Malaysian Journal of Microbiology, Vol 13(3) September 2017, pp. 210-216

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Adherence of *Streptococcus pneumoniae* and expression analysis of neuraminidase gene (NanA and NanB) after interaction of A549 human lung epithelial cells with pneumococcal strains of various serotypes

Ezarina Abdul Rahim[1,2], Zaini Mohd-Zain[2,3], Jamal Hussaini[2,3], Eng Hwa Wong[4], Lwin Lwin Nyein[2,3], N. Parasakthi[5], Ariza Adnan[2,3], Navindra Kumari Palanisamy[2,3*]

[1]Institute of Medical Molecular Biotechnology, Faculty of Medicine, UiTM, Sungai Buloh, Selangor, Malaysia.
[2]Cluster of Laboratory Medical Sciences, UiTM, Sungai Buloh, Selangor, Malaysia.
.[3]Faculty of Medicine, Universiti Teknologi MARA (UiTM), Sungai Buloh, Selangor, Malaysia.
[4] School of Medicine, Taylor's University Lakeside Campus, No. 1 Jalan Taylor's, 47500 Subang Jaya, Selangor Darul Ehsan, Malaysia.
[5]School of Medicine and Health Sciences, Monash University Malaysia.
Email: navindra@salam.uitm.edu.my

Received 7 November 2016; Received in revised form 23 February 2017; Accepted 2 March 2017

ABSTRACT

Aims: *Streptococcus pneumoniae* is the most common bacterial respiratory pathogen that can lead to invasive diseases such as pneumonia, bacteremia, and meningitis. The interaction of *S. pneumoniae* with host respiratory epithelial cells is crucial in the colonization of human respiratory tract and involve in the virulence. The aim of the study is to investigate the adherence of *S. pneumoniae* and the effect of serotypic variation on neuraminidase genes (NanA and NanB) after interaction of A549 human lung epithelial cells with *S. pneumoniae* serotypes.
Methodology and results: Six different serotypes of *S. pneumoniae* were used (1, 3, 5, 19F, 23F, and 14). A549 human lung epithelial cells were inoculated with pneumococcal strains of different serotype for 3 hours. The number of adherent bacteria was determined by serial dilution followed by spread plate technique on tryptic soy agar supplemented with 5% sheep blood. Bacterial RNA was harvested from the infected A549 cells. The differential expression level of neuraminidases was observed by quantitative real-time PCR (qRT-PCR). Based on bacterial adherence assay, serotype 14 showed highest adherence, meanwhile, serotype 23F showed lowest adherence. This suggests that serotype 14 has a better affinity to adhere to A549 cells as compared to serotype 23F. Higher NanA gene expression was observed in serotype 5, 23F and 19F, while lower expression in serotype 14. In contrast, NanB gene shows low-level expression in serotype 23F and 19F, while higher expression in serotype 14. This postulates that NanA and NanB gene may have different functions in the pathogenesis of *S. pneumoniae.*
Conclusion, significance and impact of study: Our finding on differential expression of neuraminidase gene of *S. pneumoniae* of various serotypes on A549 cells might give a better understanding of host pathogen interaction between bacteria serotypes and host cell.

Keywords: Bacillus, *Paenibacillus,* phytase, phytate, thermotolerant bacteria

INTRODUCTION

Streptococcus pneumoniae is the most crucial bacteria that cause invasive disease in children and adult. *Streptococcus pneumoniae* can cause many types of infections such as meningitis, bacteraemia, sepsis, acute sinusitis, and otitis media (William *et al.*, 2005). *Streptococcus pneumoniae* has sialidases, encoded by NanA and NanB. These sialidases have demonstrated numerous roles involved in pathogenesis and potential drug targets. NanA is a first line virulence factor for sialic acid removal, hydrolyzing sialic acid (Neu5Ac) removal from a variety of oligosaccharide, glycolipids, and glycoproteins. NanB is a trans-sialidase involved in the metabolic use of sialic acid (Xu *et al.*, 2011). Previous study showed that NanA and NanB are present in 100% and 96% of clinical isolates of *S. pneumoniae*, respectively (Pettigrew *et al.*, 2006). The sialidases or neuraminidases that are recognized as virulence determinants has the ability to cleave terminal sialic acid (neuraminic acid, NeuNAc) residues present in O-linked and N-linked glycans (Gualdi *et al.*, 2012). NanA is involved in modifying both nasopharyngeal organism and host proteins that leads to long term colonisation. NanA

*Corresponding author

ISSN (print): 1823-8262, ISSN (online): 2231-7538

has also been known to desialylate the cell surfaces of *Neisseria meningitidis* and *Haemophilus influenzae* (Shakhnovich *et al*., 2002). NanA is crucial in nasopharyngeal colonisation of the middle ear (Tong *et al*., 2000). In another study, they found that NanA plays an important role in the invasion of blood-brain barrier endothelial cells and bacterial access into the central nervous system that cause meningitis (Uchiyama *et al*., 2009). Much less is known about NanB. Evidence also suggest that pneumococcal NanA and NanB are crucial in the progress of upper and lower respiratory tract infection and sepsis (Salim *et al*., 2005). The interaction of *S. pneumoniae* with host respiratory usually found to be associated to virulence of the organism. Several virulence determinants have been identified in *S. pneumoniae*. In this present study, we investigated the adherence of *S. pneumoniae* to human lung epithelial cells and the effect of serotypic variation amongst neuraminidase genes (NanA and NanB).

MATERIALS AND METHODS

Cell line growth conditions

A549 cells were obtained from Institute of Medical Molecular Biotechnology (IMMB), University of Teknologi MARA. A549 cells were routinely maintained in 25 cm^2 tissue culture flasks and grown in complete growth media. Complete growth media consists of Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, USA) and 2% penicillin-streptomycin (Gibco, USA). The A549 human lung epithelial cell line was grown to confluent at 37 °C in humidified 5% CO_2. The cells were harvested by trypsinisation and centrifuged at 100×g for 5 min. The cell pellet was then resuspended in complete media growth and cell counting was carried out with a haemocytometer using trypan blue (Gibco, USA).

Bacterial strains growth conditions

Optimal conditions of bacterial growth were described by Restrepo *et al.* (2005). All strains were streaked and grown on tryptic soy agar supplemented with 5% sheep blood and incubated for 18-24 h at 37 °C in the presence of 5% CO_2. Then the bacterial colonies were subcultured on Todd Hewitt broth and grown until mid-log phase (OD_{600nm} = 0.5 or $1×10^8$ CFU/mL).

Morphological observation by Giemsa Staining

Giemsa staining was done to detect the adherence of pneumococci to the A549 cells. Giemsa staining method was adapted from a previous study with minor modifications (Zhang *et al*., 2015). Briefly, A549 cells were seeded into 6 well plates at $2×10^5$ cells per well (2 mL/well) and incubated at 37 °C in the presence of 5% CO_2 for 18 to 24 h. Then, the cells were infected with 2 mL of bacterial suspension in DMEM supplemented with 2% FBS for 3 h. The infected cells were washed with 1×PBS and the cells were fixed with 100% methanol for 5 min. The fixed cells were stained with 3% Giemsa (J.T. Baker) for 20 min and washed three times with 1×PBS. The adherences of *S. pneumoniae* to the host cells were observed using inverted microscope (Olympus 1×81) at 20× magnification.

Infection of A549 cell line with *S. pneumoniae*

The method used was adapted from Desa *et al.* (2008a). Bacterial pellet from overnight cultured in Todd Hewitt broth was suspended in DMEM (Gibco) supplemented with 2% foetal bovine serum (FBS) and the suspension was adjusted to the concentration of $1×10^8$ CFU/mL using Densichek Plus (Biomerieux, USA). The A549 cell line was grown in complete media growth in a 25 cm^2 tissue culture flask at 37 °C in the presence of 5% CO_2 until 90% confluency (~ $4×10^6$ cells). Next, A549 cells were washed with phosphate buffered saline (PBS) and the cells were infected with 2 mL of the bacterial suspension in DMEM supplemented with 2% FBS for 3 h (multiplicity of infection (MOI) of 50) (Hausler *et al*., 2002; Desa *et al*., 2008b). Then, A549 cell were washed three times with 1×PBS to remove non-adherent bacteria. The infected A549 cells were detached and lysed using accutase cell dissociation reagent (Gibco, USA) and 1% saponin (Calbiochem, US). For adherence assay, the final number of adherent bacteria was determined by serial dilution of the A549 cell lysate in 1× PBS followed by standard spread plate technique on tryptic soy agar supplemented with 5% sheep blood. All experiments were done in triplicates and results were averaged. For RNA extraction**,** the cells were centrifuged at 220×g for 5 min to remove cellular debris and followed by 4000 ×g for 15 min to pellet the bacteria.

RNA extraction of bacteria and host cell after infection

RNA of bacteria from the mixture of infected A549 cells and control bacteria were extracted using an RNeasy protect bacteria minikit (Qiagen, USA). Bacterial RNA was used as a control. Meanwhile, RNA from the host cell was extracted using RNeasy Mini kit (Qiagen, USA). The RNA integrity and visualization was done on a 1.2% agarose gel in 1×TBE buffer (Tris-borate-EDTA).

Quantitative real-time PCR (qRT-PCR)

The quantitative real-time PCR (qRT-PCR) was performed on a CFX96^{TM} Real Time System (Bio-Rad, US) using iTaq Universal SYBR Green One Step kit (Bio-Rad, US) according to the manufacturer's instructions. All target and housekeeping genes used in this study are listed in Table 1. *Gyr*A gene was used as the normalizing gene. Standard curves of the genes were created with four-fold serial dilutions of pneumococcal RNA extract. The slope of the standard curve gives the amplification efficiency of a qRT- PCR. The efficiency of the qRT-PCR should be between 90-110% ($-3.6 \geq$ slope ≥ -3.3). For assay reproducibility, the real-time PCR was performed in triplicates using RNA extracted from three independent

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Table 1: List of target and normalizing genes used in real-time PCR.

Primer	Sequence	References	Efficiency (E)
nanA-F	CAGTGATAGAAAAAGAAGATGTTG	(Camara *et al*., 1994)	99.0%
nanA-R	ATTATTGTAAACTGCCATAGTGAA		
nanB-F	AACTGTCCATATCTCCTATTTTTC	(Camara *et al*., 1994)	93.5%
nanB-R	TATTTCTACACCTATCTCACCAGA		
*gyr*A-F	CCA AAA TTC CCA TGA CCA TC	(Shakhnovich *et al*., 2002)	96.8%
*gyr*A-R	GGG TAA ATA CCA CCC ACA CG		

experiments. All reactions were run in 10 µL reactions consist of RNA template, 3 µM of each primer, 2× iTaq Universal SYBR Green reaction mix and iScript reverse transcriptase (Bio-Rad, US). The cycling condition comprise of one cycle of 50 °C for 10 min, 95 °C for 1 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 30 sec and subsequently, a melt curve analysis of 65 °C to 95 °C with an increasing temperature by 0.5 °C for 5 sec.

Statistical analysis

Data were obtained as threshold cycle (C_t) values using Bio-Rad CFX Manager 3.0. Relative expression ratio of the gene was determined using the $2^{-\Delta\Delta Ct}$ method described by Pfaffl, 2001.

Relative expression ratio (R)

$$= \frac{(E_{target})^{\Delta CPtarget(\text{CP deviation of control – sample of the target gene transcript})}}{(E_{ref})^{\Delta CPref(\text{CP deviation of control – sample of reference gene transcript.})}}$$

E_{target} : real time PCR efficiency of target gene transcript
E_{ref} : real time PCR efficiency of a reference gene transcript
ΔCP_{target}: CP deviation of control – sample of the target gene transcript
ΔCP_{ref} : CP deviation of control – sample of reference gene transcript.

The relative expression ratio of target gene is determined in a sample versus a control and expressed in comparison to a reference gene. Standard deviations and graphs were plotted using Microsoft Excel. All analysis was performed using IBM SPSS Statistics 20. Mann-Whitney U test was used for differences between two groups. A $p < 0.05$ was considered to be statistically significant.

RESULTS

Adherence of the bacterial isolates to A549 cells

The A549 cells that were infected by *S. pneumoniae* were observed under inverted microscope. Adherence of pneumococcal isolates was clearly observed on the surface of the infected A549 cells (Figure 1). Cytoplasm and nucleolus of A549 cells stained bluish violet while the nuclei stained purple with Giemsa staining. The results of bacterial adherence to A549 cells were expressed as colony forming unit per milliliter (CFU/mL) of adhered bacteria. The number of bacteria adherence of the various serotypes to A549 cells is shown in Figure 2. A one-way between group analysis of variance (ANOVA) was conducted to explore the number of bacteria adherence to the host cells. There was a statistically significant different at the $p < 0.05$ level among different serotypes. Based on the results, adherence of *S. pneumoniae* of different serotypes was in the range of 0.001% to 0.014%. Serotype 14 was significantly different from serotype 23F and 1. Serotype 14 shows the highest adherence with 2.82×10^4 CFU/mL, meanwhile, serotype 23F shows the lowest adherence with 3.1×10^3 CFU/mL. This suggests that serotype 14 has a better affinity to adhere to A549 cells as compared to serotype 23F. Nevertheless, the virulent property of the strains cannot be concluded as yet. Serotypes 23F and 1 appears to be having weaker adherence capability but the organism may have invaded the A549 cell and therefore the number of adhering bacteria seems lower.

RNA integrity

The integrity of RNA was checked by gel electrophoresis. Figure 3 shows the RNA (23S and 16S) that was harvested from host cells and bacteria.

Real-time PCR of neuraminidase genes

The expression of virulence gene NanA and NanB were detected by real-time RT-PCR after A549 cells were stimulated by different serotypes of *S. pneumoniae*. Expression data for each gene were normalized against *gyr*A as a housekeeping gene. In Figure 4, there is a statistically significant difference in expression level of NanA gene in serotype 3, 5, 19F and 14 compared to bacterial culture without the host as a control. NanA gene was upregulated in serotypes 5, 19F and 23F with more than two fold changes. Serotype 5, 19F, and 23F express NanA gene respectively at 2.60, 5.70 and 2.32 folds higher than the control sample. Meanwhile, serotype 14 shows downregulation of NanA gene at 3.70 fold lower than control. Serotype 1 and 3 shows expression lower than two fold changes. This suggests that serotype 19F

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

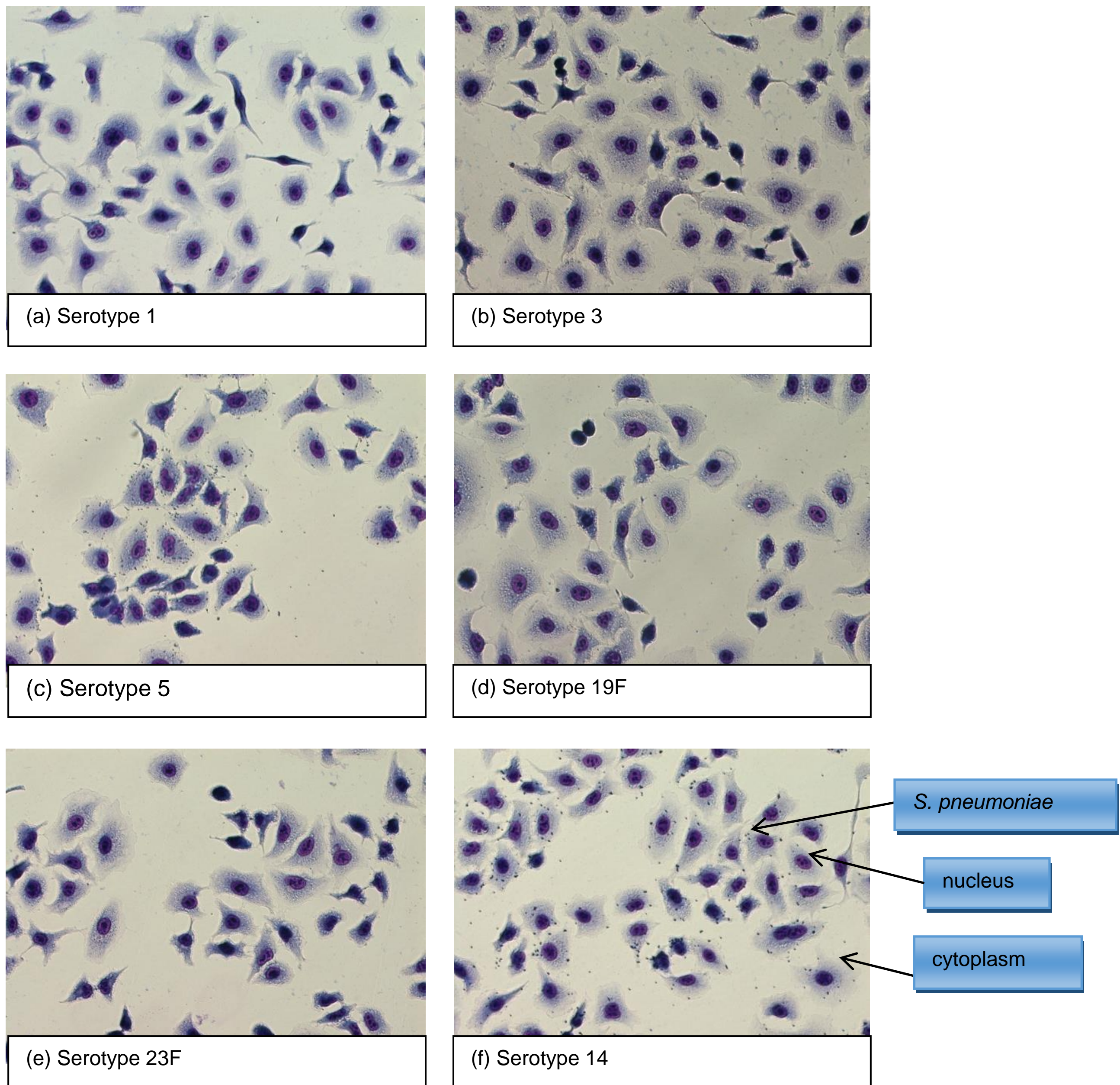

Figure 1: Giemsa staining method detected the adherence of pneumococci to the A549 cells were observed under inverted microscope at 20× magnification.

may be more virulence and invasive followed by serotype 5 and 23F, while serotype 14 is less virulent. NanA gene expression increased when host cells were stimulated with serotype 5, 19F and 23F.

For NanB (Figure 5), there is statistically significant difference between serotype 5, 19F, 23F and 14 with bacterial serotype without host cells as a control. Gene NanB was upregulated in serotypes 5 and 14 with more than two folds change. Serotype 5 and 14 express NanB gene at 3.60 and 6.00 folds higher than control, respectively. Serotype 23F showed a downregulation of NanB at 3.80 fold lower than bacteria control. Meanwhile, serotype 1, 3, and 19F shows expression lower than two fold changes. Higher expression of NanB in serotype 14 but not in NanA gene postulates that NanA and NanB gene may have different roles in the pathogenesis of *S. pneumoniae.*

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

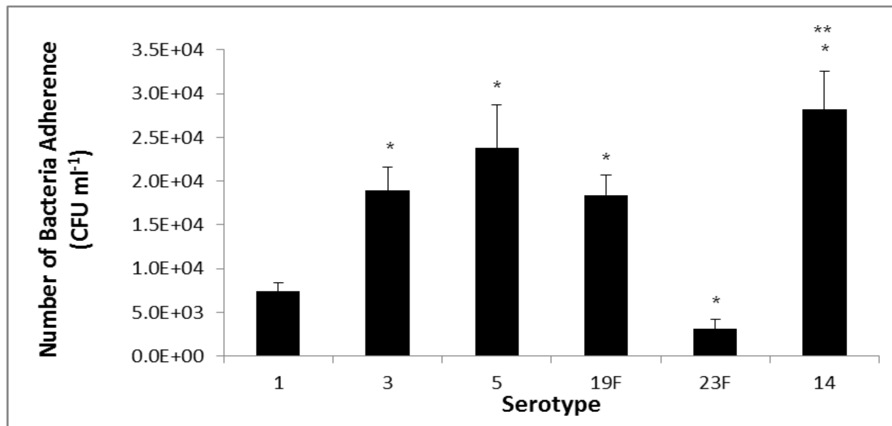

Figure 2: Comparison of adherence of different pneumococcal serotypes to A549 cells after 3 hours (multiplicity of infection of 50). Data are means ± standard deviation of each reaction. * inidicates significant when p<0.05 compared to 23F. ** indicates significant when p <0.05 compared to 14.

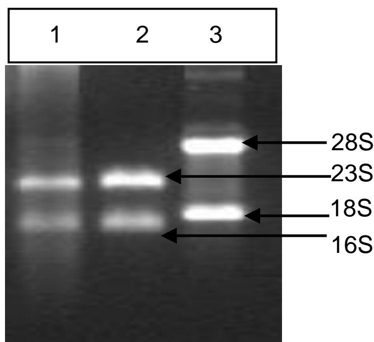

Figure 3: RNA harvested from A549 cells and bacteria. Lane 1 represents the bacteria RNA harvested from infected A549 cells. Lane 2 shows pneumococcal RNA extract from bacteria without the host cells (23S and 16S) while lane 3 shows RNA extract from A549 cell (28S and 18S).

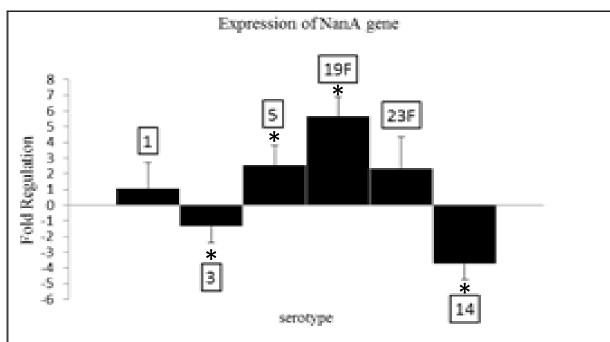

Figure 4: NanA gene expression of *S.pneumoniae* show differential gene expression among various serotypes. Relative normalized expression was determined using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001). Data are means ± SE of each reaction. Changes are considered significant when p <0.05 indicated by *.

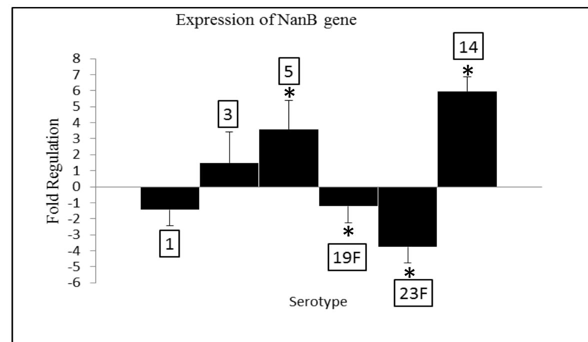

Figure 5: NanB gene expression of *S.pneumoniae* show differential gene expression among various serotypes. Relative normalized expression was determined using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001). Data are means ± SE of each reaction. Changes are considered significant when p <0.05 indicated by *.

DISCUSSION

The interaction of *S. pneumoniae* with host respiratory epithelial cells is crucial in the colonization of human respiratory tract and involved in virulence. In this study, by using Giemsa staining, A549 cells infected with bacteria were stained in order to observe the adherence of pathogenic bacteria to human cells. Therefore, pneumococcal can be seen attached and adhering to the surface of A549 epithelial cells. Cytoplasm and nucleolus of A549 cells stained bluish violet while nuclei stained purple when Giemsa staining was used. In previous studies, the bacterial growth at mid log phase was used for adherence assay as it was shown to be the optimum stage for adherence process to occur (Battig *et al.*, 2006, Desa *et al.*, 2008b . The bacterial adherence process to the host cell is a crucial step prior to penetration and infection. Once the bacteria successfully adhere, the invasion process begins. In this study, the A549 cells were

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

infected with *S. pneumoniae* for 3 h. The conditions of the adherence experiment were standardized by infecting A549 cells with bacterial inoculum size of 1×10^8 CFU/mL. Based on bacterial adherence assay analysis, strain of serotype 14 shows the highest number of adherence to the host cells compared to other serotypes. On the other hand, serotype 23F and 1 showed the lowest adherence values which may be due the bacteria have penetrated and invaded the host cells. Therefore they are mostly in the cytoplasm of the cells. Even though serotype 23F and 1 had the lowest number of adherence, but if they can internalized into the host cells this may potentially cause severe diseases. For adherence assay, care must be taken while handling the cell lines to avoid detachment of the A549 cells. If the cell detaches from the flask, there will be less bacteria adhering. Another important step is to choose the same number of washes after adhesion of bacteria. Bacteria have many virulence mechanisms and strategy that let them to conquer and adhere successfully to the human body by invading or kill the cells. All of pneumococcal serotype in this study also showed positive amplification for NanA and NanB gene using polymerase chain reaction. Expressions of these genes were also measured upon infection with human lung epithelial cells by using real-time PCR NanA gene expression was higher in serotype 5, 19F and 23F, while lower expression is detected in serotype 14. This postulates that serotype 5, 23F and 19F may show strong invasiveness compared to serotype 14, which means different level of NanA gene expression among various serotypes may cause different outcome of invasiveness. In contrast, serotype 14 had higher expression of NanB, while serotype 19F and 23F had low expression. These suggest that NanA and NanB may have different functions in the pathogenesis of *S. pneumoniae*. We may postulate that NanA and NanB has an interplay role in causing disease. The adherence study showed that strain of serotype 3,5, 19F and 14 has significant adhering capacity but the regulation of NanA and Nan B differs. The role of NanA in known for adherence but the role of NanB is still not well understood. In serotype 5 there may interplay of both neuraminidases in the disease process unlike the other serotypes which demonstrates upregulation of either one of the neuraminidases. In the other study, NanA and NanB have different roles *in vivo* because only NanA contains the LPXTG sequence and NanB has lower acidic pH optimum compared to NanA (Berry *et al.*, 1996). NanA and NanB are important in colonization for pneumococcal adherence in the respiratory tract and bloodstream. Therefore, neuraminidase genes are important target of the immune system in the interaction of *S. pneumoniae* with host respiratory epithelial cells. Our finding on differential expression of neuraminidase gene of *S. pneumoniae* of various serotypes on A549 cells might give a better understanding of host pathogen interaction between bacterial serotypes and host cell. The finding also provides some insights to the colonization and invasion process of the organism on the respiratory tract. This explains why different strains of pneumococci differ in the ability to produce disease, hence the varied efficacy of the available vaccine. However, other factors such as bacterial adaptation, survival, growth and geographic differences may also play an important role in virulence determinant. The specific roles of neuraminidase genes need to be further investigated to explore more on host pathogen interaction.

ACKNOWLEDGEMENT

This study was supported by grants provided by the Ministry of Education: 600-RMI/RAGS 5/3 (110/2012) and Geran Inisiatif Penyeliaan (GIP) provided by Universiti Teknologi MARA (UiTM): 600-IRMI/GIP 5/3 (0050/2016).

REFERENCES

Battig, P., Hathaway, L. J., Hofer, S. and Muhlemann, K. (2006). Serotype-specific invasiveness and colonization prevalence in *Streptococcus pneumoniae* correlate with the lag phase during *in vitro* growth. *Microbes and Infection* **8(11), 2612-2617.**

Berry, A. M., Lock, R. A. and Paton, J. C. (1996). Cloning and characterization of NanB, a second *Streptococcus pneumoniae* neuraminidase gene , and purification of the NanB enzyme from recombinant *Escherichia coli. Journal of Bacteriology* **178(16), 4854-4860.**

Camara, M., Boulnois, G. J., Andrew, P. W. and Mitchell, T. J. (1994). A neuraminidase from *Streptococcus pneumoniae* has the features of a surface protein. *Infection and Immunity* **62(9), 3688-3695.**

Desa, M. N. M., Navaratnam, P., Vadivelu, J. and Sekaran, S. D. (2008a). Expression analysis of adherence-associated genes in pneumococcal clinical isolates during adherence to human respiratory epithelial cells (*in vitro*) by real-time PCR. *FEMS Microbiology Letters* **288(1), 125-130.**

Desa, M. N. M., Parasakhti, N., Vadivelu, J. and Sekaran, S. D. (2008b). Differential adherence capacities of clinical isolates of *Streptococcus pneumoniae* at different growth states to human respiratory epithelial cells (*in vitro*). *Asian Journal of Cell Biology* **3(2), 61-66.**

Gualdi, L., Hayre, J. K., Gerlini, A., Bidossi, A., Colomba, L., Trappetti, C., Pozzi, G., Docquier, J. D., Andrew, P., Ricci, S. and Oggioni, M. R. (2012). Regulation of neuraminidase expression in *Streptococcus pneumoniae. BMC Microbiology* **12(1), 200.**

Hausler, K. G., Prinz, M., Nolte, C., Weber, J. R., Schumann, R. R., Kettenmann, H. and Hanisch, U. K. (2002). Interferon-γ differentially modulates the release of cytokines and chemokines in lipopolysaccharide and pneumococcal cell wall-stimulated mouse microglia and macrophages. *European Journal of Neuroscience* **16(11), 2113-2122.**

Pettigrew, M. M., Fennie, K. P., York, M. P., Daniels, J. and Ghaffar, F. (2006). Variation in the presence of

neuraminidase genes among *Streptococcus pneumoniae* isolates with identical sequence types. *Infection and Immunity* **74, 3360-3365.**

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29(9), 2002-2007.**

Restrepo, A. V, Salazar, B. E., Agudelo, M., Rodriguez, C. A, Zuluaga, A. F. and Vesga, O. (2005). Optimization of culture conditions to obtain maximal growth of penicillin-resistant *Streptococcus pneumoniae. BMC Microbiology* **5(34).**

Salim, K. Y., Cvitkovitch, D. G., Chang, P., Bast, D. J., Handfield, M., Hillman, J. D. and De Azavedo, J. C. S. (2005). Identification of group A *Streptococcus* antigenic determinants upregulated *in vivo. Infection and Immunity* **73(9), 6026-6038.**

Shakhnovich, E. A., King, S. J. and Weiser, J. N. (2002). Neuraminidase expressed by *Streptococcus pneumoniae* desialylates the lipopolysaccharide of *Neisseria meningitidis* and *Haemophilus influenzae*: A paradigm for interbacterial competition among pathogens of the human respiratory tract. *Infection and Immunity* **70(12), 7161-7164.**

Tong, H. H., Blue, L. E., James, M. A. and DeMaria, T. F. (2000). Evaluation of the virulence of a *Streptococcus pneumoniae* neuraminidase-deficient mutant in nasopharyngeal colonization and development of otitis media in the Chinchilla model. *Infection and Immunity* **68(2), 921-924.**

Uchiyama, S., Carlin, A. F., Khosravi, A., Weiman, S., Banerjee, A., Quach, D., Hightower, G., Mitchell, T. J., Doran, K. S. and Nizet, V. (2009). The surface-anchored NanA protein promotes pneumococcal brain endothelial cell invasion. *The Journal of Experimental Medicine* **206(9), 1845-1852.**

William P. H., Daniel R. F. and Keith P. K. (2005). Epidemiological differences among pneumococcal serotypes. *The LANCET Infectious Diseases* **5(2), 83-93.**

Xu, G., Kiefel, M. J., Wilson, J. C., Andrew, P. W., Oggioni, M. R. and Taylor, G. L. (2011). Three *Streptococcus pneumoniae* Sialidases: Three different products. *Journal of the American Chemical Society* **133, 1718-1721.**

Zhang, C., Yan, W., Li, B., Xu, B., Gong, Y., Chu, F., Zhang, Y., Yao, Q., Wang, P. and Lei, H. (2015). A new ligustrazine derivative-selective cytotoxicity by suppression of NF- κ B / p65 and COX-2 expression on human hepatoma cells. part 3. *International Journal of Molecular Sciences* **16, 16401-16413.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538