

SHORT COMMUNICATION

Pneumococcal replicative state in relation to its adherence capacity to A549-cell line: A preliminary *in vitro* analysis

Mohd Nasir Mohd Desa^{1*}, Navaratnam Parasakthi², Shamala Devi Sekaran³ and Jamunarani Vadivelu³

¹Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

²School of Medicine and Health Sciences, Monash University Malaysia, 46150 Bandar Sunway, Selangor, Malaysia.

³Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia.
E-mail: nasirdes@medic.upm.edu.my

Received 16 June 2010; received in revised form 14 August 2010; accepted 28 August 2010

ABSTRACT

This study was to compare the replication capacity of pneumococcal isolates (serotypes 1, 7F, 19F and 23F) with their adherence pattern to monolayer cells (A549). For standardization purposes, all isolates showed a normal growth curve in both bacteriological (THB + 0.5% yeast extract with and without 2% FBS) and cell culture media (RPMI + 2% FBS). In the former media, a shorter lag phase was observed for isolate serotypes 1 and 7F in presence of serum while in the later; growth yield was lower for all isolates with stationary phase approaching OD₆₀₀ of 0.01 as compared to 1.0 in bacteriological media. In the replicative analysis at different growth phases of the isolates in cell culture media, growth capacity at 3 h post-incubation was frequently twice as that at 1 h, and that at early-log phase was frequently higher than that at mid-log phase at both post-incubation times. Adherence was frequently the least at early-log phase although the isolates were in the most active state of replication to increase the number of pneumococcal cells to adhere. At mid- and late-log phases, pneumococcal adherence was frequently higher although the replication was reduced. This study marks the potential correlation between pneumococcal growth fitness and adherence capacity whereby the later may not be superior during the early growth phase.

Keywords: Pneumococci, adherence, THB, RPMI, Growth phase

INTRODUCTION

Adherence was the first stage of pneumococcal infection (Hammerschmidt *et al.*, 2007). The mechanisms involved are multi-factorial but pneumococcal growth capacity may also have a role as the bacterial multiplication shall determine the accumulation and persistence of pneumococcal cells at the infected sites. As a higher pneumococcal adherence has been shown to be associated with its higher invasion, a higher replication leading to a higher presence of pneumococcal cells available to adhere could also indirectly increase the risk of disease (Adamou *et al.*, 1998; Hammerschmidt *et al.*, 2007; Fernebro *et al.*, 2008). Thus, as a typical bacterial life cycle begins from the early-, mid- and late-exponential growth phases whereby the replicative state of the bacterial cell throughout the cycle may not be similar, a differential adherence capacity may also be expected at the different growth phases of the pneumococcal cell. We have measured the adherence capacity of four clinically important pneumococcal isolates at their different growth phases; early-, mid- and late-log phases, at 1 and 3 h exposure periods to human-lung epithelial cell line (A549) (Desa *et al.*, 2008). This study was to determine the replication rate of the isolates at their different growth

phases as of the adherence assay and to compare with the isolate adherence pattern at the respective growth phases for any potential correlation.

MATERIALS AND METHODS

The four clinical isolates of pneumococci were genetically distinct based on pulsed-field gel electrophoresis (PFGE) profile and exhibited capsular types 1, 7F, 19F and 23F, based on the antigenic variation of the capsular polysaccharide (referred as serotypes 1, 7F, 19F and 23F respectively) (Desa *et al.*, 2003). The former two were from invasive sites and penicillin-susceptible whereas the later two from respiratory sites and penicillin resistant. Adherence capacity of the isolates at their early-, mid- and late-log phases at 1 and 3 h exposure times to A549-cells in cell culture media (RPMI with 2% FBS) has been described (Desa *et al.*, 2008). Growth pattern in Todd Hewitt broth (THB) with 0.5% yeast extract, THB with 0.5% yeast extract and 2% fetal bovine serum (FBS), and RPMI with 2% FBS were determined by monitoring the OD₆₀₀ increments over 10 h incubation period at 37 °C in 5% CO₂ with viable counts at selected growth time points. At similar experimental parameters as of the adherence assay, pneumococcal cell pellets harvested earlier at

*Corresponding author

early-, mid- and late-log phases in THB with 0.5% yeast extract were suspended in RPMI with 2% FBS, mixed well and standardized at OD₆₀₀ of 0.08–0.15. Pneumococcal cells were then incubated at 1 and 3 h in 5% CO₂, and growth was monitored by OD₆₀₀ measurement. Experiments were done in triplicate with Kruskal-Wallis and Mann Whitney tests for statistical analysis. P ≤ 0.050 was denoted as the significant level.

RESULTS

At a standardized size of starting inocula (~6 × 10⁶ CFU/mL), all isolates showed a normal growth curve in all media conditions with lag, exponential and stationary phases; early-, mid- and late-log phases at 3, 5 and 7 h of growth time points respectively, except for isolate serotypes 1 and 7F in THB with serum. For the later, a shorter lag phase (1 to 2 h as opposed to 3 h in THB without serum) was observed with increased growth rate at significant level throughout the exponential growth as compared to their growth in THB without serum. While the effects of serum for isolate serotypes 19F and 23F in THB were negligible. Nevertheless, growth in RPMI with 2% FBS showed no significance difference at all growth time points for all isolates with stationary phase approaching only at OD₆₀₀ of 0.01 as compared to OD₆₀₀ of 1.0 in THB with and without serum.

In the replicative analysis on the four isolates at their early-, mid- and late-log phases at 1 and 3 h post-incubation times, with a starting inoculum size of ~1 × 10⁸ CFU/mL as of the adherence assay, increases of OD₆₀₀ values were observed for both post-incubation periods mainly at early- and mid-log phases. While those at late-log phases were barely observed except for isolate serotype 7F that showed quite an obvious increase. Overall, pneumococcal replication at 3 h incubation period was frequently twice as that at 1 h, and that at the early-log phase was frequently higher than that at the mid-log phase at both post-incubation times. This indicates that the isolates were able to replicate in the cell culture media, as also shown earlier in the growth curve experiment, and the growth capacity increased at a longer incubation period with the highest rate at the early-log phase. Statistical analysis showed that there was no significant difference among the four different isolates at their respective growth phases except at late-log phase at 1 h incubation time (Figure 1a). Nevertheless, in analyzing the individual isolates at their different growth phases, taking growth at early-log phase as baseline for comparison, significant decrease in replication was noted for isolate serotypes 7F and 19F at 1 h incubation and isolate serotypes 1, 19F and 23F at 3 h incubation at mid-log phase, while a very obvious decrease (P ≤ 0.050) in replication in comparison to that at early log phase was observed for all isolates at both incubation times at late-log phase (Figure 1a and 1b).

We have reported earlier the adherence capacity of the four clinical isolates but in relation to the exposure time to A549-cells (1 h vs. 3 h) at different growth phases of the respective isolates. The results showed that each

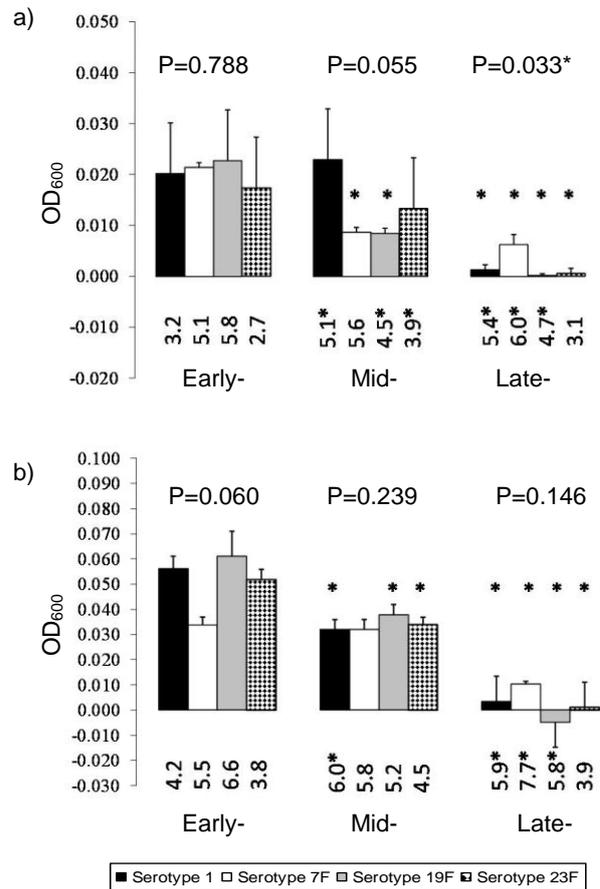


Figure 1: Graphical illustration of growth increments (based on OD₆₀₀) of the four pneumococcal isolates at their early-, mid- and late-log phases after 1 (1a) and 3 h (1b) incubation times in RPMI with 2% FBS. The P-values above the bars compare the OD₆₀₀-based growth among the four isolates at the respective growth phases. The numerals below each bar indicate the number of pneumococcal adherence (log₁₀) for the respective isolates at the respective growth phases. The sign ‘**’ indicates the P-value of ≤ 0.050 that compares the replication or adherence capacities against those at the early-log phase as the baseline for the respective isolates.

isolate had a higher adherence at 3 h as compared to that at 1-h of exposure times to A549-cells and frequently, at both exposure times, a higher adherence was observed at either mid- or late-log phase of pneumococcal growth (Desa *et al.*, 2008). In this current study, re-analysis on the adherence capacity in comparison to that at the early-log phase as the baseline showed significant increase in adherence for isolate serotypes 1 and 7F at late-log phase while that at mid-log was variable. Only isolate serotype 19F showed a contrast pattern by exhibiting a higher adherence at early- and the least at mid-log phase. In comparing the adherence pattern with the replicative

capacity of the respective isolates, adherence was frequently the least at early-log phase although the isolates were in the most active state of replication to increase the number of bacterial cells to adhere. At mid- and late-log phases, bacterial adherence was frequently higher although bacterial replication was reduced that there would not be an increase in the number of bacterial cells as much as that during the early-log phase. An obvious observation was at late-log phase whereby the highest adherence was exhibited by isolate serotypes 1 and 7F while their replications were the least at this stage at both incubation times.

DISCUSSION

Some experimental observations showed that the growth capacity of pneumococci differs between drug-resistant and drug-sensitive isolates (Mazzola *et al.*, 2003; Fernebro *et al.*, 2008; Miyashita, 2008). Comparatively, pneumococci with serotypes 6A, 6B, 8, 9V, 15, 18C, 19F, 23F, 33 and 38 have been reported to be highly associated with drug-resistance and carriage (referred as serotype-specific colonization) whereas isolate serotypes 1, 4, 5, 7F and 14 have been frequently drug-sensitive and commonly found in invasive diseases (referred as serotype-specific invasiveness) (Azouly-Dupuis *et al.*, 2000; Sandgren *et al.*, 2004; Hausdorff *et al.*, 2005; Bättig *et al.*, 2006). For the later, Bättig *et al.* (2006) has also shown that, based on *in vitro* growth in Brain Heart Infusion (BHI) broth with and without 5% fetal calf serum (FCS), pneumococcal isolates with serotype-specific invasiveness had their lag phase reduced in presence of serum. The tendencies of such serotype distribution and its connection with drug-susceptibility, site of isolation and growth pattern are merely based on epidemiological findings without any established genetic or biological explanation (Azouly-Dupuis *et al.*, 2000; Bättig *et al.*, 2006). Considering that the isolates used in this study had different serotypes, penicillin susceptibility and site of origin, their growth capacity could also vary. Thus, the isolate-*in vitro* growth characteristics were first evaluated in both bacteriological (THB with yeast extract) and cell culture media (RPML). As it is a standard to supplement the cell culture media with serum (2% FBS), which may affect the pneumococcal growth as mentioned earlier, the effect of the 2% FBS was assessed but in bacteriological media. Interestingly, the growth pattern for isolate serotypes 1 and 7F, which could be classified as the serotype-specific invasiveness, turned out to be in agreement with Bättig *et al.* (2006)'s study. The isolate growths in RPML alone without serum were not analyzed but those in presence of 2% FBS were highly comparable among the four isolates but with a lower growth yield at the stationary phase as compared to that in the bacteriological media. This suggests that different nutritional components of the media may be differentially utilized by the different isolates to result in differential outcome of the growth pattern and quality.

In the adherence assay, bacterial cells were harvested from growth in THB with 0.5% yeast extract at

3, 5 and 7 h of incubation times (representing early-, mid- and late-log phases respectively) and re-suspended in RPML with 2% FBS for exposure to A549-cells. At these harvesting times, the isolates were all in exponential growth and thus the potential discrepancy at the lag phase has not been an issue in this study. As all isolates were able to grow in the cell culture media at a comparable rate throughout the incubation time, it could be assumed that all isolates were in equal state of growth strength at the respective growth phases during the exposure to A549-cells and thus differential growth fitness that may potentially affect the adherence capacity of the isolates at the respective growth phases has also not been an issue. However, there would be a potential impact when comparing the adherence at different growth phases for the individual isolates. Clearly, the replicative capacity for each isolate differed at early-, mid- and late-log phases and thus it would be of interest to look at the adherence capacity at these different states of bacterial replication among the four isolates. If bacterial adherence was assumed to be higher when there were more bacterial cells available due to the higher replication rate, then only isolate serotype 19F would agree with such an assumption by showing a higher degree of adherence at the early-log phase. On the contrary, frequently, a higher bacterial adherence was observed at mid- or late-log phase as compared to that at early-log phase although the bacterial replication was significantly reduced at the later growth stages. Isolate serotype 7F had the highest adherence at late-log phase perhaps due to its higher replication rate as compared to others at this stage to comply with the assumption mentioned earlier but still the replication at this growth stage was not as high as that during the early-log phase. A theoretical explanation was that at the early growth stage, bacterial cells may not be mature enough to exhibit virulence phenotypes as compared to the subsequent growth stages. But for the later perhaps one could also argue that bacterial cells may be too old to do so but still, in this study, adherence was observed to be higher at either mid- or late-log phase. Another theoretical explanation for a higher adherence at late-log phase could be that, as far as pneumococcal cells are concerned, cell lysis may occur to release pneumolysin and other inflammatory components that may injure the host cell. This will expose or induce more adherence receptors available on the host cell surface for more bacteria to bind (Hammerschmidt *et al.*, 2007). Bacteria at mid-log phase was frequently used in many studies for the adherence assay as they were assumed to be in balance between the 'immature' and 'too old' (Talbot *et al.*, 1996; Adamou *et al.*, 1998; Elm *et al.*, 2004), but this study on pneumococci showed a variable result at mid-log phase. Thus this study preliminarily suggests that pneumococci at early-log phase may not be that superior to display their virulence characters as they may do at mid- or late-log phase. Nevertheless, there could be isolates that may not follow this generalization as shown by isolate serotype 19F in this study to indicate potential strain-specific properties.

As a whole, this study utilized only a limited number of isolates and thus studies on more isolates are highly warranted to establish such a correlation between growth and adherence pattern in a wider pneumococcal population. In addition, the bacterial growth curve and replication in this study were measured in absence of host cells in the bacteriological or cell culture media and thus it is not known whether the bacterial replication will be affected when the bacterial and host cells are together. Certain cellular metabolic reactions may be induced upon exposure or interaction of the bacteria and the host cells to produce different composition of metabolic products in the media as compared to the conditions whereby the bacterial or host cells are alone in the media. Different metabolic constituents may potentially alter the physiological conditions of the media to varying extents to potentially result in different impacts on the growth fitness of the different pneumococcal isolates. These matters need to be further investigated but the preliminary findings in this study raise a potential approach for preventing pneumococcal infection, which is by deterring pneumococcal growth from progressing to the mid-log phase. For such a purpose, in addition to the feasibility of vaccines, the role of probiotics could also be justified here as presence of other harmless organisms at the colonization site may suppress pneumococcal growth particularly during its early stage of growth. As adherence was shown to be better at mid- or late-log phases of pneumococcal isolates, such a prevention strategy may reduce the risk of pneumococcal adherence to the host cells that may lead to pneumococcal diseases.

ACKNOWLEDGEMENTS

This study was supported by the University of Malaya Fundamental Grant FP032/2005D.

REFERENCES

- Adamou, J. E., Wizemann, T. M., Barren, P. and Langermann, S. (1998). Adherence to *Streptococcus pneumoniae* to human bronchial epithelial cells (BEAS-2B). *Infection and Immunity* **66**, 820-822.
- Azoulay-Dupuis, E., Rieux, V., Muffat-Joly, M., Bédos, J. P., Vallée, E., Rivier, C., Isturiz, R., Carbon, C. and Moine, P. (2000). Relationship between capsular type, penicillin susceptibility, and virulence of human *Streptococcus pneumoniae* isolates in mice. *Antimicrobial agents and Chemotherapy* **44**, 1575-1577.
- Bättig, P., Hathaway, L. J., Hofer, S. and Mühlemann, K. (2006). Serotype-specific invasiveness and colonization prevalence in *Streptococcus pneumoniae* correlate with the lag phase during in vitro growth. *Microbes and Infection* **8**, 2612-2617.
- Desa, M. N. M., Lin, T. K., Yasin, R. M. and Parasakthi, N. (2003). Penicillin susceptibility and molecular characteristics of clinical isolates of *Streptococcus pneumoniae* at the University of Malaya Medical Centre, Kuala Lumpur, Malaysia. *International Journal of Infectious Diseases* **7**, 190-197.
- Desa, M. N. M., Parasakthi, N., Vadivelu, J. and Sekaran, S. D. (2008). Differential adherence of clinical isolates of *Streptococcus pneumoniae* at different growth states to human respiratory epithelial cells (*in vitro*). *Asian Journal of Cell Biology* **3**, 61-66.
- Elm, C., Rohde, M., Vaerman, J. P., Chhatwal, G. S. and Hammerschmidt, S. (2004). Characterization of the interaction of the pneumococcal surface proteins SpsA with the human polymeric immunoglobulin receptor (hplgR). *Indian Journal of Medical Research* **119**, 61-65.
- Fernebro, J., Blomberg, C., Morfeldt, E., Wolf-Watz, H., Normark, S. and Normark, B. H. (2008). The influence of *in vitro* fitness defects on pneumococcal ability to colonize and to cause invasive diseases. *BMC Microbiology* **8**, 65-76.
- Hammerschmidt, S., Paterson, G. K., Bergmann, S. and Mitchell, T. J. (2007). Pathogenesis of *Streptococcus pneumoniae* infections: Adaptive immunity, innate immunity, cell biology, virulence factors. *In: Community-Acquired Pneumonia*. Suttorp, N., Welte, T. and Marre, R. (eds.). Birkhauser Verlag Basel, Switzerland, pp. 139-181.
- Hausdorff, W. P., Feikin, D. R. and Klugman, K. P. (2005). Epidemiological differences among pneumococcal serotypes. *The Lancet Infectious Diseases* **5**, 83-93.
- Mazzola, G. J., Mortensen, J. E., Miller, L. A. and Poupard, J. A. (2003). The growth and survivability of *Streptococcus pneumoniae* clinical isolates subjected to various environmental conditions. *Diagnostic Microbiology and Infectious Diseases* **45**, 153-164.
- Miyashita, K. (2008). *Streptococcus pneumoniae* growth based on drug resistance genotype. *Kansenshogaku Zasshi* **82**, 292-299.
- Sandgren, A., Sjöström, K., Olsson-Liljequist, B., Christensson, B., Samuelsson, A., Kronvall, G. and Normark, B. H. (2004). Effect of clonal and serotype-specific properties on the invasive capacity of *Streptococcus pneumoniae*. *The Journal of Infectious Diseases* **189**, 785-796.
- Talbot, U. M., Paton, A. W. and Paton, J. C. (1996). Uptake of *Streptococcus pneumoniae* by respiratory epithelial cells. *Infection and Immunity* **64**, 3772-3777.