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Differential Adherence Capacities of Clinical Isolates of *Streptococcus pneumoniae* at Different Growth States to Human Respiratory Epithelial Cells (*in vitro*)

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Abstract: This study was conducted to evaluate the adherence and invasion capacity of four clinically important pneumococcal serotypes at early-, mid- and late-log phases at 1 and 3 h incubation times to A549 cells-a human respiratory epithelial cell. We observed that adherence capacity varied among the different isolates at all growth phases ($p \leq 0.05$) but generally, an individual isolate had a comparable adherence capacity at all levels of the log phase ($p > 0.05$). Bacterial adherence also increased after a longer exposure to the host cells but significant differences were mostly observed at only early- and late-log phases at both incubation times. No bacterial invasion was however observed at 1 h post incubation but that at 3 h, a few bacteria penetrated the host cells but the bacterial cfu numbers were statistically insufficient for analysis. More studies are required to further confirm these observations.

Key words: *Streptococcus pneumoniae*, epithelial cell, adherence, invasion, exponential growth phase

INTRODUCTION

Streptococcus pneumoniae (pneumococci) is a human lung pathogen, which lives in the respiratory tract as part of the normal microbial flora. Pneumococcal adherence to the lung epithelial cells has been long demonstrated in *vivo* and *in vitro* and is considered to be the first stage before successful colonization and invasion (Cundell *et al.*, 1995; Guilmi and Dessen, 2002; Weiser *et al.*, 1996). At the colonizing sites, pneumococci may proliferate without any apparent symptoms and enjoy a commensal relationship with the host. Only after penetrating and extending the area of infection, predominantly the respiratory lining cells, do they have potential to cause diseases (Catterall, 1999). In the progressive infection, the consequence is the pathological complications due to the host inflammatory response to the bacterial extra- and intra-cellular components such as pneumolysin (Ply), an intra-cytoplasmic toxin, which are released upon cell wall lysis (Gillespie and Balakrishnan, 2000).

The higher degree of pneumococcal adherence to human epithelial cells could be correlated with an increased risk of pneumococcal invasion leading to diseases (Adamou *et al.*, 1998). Nevertheless, such ability had been shown in many studies to vary between different strains and no correlation has

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yet to be established in relation to serotypes, antibiotic susceptibilities, sources of origin and virulence capacities of pneumococci (Robson *et al.*, 2006). The differences in the adherence ability could be due to the fact that the adherence-associated determinants at the genetic level known to be present in various pneumococcal isolates may not be equally expressed to confer the same capability for all pneumococci (LeMessurier *et al.*, 2006; Ogunniyi *et al.*, 2002). In addition, such ability may also depend on other less specific factors such as the host condition itself, the viscosity of pneumococcal capsule and the respiratory mucus, bacterial cell surface charge and hydrophobicity and sustainability of bacterial growth at the infection sites that may facilitate bacterial binding affinity to the host cells (Rayner *et al.*, 1995; Swiatlo *et al.*, 2002). Considering all the possible different mechanisms, the adherence process must be complex and multifactorial. Nevertheless, for a typical growth pattern of a bacterium, it may not remain vigorous all the times throughout its life cycle in particular the pneumococcus; it will replicate at the early growth stage and eventually undergo autolysis. Its survivability will then be maintained by its progeny at the infection site. A question arises whether all the mentioned factors in modulating the adherence are equally involved at the different stages of the pneumococcal growth during interaction with the host cells; e.g., from lag to stationary phases. A good model of experiment need to be developed to investigate this matter but in this study, we undertook a preliminary effort to evaluate the adherence and invasion capacity of clinically important pneumococcal serotypes at different levels during the bacterial exponential growth; early-, mid- and late-log phases, to human respiratory epithelial cells.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Four clinical isolates of different serotypes obtained at the University of Malaya Medical Centre from March 1999 to July 2000 were used. Genetic relatedness of the isolates was determined by PFGE in 2001 and shown to be different (Desa *et al.*, 2003). All isolates also harboured the adherence-associated genes; cbpA, cbpG and psaA, as well as ply, as confirmed by PCR and sequencing (Desa *et al.*, 2007). Data on penicillin susceptibility, site of origin and serotype of the isolates are shown in Table 1. Overnight pneumococcal culture on blood agar was suspended in Todd Hewitt Broth (THB) (Oxoid, Hampshire, England) with 0.5% yeast extract (Pronadisa, Madrid, Spain). Bacterial growth in the broth media at 37°C with CO_2 was monitored by OD measurement at 600 nm for 10 h period. Graph of growth curve was plotted to determine the time points for the early-, mid- and late-log phases, respectively. Based on the graph, bacterial suspensions at the different growth phases were harvested in the broth media by centrifugation and kept at -70°C until further use.

Infection of Epithelial Cell Line and Viable Count of the Bacterial Adherence and Invasion

Bacterial pellets were thawed at room temperature and re-suspended in RPMI 1640 (FlowLab, Sydney, Australia) supplemented with 2% Fetal Bovine Serum (FBS) (JRH, Lenexa, Kansas) to give a suspension of $\sim 1 \times 10^8$ cfu mL^{-1} (Johnston *et al.*, 2004). A549, a human pneumocyte II cell line of lung epithelial cells, was grown in RPMI 1640 with 10% FBS in 25 cm^2 Tissue Culture (TC) flask at 37°C in 5% CO_2. A549 cells were grown to confluency ($\sim 4 \times 10^6$), washed and infected with 2 mL bacterial suspension ($\sim 2 \times 10^8$ cfu) in the cell culture media for 1 and 3 h to allow for adherence at a bacterial/epithelial cell ratio of approximately 50:1 for all the isolates. For viable count, infected A549 cell monolayers were washed 3 X with PBS (Oxoid, Hampshire, England) to remove non-adherent bacteria, detached and lysed with trypsin/EDTA (FlowLab) containing 0.025% Triton X-100 (BDH, Poole, England). The total number of adherent bacteria (cfu/flask) was determined by serial dilution of the A549 cell lysates in PBS followed by standard spread plate technique on blood agar. For invasion assay, infected A549 cells were washed and incubated in cell culture media supplemented

with benzyl penicillin (10 µg mL^{-1}) and gentamicin (200 µg mL^{-1}) for 1 h prior to the cell lysis to kill the extracellular bacteria. As a control, the viability of cell culture throughout the experiments was verified by trypan blue dye exclusion to be ≥95% of the uninfected A549 cells grown in media alone and with the presence of the antibiotics. Viable counts of bacterial suspension incubated with the antibiotics were also determined to ensure that such treatment was sufficient to kill all the bacteria. All experiments were done in triplicate and results were averaged.

Statistical Analysis

Standard deviations and graphs were plotted using Microsoft Excel. SPSS 11 of Mann Whitney's test for comparison of two groups and Kruskal-Wallis's test for comparison of more than two groups was utilized for statistical analyses with a p-value of ≤0.05 was denoted as statistically significant.

RESULTS

Growth Curve

Growth curve analyses showed a similar pattern of a typical bacterial growth for all the isolates (Graph not shown). Variations were observed at the lag phase with isolates serotype 19 and 23F growing faster than isolates serotype 1 and 7F (p = 0.035 and 0.029 at 1 and 2 h time points, respectively), but growth of all isolates steadily increased at 3 h onward (p>0.05 at ≥ 3 h time points) and reached linearity after 7 h post incubation. Based on the growth patterns, 3, 5 and 7 h were chosen for incubation times to harvest bacterial growth at early-, mid- and late-log phases respectively for all the isolates.

Adherence Analysis

With respect to the early-, mid- and late-log phases for the respective isolates, the number of adherent bacteria varied with isolate serotype 1 reaching significant levels at both incubation times (Table 1). The differences for other isolates were not significant but that for isolate serotype 23F was approaching significance at 1 h post incubation (p = 0.051). At 3 h post incubation, the adherence number increased by up to 10 fold for all the isolates but mostly at early- and late-log phases; based on the p-values, the significant differences were observed for isolate serotype 1 at all phases of growth whereas other isolates at either early- or late-log phases or both. When comparing among the different isolates, significant differences were observed at all conditions except at the mid-log phase of 3 h post incubation (p = 0.206) (Table 2).

Invasion and Control Experiment

In the invasion assay, using the viable count method, none of the four isolates invaded the host cells at 1 h post incubation whereas very low amounts were noted intracellularly at 3 h at late-log phase (0-1×10^2 bacterial cfu/flask). Further analysis was not done as the bacterial cfu numbers

Table 1: Summary on the identity of isolates and statistical analyses on differences in the number of bacterial adherence at 1) different growth phases and at 2) different incubation times, of the individual isolates

Serotype	Site of isolation	[a]Penicillin susceptibility	1) p-value for difference at early-, mid- and late-log phases at the respective incubation time		2) p-value for difference at 1 and 3 h post incubation at the respective growth phase		
			1 h	3 h	Early-	Mid-	Late-
1	Tracheal	S	0.050	0.044	0.046	0.050	0.050
7F	Pleural fluid	S	0.059	0.059	0.275	0.507	0.046
19F	Sputum	R	0.066	0.082	0.050	0.513	0.050
23F	Blood	R	0.051	0.118	0.050	0.077	0.077

[a]R = resistant; S = susceptible, Significant p-values were in bold

Table 2: No. of bacterial adherence (Log_{10}) and statistical analyses of all isolates at early-, mid- and late-log phases at the respective incubation time (1 and 3 h)

Serotype	1 h			3 h		
	Early-	Mid-	Late-	Early-	Mid-	Late-
1	3.2±0.3	5.1±0.4	5.4±0.3	4.2±0.4	6.0±0.1	5.9±0.1
7F	5.1±0.5	5.6±0.1	6.0±0.1	5.5±0.3	5.8±1.1	7.7±0.2
19F	5.8±0.1	4.5±0.3	4.7±0.7	6.6±0.8	5.2±1.0	5.8±0.1
23F	2.7±0.4	3.9±0.1	3.1±0.5	3.8±0.2	4.5±0.5	3.9±0.3
p-value	0.015	0.018	0.019	0.022	0.206	0.018

Significant p-values were in bold, The numbers of bacterial adherence (cfu/flask) were reported in Log_{10}

were statistically insufficient (<30 cfu on plates). Throughout the experiments, the uninoculated A549 cells maintained the integrity during both incubation times either alone or with presence of the antibiotics in the culture media as determined by trypan blue exclusion. Viable count of the bacterial suspension pre-incubated with the antibiotics showed no growth upon plating suggesting that the exposed bacterial cells on the A549 cell surface would be killed. Plating of the infected A549 cells pre-treated with the antibiotics would allow growth of only the invading bacteria that survived from the antibiotic effect by being inside the host cells. Thus, the poor pneumococcal penetration into the epithelial cells would strongly suggest that the quantitative measurement by viable count of the infected A549 cell lysates in this study represented mostly the adherent bacteria.

DISCUSSION

In this study, we selected four non-clonal clinical isolates harbouring the vaccine-serotypes that were commonly detected from pneumococcal-associated diseases. These isolates also carried the important genes previously shown to be associated with adherence and exhibited the same growth pattern with slight inconsistency at the lag phase but a comparable level of the stationary phase. For the two growth phases mentioned, differences have been reported among different pneumococcal serotypes even by growing in the same standard growth media for pneumococci (Battig *et al.*, 2006; Mazzola *et al.*, 2003). Due to a wide genetic diversity in different pneumococcal strains (Hava *et al.*, 2003), it could possibly be difficult to obtain an identical growth pattern by different isolates even within the same serotypes. Thus, by using organisms with a comparable fitness of growth in this study, the organisms were assumed to a certain extent, to be capable of showing some levels of similarity in the adherence capacity provided that their adherence properties were to be functioning equally under the standardized experimental conditions.

Frequently, bacterial growth at mid-log phase was used for adherence assay in many studies as it was expected to be in the optimum stage for adherence process to take place (Adamou *et al.*, 1998; Talbot *et al.*, 1996). In this study, we extended to look at adherence capacity of clinical isolates at different stages during the bacterial exponential growth; early-, mid- and late-log phases. The rational for this is that there could be some variations during the early stage of the exponential growth whereby the necessary metabolic reactions are initiated to commence growth or any virulence determinants with varying rate, as well as near the stationary phase at which cell lysis begins to take place to release cellular components that could indirectly interfere with the adherence ability. For simplicity, adherence experiments in this study were standardized by infecting A549 monolayer in 25 cm^2 TC flask with bacterial inoculum size of ~10^8 cfu mL^{-1} as the number of bacteria and bacterial adherence at this condition was not too numerous for viable count and at the same time provided an ease of handling while maintaining the host cell viability as shown by the control experiment. Under these experimental conditions, we were able to assay the adherence capacity by all isolates but with very low bacterial invasion. Differences in the number of adherence at different growth stages for individual isolates were mostly not significant suggesting that during the exponential growth, adherence capacities were about

the same regardless of the phases of growth. But this may not be true for all isolates, as we have seen in this study isolate serotype 1 that showed significant differences at all the experimental conditions. Nevertheless, significant differences in the number of adherence were observed among the different isolates at almost all the experimental conditions suggesting that different isolates exhibited different adherence capacities regardless of the growth stage of the bacteria and the different incubation times. At a longer incubation period, number of bacteria that adhered increased but significant differences were observed only at certain growth levels; early- and late-log phases suggesting that the bacterial adaptation upon a longer interaction with the host epithelial cells at these two stages could be different as compared to that at mid-log phase. However, bacterial invasion was very poor suggesting that significant invasion would not easily take place regardless of the number of bacterial adherence that preceded it. Such poor penetration of host cells was reasonably expected because, although pneumococci are adapted to live and replicate in the human respiratory tract, they are not known to be intracellular pathogens, to readily invade upon attachment to the host cell surface (Hammerschmidt, 2006). Pneumococcal invasion has been shown elsewhere to be strain- and cell type-specific but was relatively low (Brock *et al.*, 2002; Talbot *et al.*, 1996). Nevertheless, the organisms are pathogenic and may invade when conditions are favorable to result in systemic diseases (AlonsoDeVelasco *et al.*, 1995).

Based on the limited number of isolates, it could be provisionally concluded that adherence ability varied among different isolates but generally, an individual isolate could have a comparable adherence capacity during the course of the exponential phase of growth. Bacterial adherence increased at a longer exposure to the host cells and as higher adherence can be correlated with invasion, such a prolonged episode of the bacterial adherence could cause an infection threat but it may as well depend on growth states of the bacteria. The bacteria were found to poorly invade but a few that were internalized into the human cells could potentially cause severe diseases. Nonetheless, discrepancies in results could be expected when using different amounts of bacterial inocula and host cell's monolayers as cellular interaction at different bacterial and host cell density could be dissimilar to affect the adherence event. On the whole, this study imparts an insight into the prospective diversity among different pneumococcal serotypes and their survivability during exposure to the host cells. More studies are warranted to further corroborate such observations and to establish any pathogenic potential in relation to these matters.

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