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Research Article

Phenotypic and Genotypic Differences in Invasive and Non-Invasive Strains of *Streptococcus pneumoniae*

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ABSTRACT

Streptococcus pneumoniae (*S. pneumoniae*) is a human pathogen and a major cause of mortality and morbidity throughout the world. *Streptococcus pneumoniae* is the causative agent of otitis media, pneumonia, meningitis and bacteremia. Children, elderly and patient with compromised immune system are at a higher risk to attain infection by these bacteria. According to immunochemistry of their capsular polysaccharide, *S. pneumoniae* has divided into more than 90 serotypes. Many surface proteins are present on *S. pneumoniae* e.g. surface protein A, pneumolycin, hyaluronate lyase etc. The capsule is a major virulent factor for pneumococci and it prevents the bacteria from phagocytosis by host's immune system. Some antigenic determinants have also studied for their potential to prompt a protective immune response in different clinical trials and experiments. The population biology of *S. pneumoniae* has poorly understood. Most of the problems arise by the molecular characterization, well-sampled population from carriage and from various discloser pneumococcal diseases. To address this, a multilocus sequence typing scheme and database by sequencing-450 bp fragments of seven housekeeping loci from 295 isolates were developed. The allelic combination of seven loci gives a sequence type or allelic profile. This typing scheme was validated using pneumococci of known genetic relatedness and could resolves >6 billion sequence type. The multilocus sequence-typing scheme allocates a powerful new approach to the characterization of pneumococci, since it delivers molecular typing data that are electronically portable between laboratories, and which may use to probe aspects of the population and evolutionary biology of these organisms. In this project, we compared two different strains of *S.pneumoniae* to check which one is growing rapidly and observed the difference between invasive and non-invasive strains based on phenotype and genotype.

Keywords: *Streptococcus pneumonia*; Bacteria; Pneumococci; Phagocytosis

INTRODUCTION

Streptococcus pneumonia (*S. pneumonia*) is gram positive, alpha or beta-hemolytic facultative anaerobic bacteria belonging to group streptococcus [1]. In 1881, a physician George Stenberg and a French scientist Louis Pasteur for its role as a causative agent of pneumonia first isolated the organism [2,3]. Later in 1886, it was known as pneumococcus [4].

From 1920, the organism was known as *Diplococcus pneumoniae*. In 1974, it was renamed as *S. pneumoniae* [5]. Depending on the strain, the genome of the genus Streptococci is closed circular DNA structure that consists of 2.0 to 2.1 million base pairs [6]. In its genome, 1553 genes are present as a core set, 154 genes in its virulome that contribute in virulence and 176 genes participate in non-invasive phenotype [6].

Transformation in *S. pneumoniae* is responsible for capsular serotype. Through the surrounding medium; the natural bacterial transformation involves the transfer of DNA from one bacterium to another. Transformation is a complex developmental process requiring energy and it is dependent on numerous genes [7].

At least, 23 genes are required for transformation in *S. pneumoniae*. In order for a bacterium to bind, take up and recombine exogenous DNA into its genome, it must enter a special physiological state called Competence [7].

DNA damaging agents such as mitomycin C, fluoroquinolone antibiotics such as norfloxacin, levofloxacin, moxifloxacin and topoisomerase induce competence in *S. pneumonia* [8].

S.pneumoniae is the member of normal microflora and an opportunistic organism but it becomes pathogenic when it acquires

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possible conditions like weak immune system. It enter the body *via* coughing or sneezing and becomes pathogenic when the immune system of the host becomes compromised.

S. pneumoniae is a major cause of morbidity and mortality worldwide [9]. The invasive pneumococcal diseases include bronchitis, meningitis, otitis media, pneumonia, arthritis, endocarditis, cellulitis and brain abscess. The major virulent factors are pneumolysin, capsule, various adhesions and immunogenic cell wall components. The body responds the inflammatory responses after pneumococcal colonization in alveoli. These inflammatory responses fill the alveoli with plasma, blood and white blood cells and this condition known as pneumonia.

S. pneumoniae has more than 90 serotype based on immunochemistry of capsular polysaccharide. Out of these 90 serotypes, 16 serotypes are responsible for causing 90% of the invasive disease [9,10].

Colony forming unit (CFU) is a unit to count viable cells in a sample. The basic purpose of plate count method is to estimate the number of cells present based on the ability to give rise new colonies. These cells give rise to new colonies under specific condition like nutrient requirement, temperature time etc. Therefore, many bacteria grow in the form of chains and clumps. The one advantage of this method is that different microbial species may give rise to new colonies that are very different from each other microscopically as well as macroscopically [11,12].

Serotyping is a universal method used to characterize serotypes obtains within the whole population. So serotyping reveals all the *S.pneumoniae* strain that variate based on genome. MLST scheme and database for *S. pneumoniae* use the method to identify major clones associated with serious invasive pneumococcal disease [13]. Due to the large number of pneumococcal serotypes, the need to study isolates from carriage (which are the bulk of the population, since carriage is common but disease is rare), and the different types of disease, as well as antibiotic-susceptible and resistant isolates, an adequate understanding of the population requires the analysis of large numbers of isolates [14]. With many molecular typing methods, the results obtained in different studies are difficult to combine. This limitation has been removed *via* development of a portable high-resolution molecular typing procedure, multilocus sequence typing, which is based on the principles of multilocus enzyme electrophoresis but characterizes the alleles present at multiple housekeeping genes directly by nucleotide sequencing, rather than indirectly from the electrophoretic motilities of their gene product [15,16]. To run the PCR of different strains of PCR with seven different housekeeping genes were used. These genes are actually constitutive genes that are required for maintenance of basic cellular functions, and express in all cells of an organism under normal and patho-physiological conditions.

The main objective of this study was to determine the growth difference of invasive and non-invasive strains of *S.pneumoniae*, genotypic and phenotypic effects as well [17-23].

MATERIALS AND METHODS

Preparation of Tod-Hewitt Broth (THY) and 1X PBS

To prepare THY, 3 g of Tod Hewitt broth and 0.5 g of Yeast extract was dissolved in 100 ml of distilled water. Then, it was autoclaved. The total volume of the solution was 500 ml. 50 ml of stock PBS 10X was added to 450 ml of distilled water followed by autoclaving.

The D39 strain of *S. pneumoniae* and TIGR4 were took from stock that was preserved at -80°C. In hood, UV light was turned on for few minutes. This was followed by transferring 10 ml of THY to two falcon tube. To one tube, with the micropipette 1 ul of bacterial culture of D39 was added. The other tube was used as negative control containing THY broth without bacterial culture. After this, the initial was taken. Then, tubes were incubated for 1 hour at 37°C. After 1 hour, first OD was taken. Then tubes were again incubated until OD reached up to 0.4.

CFU counting

180 µl of PBS was added in 8 wells of the first column. Then, 20 µl of the bacterial suspension from the same tube was taken, and mixed in first well of the column. The tip was discarded. Then, 20 µl of suspension was transferred from 1st well to 2nd. Then, from 2nd well 20 µl of the suspension was added into 3rd well and so on up to 8th well. Lastly, 20 µl from 8th well was discarded. Then, 10 µl suspension from each well was spotted on blood agar plate. After every hour, OD was checked and the blood agar plates were spotted with 8 dilution of bacterial suspension and PBS. PBS was use to maintains a constant pH, Then, plates were incubated at 37°C overnight.

DNA extraction

For MLST analysis, 20 strains of *S. pneumoniae* were streaked onto blood agar plate and incubated in a candle jar at 37°C overnight. Then, 10 ml of THY broth in falcon tubes were inoculated and placed in incubator for their optimal growth. Then tubes were spun at 3000 rpm for 15 minutes. Supernatant was discarded. Pellet was washed with 350 µl TE buffer and freeze overnight. Then, the pellet was resuspended in 2 µl of lysozyme and 40 µl of 10% SDS. After this, it was mixed and incubated at 65°C for 15 minutes. After incubation, 70.7 µl of 5 M Potassium acetate was added in it and again incubated at 65°C. After that, tubes were placed in freezer at -20°C for 20 minutes. Then pellet was spun at 12000 rpm at 4°C for 10 minutes. Then supernatant was transferred to Eppendorf's and pellet was discarded.

Then, 707 µl of 95% cold ethanol was added to the supernatant. Eppendorf's containing supernatant were tightly closed and inverted gently. Again, tubes were spun at 12000 rpm for 2 minutes and placed at -20°C for 10 minutes. Then, 707 µl of 70% cold ethanol was added and again spun for 2 minutes at 12000 rpm. Then pallet was placed for drying for 2-3 hours. Then, pellet was resuspended in 100 µl of RNase free water and dissolved completely and place at -20°C.

PCR

The isolated DNA of pneumococcal strains were taken from stock and used for PCR. The master stock was prepared for PCR reaction. Seven Housekeeping genes were amplified including Ddl, gki, recp, xpt, gdh, spi, aroE were used as primers.

RESULTS AND DISCUSSION

When strain D39 and TIGR4 were grown under same conditions, both strains showed different growth curves. The growth curve was obtained under same conditions like time and temperature. D39 strain showed slowly growth as compared to TIGR4 after 6 hours. The CFU of both strains were also bounced with each other. The following results are showing *via* graphs (Figure 1).

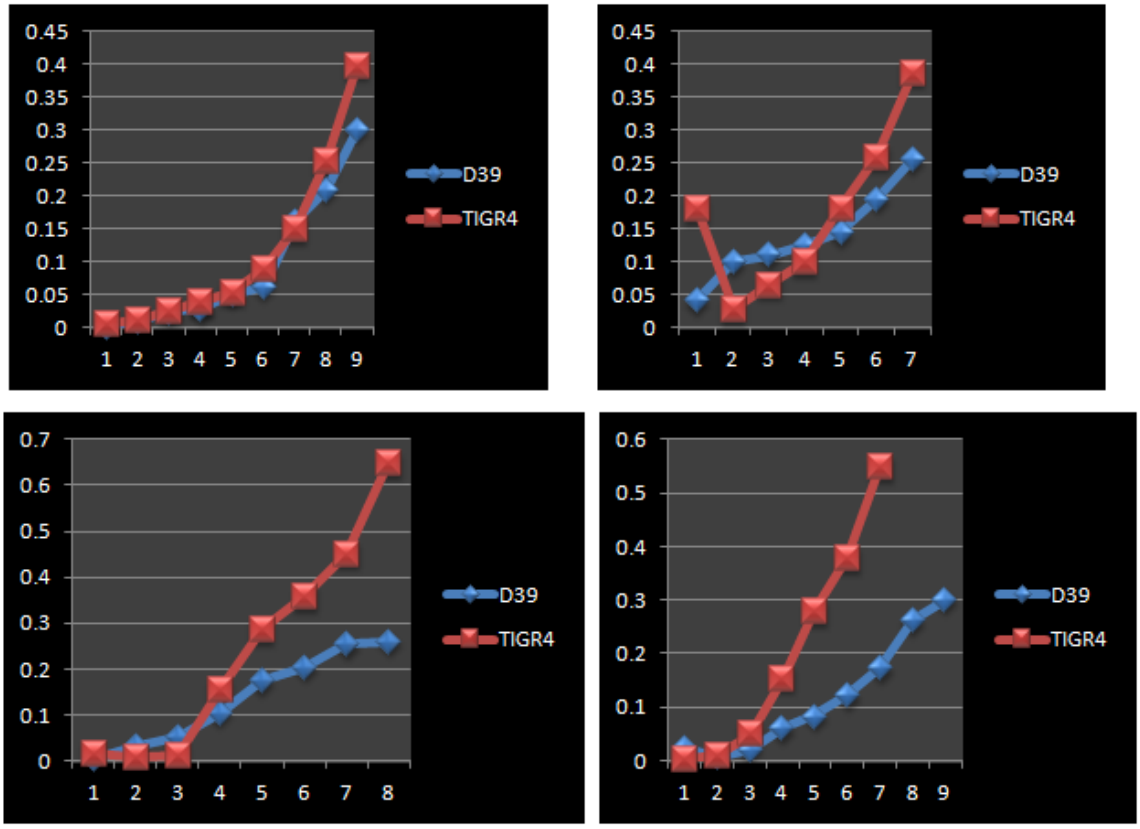

Figure 1: All figures are showing the growth difference of both strains under same conditions.

Serotyping

The 20 strains of *Streptococcus pneumoniae* were taken to determine that which antigen that strains persist. The chart below illustrate the following results (Table 1).

Table 1: Different types of strains showing its activity.

Strains	Pool sera A	Pool sera B	Pool sera O	Pool sera H
2019	-	-	+	-
196	-	-	-	-
D39	+	-	-	-
64704	-	-	-	+
5303-03	-	+	-	-
2825-06	-	-	-	+
56805-04	-	-	-	+
505	-	+	-	-
3110-06	-	-	-	-
5502-05	-	-	-	-
63805-3	-	+	-	-
63003-01	-	+	-	-
30003-05	-	+	-	-
46603	-	+	-	-
2107	-	-	-	-
13008-07	-	-	-	-
2115-05	-	+	-	-
46015	-	+	+	+
30307	-	-	-	-
0938	-	-	-	+

Strains	DNA conc.	260/280	230/260
2019	1472.8	2.10	2.36
196	72.6	1.05	1.21
D39	202.4	2.10	1.99
64704	179.3	2.07	2.07
5303-03	322.4	2.07	1.97
2825-06	21.8	2.01	1.34
56805-04	131.2	2.05	1.81
505	57.9	2.09	2.04
3110-06	58.6	1.17	1.41
5502-05	145.7	2.14	2.38
63805-3	210.7	2.11	2.11
63003-01	182.4	2.08	2.32
30003-05	27.8	1.98	1.99
46603	97.3	2.06	2.07
2107	155.2	2.01	1.76
13008-07	1158	2.05	1.84
2115-05	1017	2.11	2.27
46015	433.0	2.02	2.05
30307	1342	2.14	2.06
0938	244.1	2.09	2.17

PCR results

This shows the results of PCR (Figure 2).

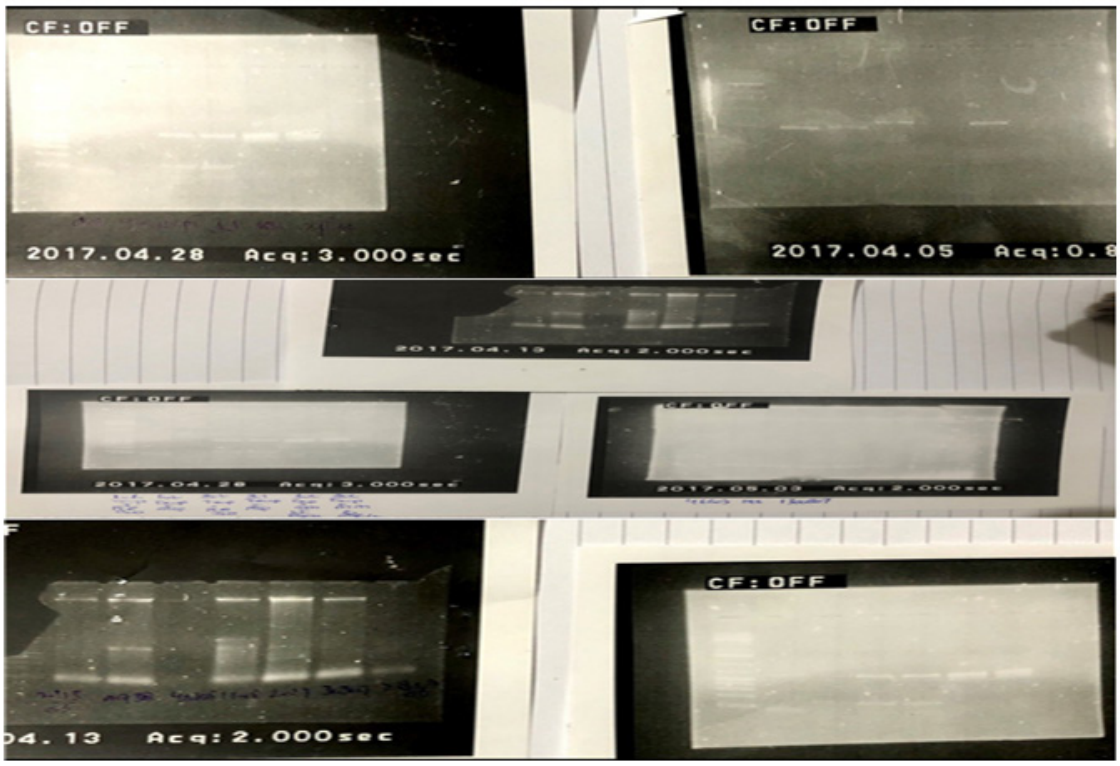

Figure 2: Different dates showing different acquisitions.

CONCLUSION

This study illustrates that D39 strains is a lab strain and it is non-virulent strains while TIGR4 is a virulent strain as well as it invasive strain. TIGR4 growths rate is much higher than D39 that's why this strain penetrates deeply in respiratory system and multiply rapidly there and cause severe illness. Serotyping of 20 strains showed different antigen persistence. All the strains have different DNA concentrations.

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