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RESEARCH ARTICLE

SEROTYPES STABILITY OF *Streptococcus pneumoniae* BEFORE AND AFTER LYOPHILIZATION

[1]Ataee Ramezan Ali, [2]Mehrabi Tavana Ali [1]Hossain Najdegerami Esmaiel and [3]Hossaini Said Mohammad, M. D.

[1]Department of Medical Microbiology, Faculty of Medicine, Baqiyatallah (a. s.) University of Medical Sciences, Tehran, I. R of Iran
[2]Department of Medical Microbiology, Faculty of Medicine and Health Management Research Center Baqiyatallah University of Medical Sciences, Tehran, I. R of Iran
[3]Molecular Biology Research Center, Baqiyatallah (a. s.) University of Medical Sciences, Tehran, I. R. of Iran

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ABSTRACT

Introduction: *Streptococcus pneumoniae* is a well known Gram positive diplococci. This bacterium has the second most important pathogen in cases of meningitis in children under 2 years and elderly too. Currently more than 90 pneumococcal serotypes have been identified based on their antigenic differences in the capsular polysaccharides. Because the factors affecting serotype variation are not well defined. The aim of this study was to determine the difference between in the types of *Streptococcus pneumoniae* before and after lyophilization.

Materials and methods: In this experimental study 50 clinical isolates of *Streptococcus pneumoniae* were collected and reidentified. Quelling reaction test as one of the serotyping methods were carried out (based on SSI protocol- Statens Serum Institute protocol).The results of the first step was recorded and then samples were lyophilized. In the second step the lyophilized samples were been reserotyped again and the results was compared.

Results: The results of this study showed that there is no difference was occurred between the serotypes before and after lyophilization. Therefore lyophilization of *Streptococcus pneumoniae* has not affect on serotype diversity. In addition, we found that the isolates were belonging to serotypes of 7, 2, 5, 1, 6, 8, 4, 20, 3, 17, 19, 14, 22 and 10. Only one isolate was nonserotypable.

Discussion and Conclusion: The finding of this study indicated that lyophilization use for maintenance samples can not change the types of *Streptococcus pneumoniae* isolates. If we haven't any preparation for serotyping of isolates, we can use lyophilization as a way for conserve of our samples. On the other hand, when the equipment and material are not available in some case condition, we would perform lyophilize the clinical isolate and do serotyping later. In this research, serotyping has been done based on immunologic reaction but for accurate recognition due to the effects of lyophilization on serotypes diversity, further study is necessary to determine existence or inexistence of eventually mutation.

INTRODUCTION

The bacterium, *Streptococcus pneumoniae* is responsible for a wide spectrum of serious diseases such as pneumonia, meningitis and bacteraemia. *S pneumoniae* can be classified into 90 serotypes based on capsular polysaccharide antigens. At this time, there is not known evidence of which factors cause serotypes produce or serotype diversity. It is show that, Genetic analysis of diverse disease-causing *S pneumoniae* indicates high levels of diversity within serotypes and capsule switching (Jefferies *et al*., 2004). Different bacteriological methods for *Streptococcus pneumonia* maintenance were reported (Wasas *et al*., 1998 and Losito *et al*., 1982). For some bacteria, freeze-drying or lyophilization treatment is one of the treatments used most commonly, allowing long-term maintenance and easy distribution (Billi *et al*., 2000). On the other hand, many freeze-dried bacterial strains have been deposited with the International bacterial culture collection, and their survival rates have been tested periodically for up to 20 years. It is probably deposited strains are of great taxonomic variety (Miyamoto-Shinohara *et al*., 2008) or minor changes in their characteristics. Some investigators thought that, to improve the survival rates during storage of these species when freeze-dried, future studies should examine which may be culture conditions suppress the production of polysaccharides and teichoic acids. However, its efficiency remains, huge diversity of cells and failures in regrowing the strains after treatment were not investigated in details (Morgan *et al*., 2006) and there is not evidence whether or not changes occurred in serotypes of *Streptococcus pneumoniae*. The aim of this study was to determine the difference between the types of *Streptococcus pneumoniae* before and after lyophilization.

Corresponding author: ataee@bmsu.ac.ir

MATERIALS AND METHODS

Material

1) Bacterial strains

Bacterial strains in this research were obtained from the hospitals of Tehran. However, *Streptococcus pneumoniae* is a fastidious bacteria the isolation, maintenance and cultivation was carried out properly

2) Mediums

For this research three medium (Blood Agar, Chocolate Agar and Thioglycolate Broth) were used:

3) Material

A) Antiserums

Antiserums by using in this study were polyclonal antibody. These antibodies were purchased from Statens Serum Institute (SSI) in Copenhagen in Denmark.

B) Phosphate Buffered Salts (PBS)

In this research we use of ready tablet PBS that was prepared before. Every tablet solved in 100 ml distilled water and use for experiments. In addition, Gram stain and biochemical tests were conducted.

pneumococcal infections and send them to central laboratory in Baqiyatallah University of Medical Sciences for further study and their characterization. All of the isolated *S. pneumoniae* strains were re-confirmed by conventional method. The samples identifeid were conducted for Quelling test as one of the serotyping methods based on Statens Serum Institut (SSI) protocol. The results was recorded. For long time preservation (Rudge R H., 1991), all isolates was send to lyophilization in Persian Type Culture Collection (PTCC). The results was recoreded and then the isolates were recoverd from and re- serotype was carried out by Quelling test method again and results were compared. The isolates were re lyophilized and stored in our laboratory.

Table 1. Frequence distribution of *Streptococcus pneumoniae* by site of infection and gender

	Num/Percent	male	female	Unknown
Respiratory infection	(16)32	9	6	1
Eye infection	(10)20	2	8	
Blood infection	(11)22	7	2	2
Trauma	(7)14	2	3	2
CSF	(6)12	4	2	

The outcome serotyping of the isolated strain before and after lyophilization have showed in Table 2.

Table 2. *Stereptococcu pneumoniae* serotype distribution before and after lyophilization

Original of isolation	Internal code	Reaction with antisera.	Serotyp identify before lyophelization	Serotyp identify afterlyophelization
Respiratory system	2	B + S	8	8
Respiratory system	106	B+Q	6	6
Respiratory system	109	A+T	2	2
Respiratory system	108	C+P	7	7
Respiratory system	3	C+P	7	7
Respiratory system	5	A+T	2	2
Respiratory system	6	C+P	7	7
Respiratory system	4	B+Q	6	6
Respiratory system	778	A+P	1	1
Respiratory system	123	G	29-34-35….	29-34-35….
Respiratory system	124	A+P	1	1
Respiratory system	125	B+S	8	8
Blood infection	102	A+R	4	4
Blood infection	103	A+R	4	4
Blood infection	115	A+T	2	2
Blood infection	116	B+Q	6	6
Blood infection	118	B+R	3	3
Blood infection	119	A+S	5	5
Blood infection	120	A+P	1	1
Blood infection	121	F+S	17	17
Eye infection	122	F+T	22	22
Eye infection	107	B+Q	6	6
Eye infection	7	H+P	14	14
Eye infection	1	B+P	19	19
Eye infection	101	B+Q	6	6
Trauma	777	Unkown	Unkown	Unkown
Trauma	104	B+Q	6	6
Trauma	105	A+S	5	5
Trauma	117	E+S	10	10
CFS	111	C+T	20	20
Blood infection	128	B+R	3	3
Blood infection	129	A+T	2	2
Respiratory system	130	C+T	20	20
Blood infection	131	G	29-34-35….	29-34-35….
Blood infection	133	A+Q	18	18

Methods

Collection and Serotyping

Base on Microbiological methods a protocol for data collection was sent to all hospitals, where patients with isolates of S. pneumoniae from respiratory, blood and ocular samples. From January 2007 to March 2010, 50 strain of S. pneumoniae were isolated from patients in different hospitals which contributed in this study. Coworkers in each hospital are asked to identify all isolates responsible for invasive

Lyophilization

Preparation of bacteria culture

The isolated strain were cultured in slant tube contain blood agar and were incubated in Candel jar 37°C with %5 CO_2 for 24h.

Preparation of bacterial suspension

Eeach tubes were added skim milk 2 ml and then was washed with pipette pasture slowly. In this step it is necessary that, suspension

prepared homogen solution from cells. The number of bacteria in suspension were approximately 1×10^7 CFU/ml.

Dispensing in ampoules

The bacterial suspension previously prepared were transmitted in tubes in a volum of 0.2 ml and then for prevention of contamination, they were fixed with a cotton plug in tube whereas that 2 Centimetere of top of over suspension. After that cotton plug was posited over of tube. Then label of contain of information of bacteria and plasmid was sticked under the first cotton plug. The prepared ampoules were transmitted in to the -70°C refrigerator for pre-freezing process for three hours.

Dehumidify of ampoules

The prepared ampoules were evicted from freezer and were transmitted to freez- drier (B1-16ChristCo., Germany). Then ampoules were posed in vacuum apparatus (0.05 mb) for 18h. The ampoules were emitted and then attach to manifold again were return to vacuum in end of 18 hours. Finally the ampoules were barred by using torch later 2 hours and were disconnected from vacuum apparatus.

RESULTS

The results of bacteriological studies show that in a period, from January 2007 to March 2010, 50 culture positive of *S. pneumoniae* were identified. These isolated strain obtain from patient wich have different infections and conferred to Tehran hospitals were serotyped. The feriquency of area infection and genus of pateints were show in Table 1.

DISCUSSION

Many works has been done for serotyping of *S.pneumoniae* in the past two decades in different countries (Ergin *et al*., 2009, Xu *et al*., 2009 and Lynch *et al*., 2009). In addition in recent years many works has been focused on molecular typing too (Calatayud *et al*., 2007). In Iran the serotyping of *S.pneumoniae* has not been investigated before. Therefore this study has been designed and the results serotyping have been compared before and after lyophilization. Finding results showed that there is no difference between serotyping of *S.pneumoniae* before and after lyophilization. There is also no published similar works to compare the findings. However, S. *pneumoniae* has been extensively studied. Much remains to be learned about the serotype distribution, pathogenesis, and epidemiology and antibiotic resistant of pneumococcal disease and serotype diversity. In this area we are asking first why currently more than 90 serotypes are recognized (InHo *et al*., 2007 and Slotved *et al*., 2004). Secondly, what is the effect of long-term maintenance on S. *pneumoniae* capsular diversity? Finally, Freeze-drying is one of the most common methods used to store microbial culture collections and also, freeze-drying is applicable to many bacteria (Shinohara *et al*., 2008), it cannot be used with some bacteria. Thus which freeze-drying methodologies have been suitable for *S. pneumonia*? To clarify some question. This study was conducted. In this research, we isolated 50 strain of Streptococcus pneumoniae from patient sample. In our laboratory, the collected isolates were reidentified again. Then, quelling reaction test as one of the serotyping methods were carried out (based on SSI protocol- Statens Serum Institute protocal).The results of the first step was recorded and then samples were lyophilized. In the second step the lyophilized samples were been reserotyped again and the results was compared.

The finding of this study indicated that lyophilization can not effect on the types of *Streptococcus pneumoniae* isolates capsule polysaccharide. Of course, we do not really know is it remains constant for very long times? If the answer be yes, we have said for how long? The more research must be done to find the answer exactly in the future.

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Conflict of Interests

Authors have no conflict of interests.

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