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Capsule Contributes to Transmigration of *Streptococcus pneumoniae* Serotype 7F Meningitis Isolates through Complex Blood Brain Barrier Models

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Abstract

Streptococcus pneumoniae is known as an opportunistic pathogen which belongs to the most common etiological agents of local and systemic diseases in humans. In immune compromised patients, pneumococci infiltrate the meninges and cause life threatening inflammation. Recently, we have established a transwell-based Blood Brain Barrier (BBB) model with human endothelial-like cells and astrocytes providing a reliable tool to study pathogenesis mechanisms of bacterial and parasitical transmigration of the cellular barrier between vascular system and cerebral tissue. We applied this model with the aim to study the role of the polysaccharide capsule of a clinical isolate from a meningitis patient identified as a highly virulent serotype 7F pneumococcus. After optimization of the transformation procedure for this clinical isolate, we successfully deleted the capsule gene locus and confirmed loss of capsule expression by southern blot hybridization and electron microscopic visualization. In consistence with the already described inhibiting effect of capsular polysaccharides on phagocytosis by macrophages, the serotype 7F strain was more efficiently endocytosed by human monocytes (U937) as the corresponding capsule deficient mutant. Infection analyses using the BBB model with the serotype 7F wild type isolate and the mutant strain demonstrated a significantly reduced transmigration activity of the capsule-deficient serotype 7F. These results were further confirmed by infection of a second blood brain barrier model using Human Brain Microvascular Endothelial Cells (HBMEC). Interestingly, a collection of serotype 7F strains isolated from cerebral liquor or blood of children suffering from meningitis revealed a higher transmigration capacity as monitored in comparable infection studies with serotype 7F isolate derived from a non-invasive colonization. These data shed new light on the role of pneumococcal capsule in invasive infection process of the cerebral tissues and underline the requirement of adequate infection models to elucidate the diversity of highly virulent clinical isolates.

Keywords: *Streptococcus pneumoniae*; Blood brain barrier; Transmigration; Capsule

Abbreviations: BBB: Blood Brain Barrier; FCS: Fetal Calf Serum; PBS: Phosphate Buffered Saline; CNS: Central Nervous System; CFU: Colony Forming Units; MOI: Multiplicity of Infection

Introduction

The facultative pathogen *Streptococcus pneumoniae* is commensally colonizing the upper respiratory epithelium of humans and is described as one of the most important pathogens worldwide [1]. According to the European Center for Disease Prevention and Control (ECDC), up to 14750 cases of invasive pneumococcal diseases in 24 EU and EEA/EFTA member states were confirmed in 2008. The clinical spectrum ranges from upper airway infections such as sinusitis and otitis media to pneumonia, invasive blood stream infections and meningitis, mostly affecting the youngest (under 5 years of age) and the elderly people (over 64 years of age) [2]. A threatening development is mirrored by the occurrence of penicillin and erythromycin-non-susceptible *S. pneumoniae* strains reaching more than 25% in southern and eastern European countries including France, Hungary and Italy [3]. Community-acquired bacterial meningitis remains an ever-present threat, even in developed countries, despite the implementation of childhood vaccination programs and effective antimicrobial agents [4,5]. The most common causes for bacterial meningitis are *Streptococcus pneumoniae* and *Neisseria meningitidis*, with the first being responsible for two-thirds of cases in Europe and the United States [4,5]. In present times, despite advances in medical care, mortality from pneumococcal meningitis ranges from 16% to 37% and neurological sequelae including hearing loss, focal neurological deficits, and cognitive impairment, are estimated to occur in up to 52% of surviving patients [6]. During the last decades of years, intensive scientific investigations are focused on the elucidation of pathogenesis mechanisms and virulence factors involved in this invasive infection disease.

The capsular polysaccharide represents one of the most important pneumococcal virulence factors and associations of capsular types with the severity of invasive pneumococcal disease have been described several times [7,8]. Amount and composition of polysaccharide capsule expression is used to distinguish between up to 94 pneumococcal serotypes (oral communication with Dr. M. van der Linden, NRZ Aachen). Each serotype is characterized by an individual polysaccharide capsule, which mediates the escape from phagocytosis or complement factor binding and prevents entrapment by neutrophil extracellular traps [9-13]. The predominant serotypes associated with severe pneumococcal diseases vary with respect to geographical and demographical situation and respective vaccination programs. In European countries, serotype

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7F isolates still belong to the leading serotypes among children [14] although capsule determinants of this serotype are included in the 13-valent conjugate vaccine (Prevnar 13®). In addition to the capsular polysaccharides several pneumococcal virulence factors have been identified mediating colonization and invasive dissemination within the different host tissue sites following the course of infection [15]. By recruitment of host-specific proteins like vitronectin, pneumococci subvert the multiple connective properties of these factors for cellular adherence and internalization into both epithelial and endothelial cells [16,17]. Some pneumococcal surface components are also directly involved in interaction with human endothelial cells. Receptor-mediated interaction was described for binding of the virulence factor PspC (also named CbpA and SpsA) to the cell surface laminin receptor, which promotes bacterial adherence to the brain endothelium and is suggested to contribute to transmigration of Bacterial Blood Brain Barrier (BBB) [18,19].

The *in vivo* BBB consists of tightly linked endothelial cells embedded in a specialized basement membrane and separates the cerebrospinal fluid from the bloodstream [20]. Representing a highly selective border, the BBB protects the Central Nervous System (CNS) from most toxic substances and pathogens. During the last decades of years, a variety of BBB models have been generated to obtain reliable *in vitro* tools to promoting drug development in preclinical studies [21-23]. Transwell-based model systems enable the investigation of the transmigration process in three dimensions compared to single endothelial monolayers cultured in well plates. The complexity of currently used transmigration models comprises up to three different cell types and different transwell membrane coatings [24].

Our recently designed and evaluated BBB models designated as ECV304-C6 BBB and HBMEC-BBB allow the analysis of transmigration mechanisms of bacterial and parasitical pathogens including *streptococci* and African trypanosomes [25,26] causing inflammatory CNS diseases like bacterial meningitis [27] and human African trypanosomiasis [28]. In this study we have used these models to investigate the role of pneumococcal polysaccharide capsule in bacterial transmigration of BBB using a highly invasive serotype 7F isolate from a meningitis patient. After successful capsule deletion mutagenesis of this clinical isolate, we have performed analysis of phagocytosis by U937 cells. Furthermore, transmigration efficiencies of 7F serotype strains derived from different clinical sources were compared using the HBMEC-BBB model.

Material and Methods

Bacterial culture conditions

Four *Streptococcus pneumoniae* serotype 7F isolates were obtained from Dr. Mark van der Linden from the National Reference Centre (NRZ) for Streptococci in Aachen, Germany including i) two cerebrospinal fluid isolates from meningitis patients (designated 7F-isolate1 and 7F-isolate4) ii) a non-invasive isolate from the nasopharyngeal cavity (designated 7F-isolate2) and iii) a blood isolate from a meningitis patient (designated 7F-isolate3). The strains were cultured to mid-log phase at 37 °C on Columbia blood agar plates (Becton Dickinson) or in Todd-Hewitt broth (Roth) supplemented with 1.0% yeast extract (THY). The 7F-isolate1 was used for capsule deletion mutagenesis and was cultivated prior to infection analyses under antibiotic pressure using 50 mg/mL kanamycin.

Deletion mutagenesis of capsule genes

The deletion mutagenesis of the clinical serotype 7F-isolate was performed with a PCR fragment amplified from the capsule deletion mutant *D39Δcps* [29]. This mutant was generated by exchange of the capsule gene locus with a kanamycin resistance gene using residual flanking regions of the *ali*A and *dex*B genes required for capsule expression [30] as homologous DNA sequences for DNA recombination [31]. After preparation of genomic DNA from the capsule-deficient mutant *D39Δcps* using Cetyltrimethylammoniumbromid (Roth), the DNA was used as template for capsule locus-specific PCR using the primer pair: dexBfor: 5´GAATCAGGCGACCTTTGGAG3´and aliArev: 5´CAAGGTAGGTTGATGGATCGGC3´ and the following PCR conditions: initial denaturation at 95°C for 1 min, amplification in 35 cycles repeating denaturation at 95°C for 45s, hybridization at 60°C for 45 s and elongation for 4 min at 72°C. The single terminal elongation was performed for 5 min at 72°C.

After cultivation of the serotype 7F isolate in THY medium at pH of 8.0 to an Optical Density (OD) at 600 nm of 0.11, 2.5 µg of the Competence Stimulating Peptide1 (CSP1) representing the peptide sequence EMRLSKFFRDFILQRKK was added an incubated for 20 min with the bacteria at 37°C. After a cold shock for 4 min on ice, the bacteria were incubated with 7.3 µg/µL of the amplified PCR product and cultivated for 30 min at 30°C followed by 90 min of incubation at 37°C without shaking. Mutants were selected after growth on blood agar plates containing 50 µg/mL kanamycin and 50 µg/mL neomycin. The positive selected kanamycin resistant clone was used for growth curve analyses in comparison to the wild type strain. After overnight culture on blood agar plates, the strains were inoculated in THY medium supplemented with 1% yeast extract, and in THY medium with 1% yeast extract, 20 µg/mL kanamycin and 20 µg/mL neomycin with a start OD at 600 nm of 0.1. The strains were cultured at 37°C and 5% CO_2 and the OD was measured in one hour time intervals up to 5 h. To confirm capsule deletion, the inserted PCR product carrying the kanamycin resistance gene was reamplified from the prepared genomic DNA from the mutant using the primer dexBfor and aliArev. These primer pair was also used to create a Digoxigenin-11-2'-desoxy-uridine-5'-triphosphate-labelled oligonucleotide to detect positive mutants by southern blot hybridization. The genomic DNA from the serotype 7F-isolate1 and the capsule-deficient mutant were prepared in addition to the DNA of a serotype 2 pneumococcus (D39) and the corresponding capsule negative mutant (*D39Δcps*). The genomic DNA from all strains was subjected to *Hin*dIII-digestion over night at 37°C with 20 U of the enzyme. The PCR product of the truncated mutagenesis fragment was used as hybridization control (ctrl). After electrophoresis the DNA was transferred by southern blotting onto a nylon membrane and subjected to hybridization with the digoxygenin-labelled probe using the DIG Nucleic Acid Detection Kit (Roche) according to the manufacturers´guidelines.

Transmigration analysis using blood brain barrier model

The BBB models were prepared as described in [25]. In brief, human endothelial cells ECV304 (ECACC 92090409), murine astrocytes C6 (ECACC 92091712, [32]) or HBMEC cells (human brain derived microvascular endothelial cells [33]) were cultivated at 37°C, 5% CO_2. ECV304 was cultivated in Quantum286 with 2 mM L-glutamin (PAA), C6 was cultivated in HAM´s F12 medium (PAA) supplemented with 2 mM L-glutamin (PAA) and 10% fetal calf serum (FCS, PAA) and HBMEC were cultivated in RPMI1640 medium supplemented with 10% FCS, NU-serum IV (Becton Dickenson), 100 mM sodium pyruvate (1:500, PAA, Germany), non essential amino acids (1:100, PAA), MEM vitamins (1:100, PAA) and 200 mM L-glutamin (1:100).

Matrigel™-precoated transwell insert systems with membranes containing pores of 8.0 µm in diameter (BD BioCoat, 354480) were used to

create a multilayer BBB model. After seeding of $5x10^4$ C6 cells underneath the PET (polyethylene terephthalate) membrane, the matrigel™ of the upper membrane side was rehydrated with 500 µL Q286 medium for 2 h according to the manufacturer (BD Biosciences). Subsequently 6 x 10^4 ECV304 cells were seeded on top the matrigel™ layer onto the transwell membrane. For generation of the HBMEC BBB, $9x10^4$ endothelial cells were seeded on top of the rehydrated matrigel™ into the transwell insert. After 5 days of cultivation the two BBB models were used for transmigration analyses. The barrier integrity of both barrier systems was controlled with the non-invasive *E. coli* strain HB101 [25].

After cultivation of the pneumococci to mid log phase, the bacteria were sedimented and adjusted to 1x108 bacteria/mL in infection medium. The BBB models were infected with 1 x 107 pneumococci for up to 6 h at 37°C and 5% CO_2. The bacteria were cultivated to mid log phase and incubated with the BBB for 6 h. Transmigration was analyzed by determination of the number of pneumococci transmigrated through the cellular barrier model after counting of bacterial CFU from the lower transwell compartment. In addition the amount of positively transmigrated barrier inserts was presented as percentage of all infected inserts (transmigration rate). The amount of nine inserts was set to 100% and the number of transmigrated inserts was calculated as percentage in relation to the total insert number.

Phagocytosis by U937 cells

The human monocytic macrophage-like cell line U937 was cultivated in RPMI1640 (PAA) supplemented with 2 mM L-glutamin (PAA) and 10% FCS (PAA). Prior to infection analyses, the grown monocytes were sedimented and resuspended in RPMI1640 medium. In order to stimulate differentiation of the monocytes, 1 x 10^6 cells/mL were incubated with 10 nM Phorbol Myristate Acetate (PMA) for up to 48 h at 37°C and 5% CO_2. The differentiated U937-macrophages were infected with either serotype 7F wild type pneumococci or the capsule deficient mutant using an MOI of 10 for 1 h at 37°C and 5% CO_2. After washing with prewarmed PBS, the cells were incubated with RPMI1640 supplemented with 10 mg/mL gentamycin and 2 mg/mL penicillin for 1 h at 37°C and 5% CO_2. After incubation the cells were washed three times with PBS and after incubation with 1% saponin solution for 10 min, the cell lysates were plated in serial dilutions on THY agar plates with and without kanamycin to determine the CFU of phagocytosed pneumococci. In parallel infected U937 cells were subjected to saponin treatment and CFU determination without incubation with gentamycin and penicillin.

Statistical analysis

Transmigration experiments were repeated in at least two independent test series with a minimum of three parallels each. Results were expressed as mean ± SD. Statistical significance was calculated with Student's t-test (Microsoft Excel 2007) and is represented in the figures by asterisks (*) indicating different *p*-values (*$p \leq 0.05$; **$p \leq 0.01$).

Electron microscopic visualization of capsule

After sedimentation of pneumococci, the pellet was resuspended in 200 µL cacodylate buffer (pH 2.5) and incubated with cationiced nanogold-particles (10 nm in diameter) for 15 min at room temperature. After washing twice with cacodylate buffer bacteria were fixed with 3% formaldehyde for 30 min at 4°C and washed with TE-buffer. 2 µL of the resuspended bacteria in TE-buffer was placed onto butvar-coated 300 mesh copper grids and allowed to settle for 3 min. Grids were then washed on a drop of TE-buffer and on drops of distilled water. Grids were blotted dry on filter paper, air dried and examined in a Zeiss EM 910 transmission electron microscope (Zeiss, Oberkochen, Germany) at an acceleration voltage of 80 kV. Images were recorded digitally at calibrated magnifications with a Slow-Scan CCD-Camera (ProScan, 1024x1024, Scheuring, Germany) with ITEM-Software (Olympus Soft Imaging Solutions, Münster, Germany). Contrast and brightness were adjusted with Adobe Photoshop CS3.

Results

Capsule deletion of the clinical meningitis 7F-isolate

In contrast to the naturally competent laboratory strains with well known competence peptide signatures, transformation of clinical pneumococcal isolates remains a challenge. After successful transformation of the clinical serotype 7F-isolate with a DNA sequence comprising the kanamycin cassette flanked with residual sequence stretches from the capsule genes used for homologous recombination, capsule deficient clones were selected. After growth on blood agar plates containing kanamycin as selection marker, a rough colony morphology of the transformants indicated insertion of the kanamycin cassette and loss of capsule expression in contrast to the shiny and mucous colony morphology of the wild type strain (data not shown). The mutant strain could be cultivated in medium supplemented with kanamycin and showed typical growth behavior similar to the wild type strain grown in medium without antibiotics (Figure 1A). Both strains reached the exponential phase after 240 min of cultivation and swiveled into the lag phase at an OD of 1.0. Moreover, southern blot analysis confirmed the insertion of the DNA fragment containing the kanamycin resistance cassette and demonstrated a significant reduction of DNA sequence size of capsule gene locus (Figure 1B). In contrast to the thick capsule layer marked by cationic gold particles of the 7F wild type isolate (Figure 1C, black arrows), only minor amounts of cationic nanogold-particles were detected directly bound to the cell wall of the capsule-deficient mutant being indicative for the absence of a capsule (Figure 1C, arrow heads). Therefore, molecular, morphological and visual approaches confirmed successful capsule deletion of the clinical serotype 7F-isolate, which is named 7F-isolate1Δ*cps* and was used for further characterization of virulence properties.

Polysaccharide capsule protects against phagocytosis

It has been known that bacterial capsules protect against clearance by the immune defense due to masking immunogenic surface components [10]. In order to confirm the role of the polysaccharide capsule as a surrounding coat preventing uptake of the serotype 7F strain, phagocytosis analyses were performed using the PMA differentiated macrophage-like cell line U937. In contrast to the amount of 6.67 x 10^3 internalized bacteria of the highly encapsulated wild type strain, phagocytosis of the 7F-isolate1Δ*cps* mutant was significantly higher reaching an amount of 5.5 x 10^5 bacteria after 2 h of infection (Figure 2). These data confirm the protective function of the polysaccharide capsule against bacterial uptake by phagocytic cell line U937.

Capsule deletion reduces pneumococcal transmigration capacity

The role of the pneumococcal capsule of the 7F-serotype in transmigration of the blood brain barrier and subsequently in induction of severe inflammation of the meninges was analyzed using two established *in vitro* models of the blood brain barrier. The ECV304-C6 BBB was composed of a confluent endothelial-like cell layer grown onto matrigel-coated transwell membranes and C6 astrocytes that were

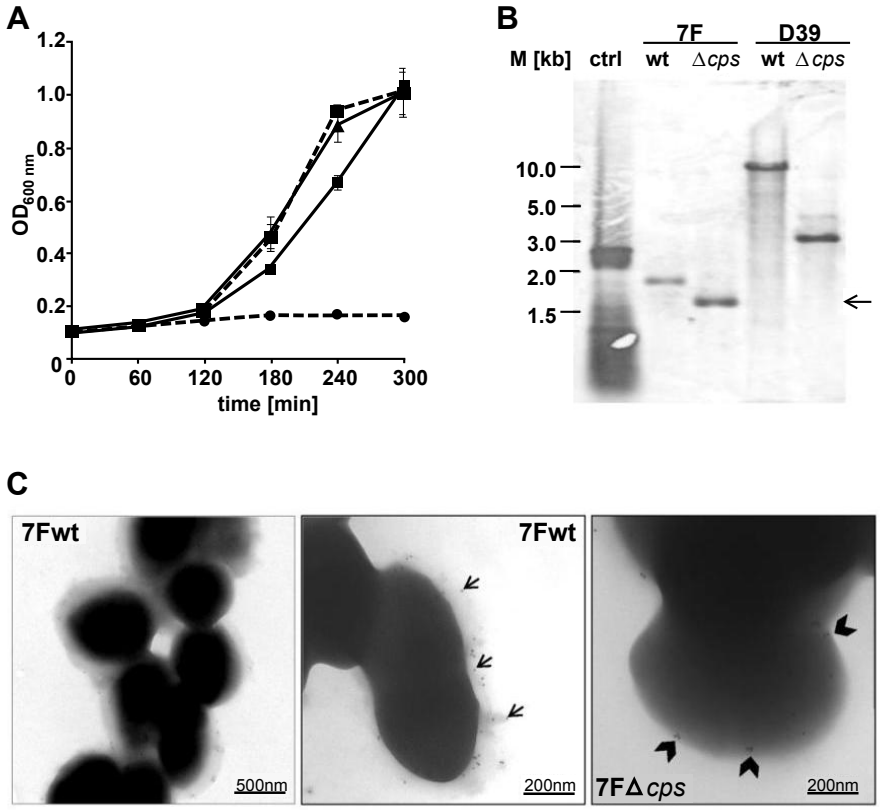

Figure 1: Molecular visualization of capsule deletion of the serotype 7F meningitis isolate.
A) Both wild type (wt, filled line with squares) and capsule deficient mutant (*Δcps*,filled line with triangels) showed similar growth behavior in complete medium. In contrast to the wild type strain (wt+AB, dotted line with circles) growth behavior of 7F-isolate1*Δcps* (*Δcps*+AB, dotted line with squares) was not impaired in medium supplemented with kanamycin. Standard deviation was calculated from triplicates, the growth curves were repeated twice.
B) Southern blot hybridization was performed after separation of *Hin*dIII-digested genomic DNA from the 7F-isolate1 (7F wt, lane 2), the capsule mutant (7F *Δcps*, lane 3). The pcr product of the mutagenesis fragment was used as control probe for hybridization in lane 1 (ctrl). *Hin*dIII-digested genomic DNA of D39 (D39 wt) and corresponding capsule negative mutant (D39 *Δcps*) representing well described comparative controls were probed in lane 5, 6. Southern blot analyses confirmed insertion of the kanamycin resistance cassette and loss of capsule gene locus resulting in reduced fragment size (arrow).
C) Transmission electron microscopic visualization of capsule expression after labeling with cationic nanogold-particles. Capsule surrounded the 7F wild type bacteria (7Fwt, black arrows). Deletion of capsular polysaccharides resulted in loss of carbohydrate structures covering the cell wall (7F*Δcps*, arrow heads). Size scale represents 500 nm (left panel) and 200 nm (middle and right panel).

cultivated underneath the transwell membrane in order to promote confluence of the endothelium. After incubation of this model with 7F-isolate1 wild type pneumococci or the capsule deficient mutant, the amount of bacteria transmigrated through the cellular barrier model was determined by counting of bacterial CFU from the lower transwell compartment. In addition the amount of positively transmigrated barrier inserts was determined and presented in percentage of all infected inserts (transmigration rate). The capsule deletion mutant was not able to transmigrate through the barrier at all, although nine barriers have been infected in total (Figure 3A). In contrast a high transmigration rate of 66.7% was reached by the serotype 7F wild type strain, indicating an effective bacterial transmigration of six from nine infected inserts. The transmigration rate of the strains was also determined using a second BBB model consisting of a confluent HBMEC cell layer cultivated on top of a matrigel coated transwell membrane. In accordance with the result obtained with the ECV304-C6 BBB, all barrier inserts were transmigrated by the 7F-isolate1 wild type strain reaching a transmigration rate of 100% (Figure 3B). In this model the corresponding capsule-deficient mutant only transmigrated 50% of the barrier systems (Figure 3B). These results indicated that the capsule expression not only protects against cellular internalization but also promotes transmigration of the serotype 7F strains.

Similar transmigration of different serotype 7F pneumococci in HBMEC-BBB model

A high amount of serotype 7F strains have been isolated from patients suffering from invasive systemic infections during the last five years [34]. Since 7F-serotypes were also known as harmless colonizers of the human nasopharynx, transmigration analyses of four serotype 7F strains derived from different sources were performed to assess differences in BBB transmigration. A cerebrospinal fluid isolate from meningitis patients (designated 7F-isolate4), a non-invasive isolate from the nasopharyngeal cavity (designated 7F-isolate2) and a blood isolate from a meningitis patient (designated 7F-isolate3) were included in this study. The cerebrospinal fluid meningitis isolate 7F-isolate1 previously used for capsule deletion mutagenesis was also employed. Results of transmigration analyses using the HBMEC BBB indicated very similar amounts of transmigrated bacteria for all tested isolates

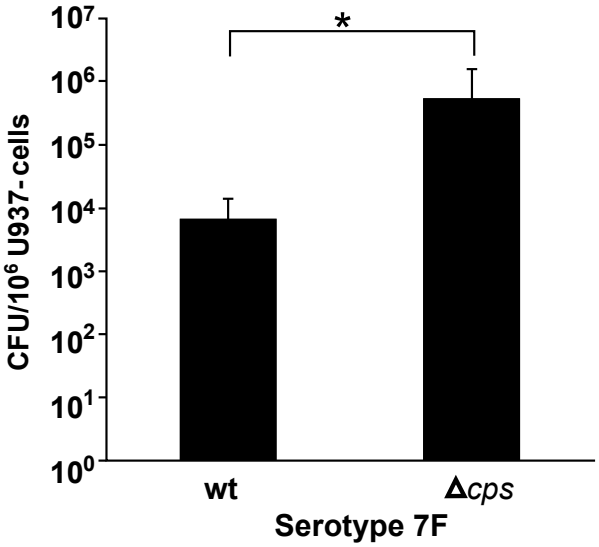

Figure 2: Phagocytosis of capsulated and non-capsulated meningitis isolates by human phagocytic cell line U937.
Uptake of highly capsulated serotype 7F pneumococci and the 7F-isolate1*Δcps* by the macrophage-like U937 cells was analyzed after *in vitro* infection of 1 x 10^6 U937 cells with 1 x 10^7 pneumococci (MOI of 10). Phagocytosis of the non-capsulated mutant was significantly higher as uptake of the wild type strain. Standard deviation was calculated from triplicates from two independent standardized experimental setups.*) $p < 0.05$.

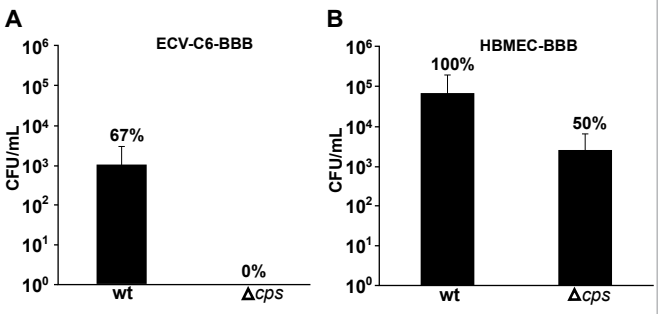

Figure 3: Transmigration of serotype 7F and corresponding capsule mutant through blood brain barrier models.
Serotype7F wild type bacteria and 7F-isolate1*Δcps* were incubated with A) the ECV304-C6-BBB and B) the HBMEC-BBB for 6 h using an MOI of 10. Transmigration rates were determined by counting of CFU from the bottom well. Calculation of transmigration efficiency was performed based on the amount of positive transmigration events and presented as percentage from the total number of tested inserts. A total number of nine ECV304-C6-BBB transwells and of nine HBMEC-BBB transwells were used for calculation for each strain. Standard deviation represents triplicate data from two independent test series.

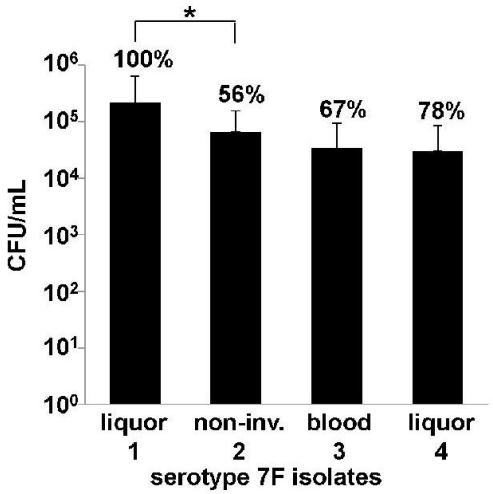

Figure 4: Comparison of transmigration efficiency of different serotype 7F isolates using the HBMEC-BBB model.
In addition to the 7F-liquor isolate1, a further cerebrospinal fluid isolate from meningitis patients (7F-isolate4), a non-invasive isolate from the nasopharyngeal cavity (7F-isolate2) and a blood isolate from a meningitis patient (7F-isolate3) were incubated with the HBMEC-BBB for 6 h using an MOI of 10. Transmigration rate and efficiencies were calculated by determination of transmigrated CFU after plating and overnight culture on blood agar plates. Results indicated higher transmigration efficiency for the cerebrospinal and blood isolates derived from invasive meningitis in contrast to less transmigration determined for the serotype 7F isolated as non-invasive colonizer from a healthy child. Statistical significance calculation determined a p-value of < 0.05 for differences between the non-invasive isolate 2 and the liquor isolate 1 from a meningitis patient.

(Figure 4). Interestingly, the transmigration rate of the non-invasive isolate2 (55.6%) reaches only half of the transmigration rate of the liquor isolate 1 (100%) and was lower compared to the blood isolate 3 (66.7%) and the liquor isolate 4 (77.8%) (Figure 4). These data point to differences in the transmigration efficiencies of isolates of the same serotype but from different clinical backgrounds.

Discussion

The outbreak of invasive pneumococcal disease in human is attributed to special prerequisites including colonization by a pneumococcal strain that has not yet been recognized by host immunity and to alterations of the natural barriers or immune defense components [35,36]. Thereby the efficiency of pneumococcal colonization is dominantly affected by the biochemical composition and the expressed amount of capsular polysaccharides, which are used as determinants to discriminate between different pneumococcal serotypes [37]. Despite the implementation of childhood vaccination programs, the serotypes of most isolates from invasive diseases share remarkable consistency with respect to (i) their tendency to cause invasive or mucosal disease [38-41], (ii) their age distribution [42] and (iii) their antimicrobial resistance profiles [43]. With the aim to expand the immune protection against different pneumococcal seroytpes and to minimize contradictory effects like serotype replacement, the amount of capsule determinants included in conjugate vaccines continuously increased by generating up to 13 valent vaccine (Prevnar13®, Wyeth/Pfizer) during the last ten years. In contrast to serotypes such as 19F and 23F, which are predominantly present in healthy carriers, serotype 7F isolates belong to the strains that are mainly observed to cause invasive disease and are rarely observed among carriers. Moreover, the serotype 7F remained one example of a predominant serotype constantly isolated from invasive infections from children [34]. Due to the high prevalence, serotype 7F isolates were chosen in this study to characterize pathogenic properties that may give an explanation for the endemic persistence of this serotype. The successful deletion of the capsule gene locus of a serotype 7F meningitis isolate resulted in a significant change of colony morphology and was visualized by transmission electron microscopy after labeling with cationic nanogold particles. This insertion deletion mutagenesis conveyed an adequate capsule-deficient mutant to study

the function of the polysaccharide capsule in phagocytic uptake and barrier transmigration of this serotype. Wood and Smith were the first describing the anti-phagocytic activity of pneumococcal polysaccharide capsule of a serotype 3 strain [44]. In consistence with already investigated serotypes, the serotype 7F capsule also confers protection against phagocytosis in our studies. Non-capsulated mutants rarely cause invasive infection and are highly attenuated in systemic infection models due to more efficient opsonophagocytic clearance [45]. In order to assess the function of the capsular polysaccharides in pneumococcal transmigration of a cellular BBB, we performed infection analysis with two BBB model systems. Our barrier model systems employed in this study combined the advantage of multicellular complexity with functional specificity. The ECV304-C6 system has already successfully established as an experimental model for analysis of BBB penetration by African trypanosomes causing human trypanosomiasis and has been confirmed as reliable analytical method for the quantification of drug penetration properties [25,26]. In addition to this multicellular model we employed a second system based on a Brain Derived Endothelial Cells (HBMEC) in our assays in order to elucidate transmigration efficiencies with this brain-tissue specific barrier system. Although HBMEC comprise a higher proportion of endothelial properties than ECV304 cells, both cell types express the endothelial laminin receptor (data not shown), which has been demonstrated to be involved in pneumococcal adherence [18,19].

Nelson and coworkers have already discussed the variable functionality of polysaccharide capsules extending its antiphagocytic activity to distinct functional roles during infection and colonization. Immunohistochemical studies indicated an efficient capsule-driven luminal mucus translocation of pneumococci to epithelial cell surfaces [46]. In accordance with the results of luminal mucus transmigration published by Nelson, our data demonstrated a significantly higher transmigration of capsulated serotype 7F strains from blood and liquor of meningitis patients in contrast to the low amounts of transmigrated bacteria of the capsule-negative mutant and the non-invasive serotype 7F isolate. These results indicate that the polysaccharide capsule promotes transmigration of serotype 7F pneumococci through BBB systems. Nevertheless, the function of capsule in pneumococcal adherence to endothelial and epithelial cells is contradictory discussed to its role in transmigration. Several infection analyses provide strong body of evidence that the capsule prevents attachment of pneumococci to epithelial cells, as well as to endothelial cells [47]. These results are supported by *in vivo* mouse colonization studies demonstrating a more efficient colonization of mucosal nasopharyngeal surfaces by a transparent phenotype of pneumococci, which produces smaller amounts of capsular polysaccharide, whereas the capsulated opaque phenotypes were more virulent in systemic infections [48,49]. It has been suggested that the increased cell adherence and surface colonization properties of less capsulated, transparent phenotypes is attributed to the availability of adhesive surface determinants, which are not hidden below a thick capsule layer [46-49]. Pneumococci express several adhesive surface components mediating a direct receptor-mediated cell surface adherence. The group of pneumococcal adherence factors include the pneumococcal surface protein C (PspC, SpsA) [50], which binds the polymeric immunoglobulin receptor (pIgR) and the phosphorylcholine moieties of cell wall teichoic acids interacting with the Platelet Activating Factor Receptor (PAFR) [51]. In addition, the intimate approaching to the cell surface by pneumococci is promoted by the ability of pneumococci to recruite extracellular matrix proteins like fibronectin and vitronectin, which mediate subsequently the internalization into the cytoplasm of epithelial and endothelial cells in an integrin-dependent process [52,16]. Interestingly, electron microscopic studies of pneumococcal encapsulation status after epithelial cell culture infections clearly visualized a substantially reduced capsule layer of highly encapsulated pneumococci at the sites of direct bacterial contact to cell surfaces [53]. Moreover, in contrast to highly encapsulated pneumocccal strains recovered from bloodstream of infected mice, bacteria recovered from the intracellular cell environment were less encapsulated. These data provided evidence for the presence of response mechanisms regulating capsule expression in environment-dependent manner. Referring to the observation of reduced capsule production at cellular contact sites of epithelial monolayers as described by Hammerschmidt and coworkers, serotype 7F pneumococci may decrease their capsule expression in similar manner in course of the *in vitro* infection process.

Moreover, the high transmigration rates of the serotype 7F wild type bacteria may be a result of direct transmigration events without an intermediate adherence step. In addition to the intracellular route of pneumococcal transmigration, which is initiated via an intimate cellular adherence followed by receptor-mediated internalization, cell culture infection analysis and mouse infection studies indicated that pneumococci also perform paracellular transmigration by intercellular junction cleavage [54,55]. In contrast to the inhibiting effect of the capsule with respect to cellular adherence, the reduced amount of bacterial attachment sites due to coverage by capsule may be of advantage for a paracellular penetration of the cell layer. A paracellular transmigration mechanism may also be supported by additional proteolytic activities of pneumococci. We have shown previously that pneumococci subvert proteolytical activity of human fibrinolytic plasmin to degrade fibrin thrombi and proteins of the extracellular matrix like laminin [56,57]. Furthermore, clinical studies indicated a direct correlation between pneumococcal induced breakdown of BBB and pleocytosis with high levels of the plasminogen activator urokinase in meningitis patients [58]. Although transmigration analysis using cell-free matrigel-coated transwell inserts confirmed that the extracelluar matrix alone represents no barrier function (data not shown), the recruited proteolytical activity may facilitate the transmigration process. In addition, pneumococci express a cytotoxic and cytolytic pneumolysin, which has also been discussed as potent cofactor mediating pneumococcal transmigration through a destroyed endothelial barrier in paracellular manner [59,60].

Our study generated important information on the contribution of 7F polysaccharides to pneumococcal transmigration of BBB including data of a highly virulent clinical isolate and the capsule-deleted mutant. A further detailed investigation of each single step of the transmigration process including amount of capsule expression at different transmigration steps and proteolytic cleaving activities will provide deeper insights into the specific mode of transmigration mechanisms. The presented data shed new light on the broad versatility and dominant role of pneumococcal polysaccharide capsule with regard to the efficiency of pneumococcal transmigration of blood brain barrier.

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