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Serotyping of Brunei pneumococcal clinical strains and the investigation of their capability to adhere and invade a brain endothelium model

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ABSTRACT

Introduction: Pneumococcal infections have caused morbidity and mortality globally. *Streptococcus pneumoniae* (pneumococci) are commensal bacteria that colonize the nasopharynx, asymptomatically. From there, pneumococci can spread in the lungs causing pneumonia and disseminate in the blood-stream causing bacteremia (sepsis) and reach the brain leading to meningitis. Endothelial cells are one of the most important components of the blood-brain barrier that separates the blood from the brain and plays the first protective role against pneumococcal entry. Thus this study aimed to investigate on the ability of non-meningitis pneumococcal clinical strains to adhere and invade a brain endothelium model.
Methods: Two pneumococcal Brunei clinical strains were serotyped by multiplex PCR method using oligonucleotide sequences derived from Centers for Disease Control and Prevention. A validated immortalised mouse brain endothelial cell line (bEnd.3) was used as a brain endothelium model for the study of the pneumococcal breach of the blood-brain barrier using an adherence and invasion assay.
Results: Both of the pneumococcal clinical strains were found to be serotype 19F, a common circulating serotype in Southeast Asia and globally and possess the ability to adhere and invade the brain endothelial cells.
Conclusion: In addition, this is the first report on the serotype identification of pneumococci in Brunei Darussalam and their application on a brain endothelium model. Further studies are required to understand the virulence capabilities of the clinical strains.

1. Introduction

Streptococcus pneumoniae (the pneumococcus, or pneumococci in plural), a Gram positive and alpha-haemolytic bacterium, can spread from nasal location causing mild infection such as otitis media and sinusitis or severe infections, such as pneumonia, bacteremia and meningitis. The risk groups who are easily infected with pneumococcus include children, immunocompromised and the elderly. To date, pneumococcus is the leading cause for bacterial meningitis, a disease with high attributable mortality and frequent permanent neurological sequelae [1]. In most of the meningitis cases, the disease is caused by pneumococcus that initially acts as extracellular commensals of the nasopharynx [2]. World Health Organization reported that the mortality rate caused by pneumococcal infections for children who are less than five years old in Brunei Darussalam is nine in every 1000 live births [3].

Following nasopharyngeal colonization, pneumococci breaches the lung alveolar endothelial barrier, which then follows the flow of blood circulation towards the brain. At this stage, it has been proven that a control strain of pneumococcus has the ability to cross the blood–brain barrier (BBB) on an animal model. This is a key feature of meningitis, and their adherence to the BBB is spatiotemporally controlled at different sites throughout the brain [4,5]. Thus BBB models that consisted of a brain endothelium model (a monolayer of endothelial cells) co-cultured with other neighbouring neurovascular cells (astrocytes, pericytes and basement membrane) have been established to closely resemble the physiological structure of a BBB *in vivo* [6]. The monolayer of endothelial cells of the brain

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endothelium model is highly responsible to form the physical barrier that separates the brain from the blood [7,8].

There are more than 90 known serotypes of pneumococcus globally, but only few are found to be pathogenic to humans, thus pneumococcal vaccines have been created to contain antibodies to serotype-specific capsular polysaccharide antigens of pneumococcus [9–11]. These vaccines were created based on the most prevalent and pathogenic serotypes in the Western countries [10]. Even though the majority of pneumococcal serotypes in Asia are included in the vaccine formulation, these vaccines are unfortunately not included in the routine childhood immunisation schedules of all Asian countries [12]. The serotype distribution of pneumococcus in the Southeast Asian region have been reported to date [13], however this does not include Brunei Darussalam. Therefore, we aimed to identify the serotype that has caused pneumococcus infections in Brunei Darussalam, following which we aimed to investigate whether the serotype was able to infect a brain endothelium model to cause potential meningitis.

2. Materials and methods

2.1. Bacterial strains used in this study

Streptococcus pneumoniae clinical strains used in this study were isolated from gastric infection (designated as RSP1) and ear infection (designated as RSP2), collected from Raja Isteri Pengiran Anak Saleha (RIPAS) Hospital, Bandar Seri Begawan, Brunei Darussalam in March 2016. These strains have been maintained in 20% glycerol (v/v in cation-adjusted Mueller Hinton broth with 10% lysed human blood v/v in sterile distilled water) at −20 °C. *Streptococcus pneumoniae* ATCC 49619 was used as the control strain.

2.2. DNA extraction

Bacterial DNA was extracted using GenElute™ bacterial genomic DNA kit (Sigma, NA2120), following the manufacturer's instructions.

2.3. Serotyping

The serotypes of pneumococcal strains were identified by multiplex polymerase chain reaction (PCR) method adapted from previous studies [14–16]. The oligonucleotide primer sequences were adapted from Centers for Disease Control and Prevention [17] (Table 1). In brief, 12.5 μl of multiplex PCR mastermix (Qiagen, Germany), 1 μl of each primer, 9.3 μl of RNAse-free water and 1.2 μl of DNA were prepared to make a total PCR reaction volume of 25 μl. A primer pair targeting the *cpsA* locus was included as an internal control since it is found in all pneumococci [18]. A water control was also included as a negative and sterility control. The thermal cycle was set at 95 °C for 15 min, followed by 37 cycles of 94 °C for 40 s, 61 °C for 50 s, and 72 °C for 60 s and then at 72 °C for 10 min and held at 4 °C using Veriti 96 Well thermocycler (Applied Biosystem, Singapore). The PCR products were run on a 2% agarose gel (v/v in Tris-borate-EDTA buffer) at 90 V for 75 min. The gel was viewed using Molecular Imager VersaDoc™ 4000 MP (BioRad, US) and the images were captured using Quantity One software version 4.6.9 (BioRad, US).

2.4. Cell culture

Immortalised mouse brain endothelial cell line (bEnd.3) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented by 10% fetal bovine serum (FBS), 1% non-essential amino acid and 1% of penicillin-streptomycin at 37 °C in 5% CO_2 [19–21]. The cells were grown on coverslips in 6-well plates for immunofluorescence studies and 12-well plates for the adherence and invasion assay at a seeding concentration of 1×10^4 cells ml^{-1} and cultured for 3 days until 90–100% cell confluency. The cell passages used in this study were between passage 31 to passage 39.

2.5. Immunofluorescence staining

For the validation of bEnd.3 cells as brain endothelial cells, the cells must positively express the endothelial marker, von Willebrand marker (vWF) and the tight junction proteins (occludin and claudin-5). Uninfected bEnd.3 cells at 90–100% confluency were fixed using cold acetone for five minutes. Cells were washed three times with phosphate-buffered saline (PBS). Primary antibodies used were anti-von Willibrand factor (abcam, ab6994), anti-occludin (abcam, ab31721) and anti-claudin-5 (abcam, ab15106) at a concentration of 1:1000 in 1% bovine serum albumin (BSA) v/v in PBS. These antibodies were applied onto the cells for one hour at room temperature. Cells were then washed three times with PBS. Secondary antibodies of anti-mouse conjugated with Alexa Fluor 488 (Cell Signaling Technology, 4412S) at a concentration of 1:1000 in 1% BSA v/v in PBS were then applied for one hour at room temperature. The cells were washed again three times with PBS, and finally mounted using a fluorescent mounting medium (Dako, S3023). The cells were viewed using a fluorescence microscope (Eclipse 90i, Nikon), and images were captured using a digital image analysis (NIS Elements 3.0, Nikon).

2.6. Preparation of pneumococci for adherence and invasion assay

Pneumococcal isolates were cultured in Todd-Hewitt broth supplemented with 5% yeast extract (THY) to their mid-log phase (four-hours growth at 150 RPM and 37 °C). The pneumococcal pellets were suspended in fresh maintenance medium (2% of FBS in DMEM with no antibiotics added). The calculation of colony forming unit (CFU) before experiment was done by suspending pneumococci using ten-fold and hundred-fold dilutions in PBS. 10 μl of the diluted suspension were transferred into blood agar and incubated at 37 °C overnight.

2.7. Adherence and invasion assays

Pneumococcal adherence and invasion studies were performed as described previously [22–24]. For the adherence assay, endothelial cells were incubated with pneumococci (approximate multiplicity of infection (MOI) of 1) at 37 °C in 5% CO_2 for 30 min and 1 h. The cells were washed with PBS three times, trypsinised and diluted ten-fold and hundred-fold in PBS. 10 μl of the diluted suspension was plated on blood agar plates and incubated overnight at 37°. Meanwhile for the invasion assays, endothelial cells were prepared in the same manner as adherence assays whereby the cells were incubated with pneumococci (approximate MOI of 1) for 2 h at 37 °C in 5% CO_2. Then, the cells were incubated with L-glutamine and penicillin/streptomycin for 1 h to kill the extracellular pneumococci and washed with PBS for three times to remove the antibiotics. The cells were trypsinised and lysed with Triton X-100 (0.0125% v/v in PBS). Ten-fold and hundred-fold dilution were performed in PBS, and 10 μl of the diluted suspension was plated on blood agar plates and incubated overnight at 37 °C. CFU were calculated the following day as previously described [25]. CFU of the original pneumococcal suspension (total CFU) and of the suspension after the adherence assay (final CFU) were estimated by the CFU-counting method. The pneumococcal concentration (CFU mL^{-1}) was calculated as CFU x dilution x final total volume of cells. The adherence and invasion percentages were calculated as final