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MENINGOCOCCAL PRE-VACCINATION SURVEY ON CARRIAGE OF NEISSERIA SPECIES

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ABSTRACT

Introduction and aims: the carriage rate concurrency of *N. meningitidis* serogroups and *N. lactamica* in oropharynx of volunteers preparing for military service before vaccination are remained unknown. The current study aimed to investigate the frequency of *N. meningitidis* serogroups and *N. lactamica* in the oropharynx of young adults.

Materials and Methods: A total of 300 oropharyngeal swab samples of the young healthy adults were studied during August 2014 to September 2015. Swabs were plated onto enriched selective media. Gram-negative and oxidase-positive diplococci were phenotypically, genetically and biochemically identified. The PCR products were subjected sequencing in order to confirm the accuracy of the results. **Results:** Among 300 young healthy adults with the mean age of 24 years, identified: 25 *N. meningitidis* strains (8.3%) which highest frequency was belonging to serogroup C with 12 cases (4%). in 121 cases (40.3%) the *N. lactamica* was isolated from oropharynx of the subjects. Analyses of the results indicate that in 154 cases (51.3%), none of the mentioned neisseria had not isolated from the orophrynx.

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Conclusions: Determination of the healthy carriage rate and screening of *N. meningitidis* with concomitant colonization of the *N. lactamica* in oropharynx may be crucial for design of meningococcal disease prevention methods.

INTRODUCTION

It is well known that, the genera of Neisserial bacterium are habitat the upper respiratory tract of humans (1). However, the most species are commensally and nonpathogenic state. While, two species; *Neisseria gonorrhoea* and *Neisseria meningitides* (*N. meningitides*) are strictly human pathogen and asymptomatic carriers are presumably the major source of the pathogenic strains (2). The *N. meningitides* have 13 serogroups which includes A, B, C, W-135, X and Y are highly prevalent in the world populations and associate with invasive meningococcal disease worldwide (3). Thus, vaccination is recommended for at risk people for meningococcal disease such as: military recruits (4); the traveler to hyper endemic or epidemic region (5) and people who have terminal complement component deficiencies (6). Recently, based on cross reaction between *Neisseria lactamica* (*N. lactamica*) outer membrane vesicles (OMV) antigens with *N. meningitides*, the researchers have prompted to study the effect of inhibiting the growth of *N. meningitides* by *N. lactamica* (7). On the other hands, the results of colonization in the nasopharynx by *N. lactamica*, has been suggested and lead to the acquisition of natural immunity against *N. meningitidis* in young adults. However, it is not clear how *N. lactamica* oropharynx colonization can inhibit all serogroups of *N. meningitides* or not. In this case, the carriage rate may be a central key to understanding of the meningococcal infection epidemiology and determine the pattern of the meningococcal carriage before the introduction of the appropriate vaccine. The aim of this cross-sectional survey was designated to determine the relatively carriage rate of oropharynx *N. lactamica* and *N. meningitides* before vaccination in IR Iran.

METHODS

In this study, Glucose, Maltose, Lactose, Nutrient Agar, Nitrate broth, Thayer Martin Agar, Tris-HCl, Acetic acid, and ethylenediaminetetraacetic acid (EDTA), H₂O₂, oxidase were purchased from Merck (Germany). Vancomycin, Trimethoprim Lactate,

Colistin Sulfate, and Nystatin were obtained from Mast Co (England).

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

Oropharyngeal Sampling and Isolation of Niesseria

From August 2014 to September 2015, 300 volunteers who had been referred to the Shemiranat Health Center at Shahid Beheshti Medical University Tehran IR Iran 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 Agar containing 3mg/lit vancomycin, 5mg/lit trimethoprim lactate, 7.5mg/lit colistin sulfate and 12,500 unit nystatin and kept in a 35–37°C cabinet with a 3–5% CO₂ atmosphere then transported to the microbiology laboratory (Baqiyatallah University of Medical Sciences, Tehran IR Iran) within 2- 4 hours of collection. The plates 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 3–5% CO₂ atmosphere. Subsequently, morphological evaluations of bacterial colonies on selective media were subjected to biochemical assay. 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 3–5% CO₂ atmosphere. If necessary, any colony that showed up was repeated in order to obtain pure colonies by the end of the procedure. The primary bacterial identification was based on colony morphology on selective medium and biochemical tests. The recognized colonies were further sub-cultured on blood agar containing 5% defibrinated sheep blood for further assessments. The Gram negative colonies were tested for oxidase activity and Carbohydrates fermentation (Glucose, Maltose, and Lactose). 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 considered diagnosis as *N. meningitidis* and were subjected to serogrouping.

Primers: The oligonucleotide primers selected for identification and serogroup determination with specific sequences from earlier studies are listed in Table 1.

Table 1. Characteristics of primers used in this study.

Purposes	Primer name	Sequences	TM°C	Product Size(bp)	Ref
Neisseria Genus	<i>ctrA</i>	Forward: 5'-gtagtggttcaacggcaaa-3' Reverse: 5'-tcgcggatttgcaactaaat-3'	58.4	101	19
Neisseria lactamica	<i>pdhC</i>	Forward: 5'-aatgtttggacggcgactac-3' Reverse: 5'-gtacaacttttgcgggtcgt-3'	57	161	16
Serogroup A	<i>orf-2(A)</i>	Forward: 5'-cgcaataggtgtatatattctcc-3' Reverse: 5'-cgtaatagtttcgtatgccttctt-3'	60.1	400	
Serogroup C	<i>siaD(C)</i>	Forward: 5'tcaaatgagtttgcgaatagaaggt-3' Reverse: 5'-caatcacgatttgcceaattgac-3'	60.9	250	
Serogroup Y	<i>Synf (Y)</i>	Forward: 5'-cagaaagtgagggattccata-3' Reverse: 5'-cacaaccattttcattatagttactgt-3'	60.3	75	20
Serogroup W-135	<i>siaD(W-135)</i>	Forward: 5'-cagaaagtgagggattccata-3' Reverse: 5'-cacaaccattttcattatagttactgt-3'	58.5	120	
Serogroup X	<i>Ctr A(X)</i>	Forward: 5'-aatgcaaattcaattggtt-3' Reverse: 5'-cttggccttatacaagac-3'	51	190	

Bacterial DNA Isolation

Genomic DNA was extracted as previously described (8). Briefly, the DNA from each bacterial strain was extracted by suspending one loop of bacteria in 500µl 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 180µl of 2% sodium dodecyl sulfate (SDS). After that, 375µl of the 0.3 M sodium acetate (pH=5.2) was added and the tube was gently mixed by upside-downside move. The tubes were then centrifuged (13,000×g for 5 min at 4°C) and the supernatant were discarded. The cold isopropanol (750µl) was added to the sediment and kept in a freezer at -20°C overnight and the next day was centrifuged (13,000×g for 5 min at 4°C). The supernatant was discarded, and 400µl 70% ethanol was added. Centrifugation was repeated (13,000×g for 5 min at 4°C), and the sediment was placed in 25µl 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 190bp fragments in a total volume of 25µl, which included 1µl (50 ng/µl) of template DNA, 1.5 U Taq polymerase DNA, 1µl of

0.12 mM dNTP Mix, 10 pmol/µl of each primer, 2µl of the 2mM MgCl₂, and 18µl of the DNA-free H₂O.

DNA amplification was performed in a thermal cycler (Eppendorf AG 22331) 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 aliquot of the PCR product and 1 µl FluoroDye DNA Fluorescent 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, 300 oropharyngeal swab sample of participants were assayed. The results of the demographic analysis indicated that participants (the junior volunteers of conscription) prior to vaccination were totally male and with 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 300 throat swabs for bacteriological culture and specific biochemical diagnostic tests yielded 25 *N. meningitidis* strains. According to this frequency, the rate of meningococcal carriage in this study was estimated at nearly 8.3%.

Serogroup determination: The results of bacteriological assay of 300 nasopharyngeal soap and the

frequency of isolated bacterial are illustrated in Fig 1. A total of 25 *N. meningitidis* isolated strains were subjected to serogrouping by PCR-based methods and the sequencing has also confirmed the accuracy of the results. The results of the genotyping-based PCR frequency of the isolated Neisseria genus, *N. lactamica* and *N. meningitidis* are shown in Table 2.

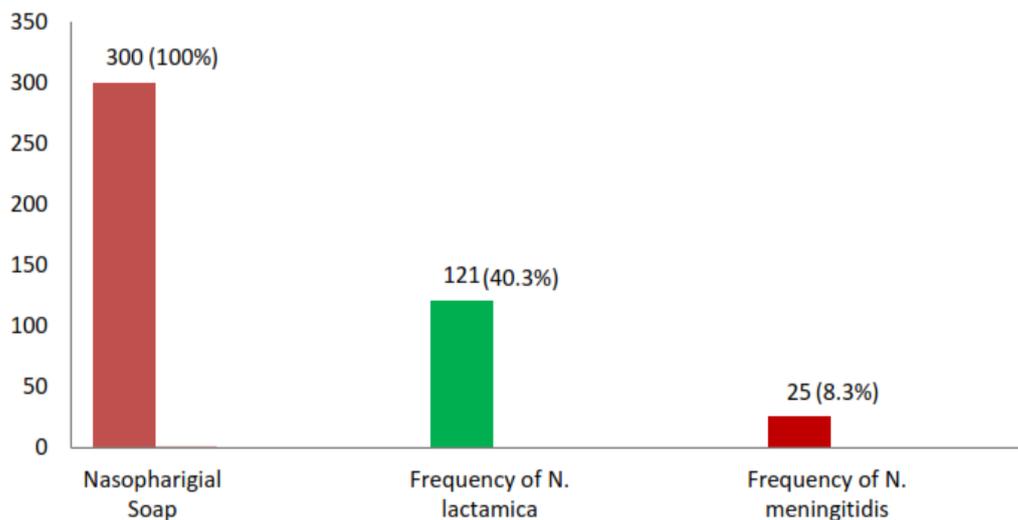


Figure 1. The frequency of isolated of Neisserial species is shown.

Table 2. The results of the frequency of *N. meningitidis* serogroups and co- isolated *N. lactamica* and also Neisseial Genus determinants are shown.

Bacteria	Serogroups or species						Total
	A	C	Y	W- 135	X	<i>N. lactamica</i>	
<i>N. meningitidis</i>	5	12	4	3	1	-	25
<i>N. lactamica</i>	5	11	4	2	1	98	121
Neisseria Genus	+	+	+	+	+	+	146

DISCUSSION

It is well known that at least 13 serogroups of *N. meningitidis* have been identified. The highest incidence serogroups associated with human disease are named as A, B, C, X, Y and w- 135 serogroups (9).

All pathogenic and commensal neisseiral strains are able to colonize onto the upper respiratory surface mucosa. The study results showed that 5- 30% of the normal population may harbor one or more of *N. meningitidis* serogroups in their nasopharynx. From the nasopharynx, the bacterium may invade the bloodstream and cerebrospinal tissue. However, the carriage rates and frequency are

geographically differing. It is not clear why in 70% of the population have not been colonized by pathogenic *N. meningitidis* serogroups. In addition, the serogroup distributions around the globe are not the same. For example, the meningococemia causative serogroups in Europe are mainly by B, C, W-135 and Y serogroups. The most prevalent meningococcal serogroups in the American continent are the A and C groups and in Africa, the serogroups A, C, Y and W-135 are the most prevalent of causative meningococcal disease. However, the quantitative roles of colonized commensal *N. lactamica* on the nasopharynx are not known. Thus, this study was designed to investigate the frequency of

N. meningitidis serogroups and *N. lactamica* in the nasopharynx of the candidates receiving vaccine in purpose to understand how to make the prevention and control better strategy the meningococcal disease in high risk population such as student campus or military solders.

The finding of this study was that, in 25 cases (8.3%) *N. meningitidis* were isolate and characterized. The highest frequency was belonging to serogroup C with 12 cases (4%). While, in 121 cases (40.3%) the *N. lactamica* was isolated from oropharynx of the subjects. Analyses of the results indicate that in 154 cases (51.3%), none of the above mentioned neisseria had not isolated from the oropharynx. The reason is not clear, it may be related to the inhibitory roles of other colonized commensally neisseria on the oropharynx. To clarify this fact, it requires more comprehensive researches. However, in recent years the similarities of surface protein antigens between *N. meningitidis* serogroups and *N. lactamica* have been shown (10). Therefore, this similarity can provide immunity cross- reactivity and or induced mucosal immunity which resulted is preventing the colonization of pathogenic *N. meningitidis* serogroups. During a report research, this fact has been experimentally shown by nasal inoculation of the commensal *N. lactamica* which reduced carriage rate of *N. meningitidis* by young adults. That controlled human infection study, where the authors have attempt to evaluated the effects of controlled infection of human volunteers with *N. lactamica* prevents colonization by *N. meningitidis* and conclud that the inhibition of meningococcal carriage by *N. lactamica* is even more efficacious than after glycoconjugate meningococcal vaccination (11). Nevertheless, the role of other saprophytic neisseria in human oropharyngx remains unknown. However, researchers had reported the important of the Meningococcal carriage studies and improved their understanding of the epidemiology of the meningococcal disease (12- 14). several research results have shown the natural immunity to *N. meningitidis* may be occurred subsequent nasopharyngeal carriage of closely related commensalism, *N. lactamica* (15, 16). According to the data reports in this area and also our experiences (17, 18), we do not really know how relationship between pathogenic and non-pathogenic strains of neisseria genus or even between varieties or clons of a bacteria species on the animal oropharynx. In fact the competition between bacterial strains in oropharyngeal mucosa is not understood. Despite, several reports pertaining to nasopharyngeal carriage and colonization inhibition of the Neisseria genus have been

published. Internationally comprehensive research must be performed in order to find out the genuine, precise and reliable data to reduce the waste of investment and resources, in order to achieved efficient method of control and preventing of the Neisseria disease.

CONCLUSION

Determination of the healthy carriage rate and screening of *N. meningitidis* with concomitant colonization of the *N. lactamica* in oropharynx may be crucial for design of meningococcal disease prevention methods.

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