection with

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N. meningitidis occur every year (8). The reported mortality remains at approximately 10% in developed countries and is higher (approximately 20%) in the developing countries. In addition, the morbidity of meningococcal disease is an additional 11–19% and includes digit and limb loss, scarring, hearing loss, cognitive dysfunction, visual impairment, educational difficulties and developmental delays, motor nerve deficits, seizures, and behavioral problems (9).

Due to the broad spectrum of human diseases caused by *N. meningitidis*, there are several high-risk population groups for meningococcal disease; close living quarters in military barracks, dormitories, homeless shelters, long-term care facilities for the elderly or mentally handicapped, and prisons are the most at risk communities. Human exposure to meningococcal infection depends on having close contact with sources of infection or healthy carriers within the environment or the promotion of person-to-person transmission (10). The results of research that evaluated the effectiveness of immunoprophylaxis in suppressing the carriage of *N. meningitidis* in a military environment showed that asymptomatic carriers are the major source of meningococcal infection (11).

The prevalence of meningococcal carriage in the United States is 5–10% under non-epidemic conditions. in closed populations, such as amongst military recruits, carriage rates can reach up to 40–90%. People may carry the bacteria for a long time (several months), which is an important factor that may lead to the development of invasive disease by increasing the risk of transmission of the bacteria to susceptible individuals (12). However, a serology and biochemical study of capsular polysaccharides have classified *N. meningitides* capsular polysaccharides into 13 serogroups: A, B, C, D, E, H, I, K, L, W-135, X, Y, and Z (13); while A, B, C, W-135, X, and Y were the most commonly isolated serogroups from all types of invasive meningococcal disease (14). In addition, the incidence of meningococcal disease outbreaks in military recruits and the appearance of antibiotic resistance in *N. meningitidis* have renewed researchers' interest in meningococcal vaccine development (15).

As a result, vaccination is recommended for adults at risk for meningococcal disease, including the following: military recruits (16); those who travel to or reside in countries in which *N. meningitidis* is hyper endemic or epidemic (17); people who have terminal complement component deficiencies (18).

Currently, a bivalent (A/C) meningococcal vaccine is a routine requirement for Iranian soldiers upon their arrival at any military garrison. During the past three decades, the meningococcal vaccine has effectively reduced the incidence of meningococcal meningitis in conscripts papulation. However, the need for this particular vaccine and the mandatory vaccination of new arrival soldiers continues to be evaluated. The aim of this study was to determine the incidence of oropharyngeal carriers of *N. meningitidis* to prepare troops had military service before vaccination.

MATERIALS AND METHODS

Mueller-Hinton broth, Mueller-Hinton Agar, Thayer Martin Agar, Brucella Agar, Tris- HCl, Acetic acid, and ethylene diamine tetra acetic acid (EDTA) were purchased from Merck Co. (Germany). Vancomycin, trimethoprim lactate, colistin sulfate, and nystatin were obtained from Mast Co. (England).

This project (meningococcal carriage study) was approved by the Ethics Committee of Baqiyatallah University of Medical Sciences (November 2, 2014, Code No: 37).

Isolation of *N. meningitidis* strains from individual carriers. From August 2014 to September 2015, 226 volunteers who were ready to enter military service and who had been referred to the Shemiranat Health Center at Shahid Beheshti Medical University for meningococcal vaccination were enrolled in this study. Before the vaccine injection, pharynx sampling of these participants was carried out separately. Two pharyngeal swabs were taken from each subject and inoculated directly onto a modified Thayer-Martin, Brucella Agar plate containing 3 mg/lit vancomycin, 5 mg/ lit trimethoprim lactate, 7.5 mg/ lit colistin sulfate and 12,500 U nystatin and kept in a 35–37°C cabinet with a 5–10% CO_2 atmosphere then transported to the microbiology laboratory (Baqiyatallah University of Medical Sciences) within 4 hours of collection. The plates and tubes were then continued to be incubated at 35–37°C for a period ranging from 24, 36, 48, and 72 h (with the time depending on the growth of the colonies) in a 5–10% CO_2 atmosphere. Subsequently, a morphological evaluation of bacterial colonies on selective media

was performed. All colonies recognized as possible of *N. meningitidis* were sub cultured onto chocolate agar and were then incubated at 35–37°C for 24 h in a 5–10% CO_2 atmosphere. If necessary, any colony that showed up was repeated in order to obtain pure colonies by the end of the procedure. Bacterial identification was based colony morphology on selective medium agar. Isolated colonies were further sub-cultured on blood agar containing 5% defibrinated sheep blood for purification. The Gram negative colonies were tested for oxidase activity and Carbohydrate fermentation. Oxidase-positive Gram-negative diplococci were tested for β-galactosidase activity using O-nitrophenyl-β- galactopyranoside (ONPG) (Rosco Diagnostica, Taastrup, Denmark). Based on the results of phenotypic tests, isolates were presumptively identified as *N. meningitidis* and were subjected to genogrouping.

Primers sequences. The oligonucleotide primers selected for identification and serogroup determination with specific sequences from earlier studies are listed in Table 1 (24–25).

Bacterial DNA isolation. Genomic DNA was extracted using the modified boiling method (21). Briefly, the DNA of each bacterial strain was isolated by suspending one loop of bacteria in 500 µl of Tris-EDTA buffer (pH 8.0). The suspension was heated at 95°C for 10 min and then centrifuged (5000×g for 5 min, in room temperature). The supernatant was then transferred to a new DNA-free micro tube containing 180 µl of 2% sodium dodecyl sulfate (SDS). After that, 375 µl of the 0.3 M sodium acetate (pH=5.2) was added and the tube was gently mixed by upside-downside moves. The tubes were then centrifuged (15,000×g for 5 min at 4°C) and the supernatant was discarded. The cold isopropanol (750 µl) was added to the sediment and kept in a freezer at -20°C overnight and the next day was centrifuged (15,000×g for 5 min at 4°C). The supernatant was discarded, and 400 µl of 70% ethanol was added. Centrifugation was repeated (15,000×g for 5 min at 4°C), and the sediment was placed in 25 µl Tris EDTA and stored in the freezer at 20°C until used.

DNA amplification. Polymerase chain reaction (PCR) was performed under optimum conditions for the amplification of 101, 400, 250, 75, 120, and 190 bp fragments in a total volume of 25 µl, which included 1 µL (50 ng/µl) of template DNA, 1.5 U Taq DNA polymerase, 1 µL of 0.12 mM dNTP Mix, 10 *p*mol/µL of each primer, 2 µL of the 2 mM $MgCl_2$, and 18µL of the DNA-free H_2O.

DNA amplification was performed in a thermal cycler (Eppendorf AG 22331 Germany) using the following conditions: initial denaturation for 4 min at 94°C followed by 35 cycles of denaturation (92°C for 40s), annealing at 55°C for 30s, and extension at 72°C for 20s. The final extension step at 72°C for 5 min was performed after completing the cycles. As a positive control, PCR containing template DNA was extracted from bacteria.

Visualization of the amplified DNA. A 5-µl

Table 1. Characteristics of primers used in this study.

Purposes	Primer name	sequences	TM°C	Product Size (bP)	Ref
Neisseria Genus	*ctrA*	Forward: 5'-gtaggtggttcaacggcaaa-3' Reverse: 5'-tcgcggatttgcaactaaat-3'	58.4	101	19
Serogroup A	*orf*-2(A)	Forward: 5'-cgcaataggtgtatatattcttcc-3' Reverse: 5'-cgtaatagtttcgtatgccttctt-3'	60.1	400	
Serogroup C	*siaD(C)*	Forward: 5'-tcaaatgagtttgcgaatagaaggt-3' Reverse: 5'-caatcacgatttgcccaattgac-3'	60.9	250	20
Serogroup Y	*Synf (Y)*	Forward: 5'-cagaaagtgagggatttccata-3' Reverse: 5'-cacaaccattttcattatagttactgt-3'	60.3	75	
Serogroup W-135	*siaD(W-135)*	Forward: 5'-cagaaagtgagggatttccata-3' Reverse: 5'-cacaaccattttcattatagttactgt-3'	58.5	120	
Serogroup X	*Ctr A(X)*	Forward: 5'-aatgcaaattcaattggttg-3' Reverse: 5'-cttgggccttatacaaagac-3'	51	190	

aliquot of the PCR product and 1 µl FluoroDye DNA Fluoresent Loading Dye 6X (SMOBiO, DL 5000) were analyzed on 1.5–2% TBE agarose. The electrophoresis was carried out in horizontal gel tanks at 100 mV for 45 min or until the desired resolution was obtained. Then, the agarose slab gels were viewed by UV Trans illumination and photographed.

RESULTS

Demographic and carriage rates. In this study, 226 oropharyngeal swab sample participants were assayed. The results of the demographic analysis indicated that participants (the junior volunteers of conscription) prior to vaccination were totally male and 19–28 years of age with a mean age of 24 years old. All of them were in healthy condition and had been sent a military service registration form. The results of 226 throat swabs for bacteriological culture and specific biochemical diagnostic tests yielded 18 *N. meningitidis* strains. According to this frequency, the rate of meningococcal carriage in this study groups was calculated as nearly 8%.

Serogroups and types sequencing. A total of 18 different *N. meningitidis* strains were identified by PCR-based genogrouping. The results of the genotyping-based PCR frequency of the isolated *N. meningitidis* are shown in Fig. 1. The results of PCR product sequencing have also confirmed the accuracy of the methods. The results of genogroups determination based on the PCR product electrophoresis are shown in Fig 2.

DISCUSSION

Iranian has managed meningococcal meningitis among conscripts and has brought the condition successfully under control during the past three decades (22). A few reported cases of this disease is attributed to defects in the complement components (23), and no further cases of meningococcal disease were reported in military services. The results of bacteriology showed the number of cases with meningococcal meningitis has been reduced (24)

During the same period of time, several outbreaks of this disease have been reported in different parts of the world. Meningitis outbreaks with serogroups A and C in the United States military in the 1960s, at UK

Fig. 2. The results of PCR product electrophoresis of *N. meningitides* strain genotypes was shown. Line 1 is a 50-bp DNA ladder; line 2 is extracted DNA; line 3 indicates serogroup X; line 4 is serogroup Y; line 5 illustrates a 100-bp DNA ladder; line 6 represents serogroup A; line 7 is serogroup W-135 and line 8 represents serogroup C.

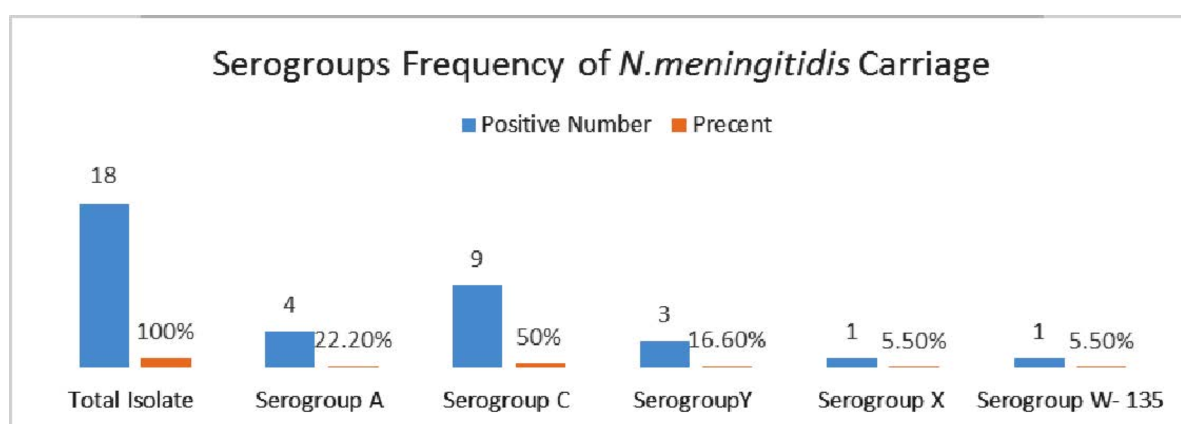

Fig. 1. Genogroups frequency is shown. The most common serogroups were C, A and Y, respectively

universities during the 1990s, a global Hajj-associated serogroup W (MenW) outbreak from 2000–2001 and subsequent MenW epidemics in sub-Saharan Africa and in South Africa (25) were pointed out and have added global concern.

However, due to the presence of a high level of multinational papulation in our region and increases in international travel, the possibility of new risks for meningococcal disease by new serogroups has arisen. Thus, carriage studies are important to improve our understanding of the *N. meningitidis* serogroup distribution and also the epidemiology of meningococcal disease control. Thus, it is important to determine the percentage of carriage rates. If the rate of carriers were identified, then tools to reduce personal contacts could be provided in populations with a high carrier rate. This process may include avoidance of crowding, reconstruction of the air-condition systems of the dorms, personal health education (26), or the administration of vaccines.

Few researchers have recently attempted to evaluate and determine the rate of *N. meningitis* carriers in defined populations in Iran. For example, in 2008, a group of researchers determined the carriage rate of *N. meningitidis* before and after Hajj pilgrimage (where it was obligatory to receive a tetravalent meningococcal vaccine) among 674 randomly selected Iranian pilgrims. The carriage rates of *N. meningitidis* were 5.2% before and 4.6% after the pilgrimage. Their results identified three new serogroups Z, Z' and A (27).

Another cross-sectional study was conducted on 1289 students in Kashan during 2011–2012 and revealed that the highest rate of carriers (12.3%) occurred in people from 15–19 years of age and they revealed that only *N. meningitidis* groups B (8 cases) and C (107 cases) were predominantly detected (28). Another study examined the prevalence, risk factors, and molecular characteristics of meningococcal carriage among Brazilian adolescents (29).

However, the carriages rate of meningococcal serogroups in volunteers for military service are not known, this study was conducted.

The results showed that, only in 18 cases (100%) *N. meningitidis* isolated and the frequency for group C 50% (9 cases) and A 25% (4 cases) were predominantly detected. Another groups frequency of *N. meningitidis* group Y 13% (3 cases), W-135 (1 cases) 6%, and X 6% (1 cases) were identified respectively,

However, humans are the exclusive reservoir of *N. meningitides* and of the broad range of species that comprise the *Neisseria* genus, only five are frequently pathogenic and reside within the nasopharynx. Although, *N. meningitidis* can cause severe disease if it invades the bloodstream, the vast majority of interactions between humans and Neisseria are benign, with the bacteria inhabiting its mucosal niche as a non-invasive commensal (30). However, the epidemiology and serogroup distribution of *N. meningitis* can change very quickly; the incidence of meningitis infection varies from very rare to more than 1000 cases per 100,000 of the population yearly. The carriage of *N. meningitidis* represents an exclusive human commensal; in rare cases, bacteria proliferate in the central nervous system and rapidly lead to the death for the affected subjects (31).

In this way, continuous surveillance of serogroup carriage is necessary to control any possible diversity and emerging virulent strains in high-risk populations as well as to predict the epidemiology of meningococcal infections and the clinical spectrum of affected populations.

The results of this study have revealed that soldiers are at high risk of contracting meningococcal disease; therefore, special attention is required in this population. Our results is as the same of others. For example, there is a report of two meningococcemia and meningitis cases due to *N. meningitidis* W-135 that developed in people who had been vaccinated with the bivalent (A/C) meningococcal vaccine (32).

CONCLUSION

This study was designed and carried out to determine the *N. meningitides* carriage rate among volunteers preparing to enter military service. The results of this study indicated that the rate of *N. meningitides* carriers in soldiers was lower than that in the general population. Another important finding was that the carriage rates for serogroups A and C were dominant; but the existence of serogroups Y and W indicated the need to revise the A/C vaccine so that it covers a wider variety of serogroups. However, more research must be performed in a large sample in order to determine the exact carriage rates before and after vaccination.

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