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Effect of epithelial cell type on *in vitro* invasion of non-typeable *Haemophilus influenzae*

Neeraj Kumar Singh, Dale A. Kunde, Stephen G. Tristram *

School of Health Sciences, University of Tasmania, Launceston, Tasmania, Australia

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ABSTRACT

Non-typeable *Haemophilus influenzae* (NTHi) have been shown to have variable ability for *in vitro* invasion with a range of epithelial cells, and increased invasion of BEAS-2B cells has been associated with altered penicillin binding protein3 (PBP3), which is concerning as these strains are increasing worldwide. The aim of the study was to investigate the effect of respiratory cell type and the presence of altered PBP3 on the *in vitro* invasion of NTHi. A collection of 16 clinical NTHi isolates was established, 7 had normal PBP3, and 9 had altered PBP3 as defined by an N526K substitution. The isolates were tested for invasion of BEAS-2B, NHBE, A549 and NCI-H292 respiratory epithelial cells *in vitro* using a gentamicin survival assay, with invasion measured as the percentage of intracellular organisms relative to the initial inoculum. The overall median invasion for the 16 NTHi isolates for cell types BEAS-2B, NHBE, A549 and NCI-H292 cells were 3.17, 2.31, 0.11 and 1.52 respectively. The differences were statistically significant for BEAS-2B compared to A549 ($P = 0.015$) and A549 compared to NCI-H292 ($P = 0.015$), and there were also very marked differences in invasion for some individual isolates depending on the cell type used. There was a consistent bias for invasion of isolates with normal *versus* abnormal PBP3: and this was statistically significant for BEAS-2B (0.07 to 9.90, $P = 0.031$) and A549 cells (0.02 to 1.68, $P = 0.037$). These results show that NTHi invasion of respiratory epithelial cells *in vitro* is both strain dependant and influenced significantly by the cell line used, and that the association between altered PBP3 and increased invasion is conserved across multiple cell lines.

1. Introduction

Non-typeable *Haemophilus influenzae* (NTHi) is a commensal of the human nasopharynx and is also associated with a large burden of disease attributable to its role in a range of opportunistic infections, such as sinusitis (41% of cases in children), otitis media (55–95% of cases in children), community acquired pneumonia (20–94% of cases) and acute exacerbations of chronic obstructive pulmonary disease (>90% of cases) (Van Eldere et al., 2014; Clementi and Murphy, 2011; King, 2012; López-Gómez et al., 2012). Although NTHi has traditionally been considered an extracellular pathogen, there is significant *in vivo* and *in vitro* evidence that the organism can invade and persist within host epithelial cells (Ketterer et al., 1999; Clementi and Murphy, 2011; King, 2012; López-Gómez et al., 2012). Adhesion and invasion are thought to be key events in the pathogenesis of NTHi, and the ability to enter and survive in the intracellular space has been proposed as a means by which the organism evades both the host immune response and the inhibitory effects of antibiotics during persistent, recurrent or intractable infections (Swords et al., 2000; Hotomi et al., 2010; Clementi and Murphy, 2011; King, 2012).

NTHi entry into epithelial cells appears to be a complex process that involves bacterial adhesins, host cell receptors, signalling, endocytosis and trafficking pathways (Clementi and Murphy, 2011; López-Gómez et al., 2012). A range of outer membrane proteins and lipooligosaccharides have been identified as adhesins (Swords et al., 2000; Raffel et al., 2013), and there is some evidence to suggest that altered penicillin binding protein 3 (PBP3) may also be involved in adhesion and subsequent invasion. Two recent studies found that isolates with altered PBP3, which is associated with decreased binding affinity and susceptibility to β-lactam antibiotics, are more invasive in BEAS-2B cells than isolates with normal PBP3 (Okabe et al., 2010; Atkins et al., 2014) but the mechanism has not been clarified. The relationship between altered PBP3 and invasion is particularly concerning because the prevalence of these strains is increasing worldwide (Kishii et al., 2011; Witherden et al., 2011).

Despite increased understanding of some mechanisms involved with invasion, the relationship between intracellular NTHi and pathogenesis is still unclear and many studies have shown enormous strain-to-strain variation in the *in vitro* invasive ability of clinical isolates (Swords et al., 2000; Ahren et al., 2001; Clementi and Murphy, 2011;

* Corresponding author.
E-mail address: Stephen.Tristram@utas.edu.au (S.G. Tristram).

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Swords, 2012; Goyal et al., 2015). More specifically, Atkins et al. (2014) was unable to demonstrate a significant difference in invasion between isolates from various clinical sites and those isolated from the nasopharynx of healthy carriers, and when Hotomi et al. (2010) studied five isolates from cases of intractable otitis media, two of these were non-invasive.

One of the limitations in understanding the relationship between intracellular NTHi and pathogenesis, is a lack of a standardised model for studying invasion. Most studies have used a simple monoculture method where the model is restricted to epithelial and bacterial cells, whereas coculture methods, that combine epithelial, bacterial and other host cells may better reflect the *in vivo* environment (Duell et al., 2011). Clementi and Murphy (2011) acknowledges that careful selection of both bacterial strains and host cell types for *in vitro* invasion studies is required, yet even a brief review of the literature reveals that a very large range of both respiratory and non-respiratory, and primary and immortal cell lines have been used, often without explanation or justification for the cell type used (Ketterer et al., 1999; Swords et al., 2000; Ahren et al., 2001; Hotomi et al., 2010; Okabe et al., 2010; López-Gómez et al., 2012; Raffel et al., 2013). It is unclear whether an isolate that shows *in vitro* invasion in one cell type will be similarly invasive in another cell type, and this makes comparisons between studies very difficult.

The aim of this study is to compare the ability of a range of NTHi strains to invade a variety of respiratory cell types *in vitro* using a monoculture model and also to see if the increased invasion associated with altered PBP3 and BEAS-2B cells extends to other cell types.

2. Methods

2.1. Bacterial strains and culture conditions

A collection of 16 clinical isolates of NTHi from de-identified patients was established, from the following sites and clinical conditions: ear (otitis media, $n = 4$), eye (conjunctivitis, n = 4), sputum (lower respiratory tract infection, $n = 5$) and oropharynx (normal flora, $n = 3$). Bacterial identity was confirmed using a PCR algorithm for hpd, fucK and SodC as described previously (Witherden and Tristram, 2013) and characterised as having normal or abnormal PBP3 using the PCR method of Witherden et al. (2011). Isolates were stored in glycerol broth in liquid nitrogen and sub-cultured at least twice on chocolate agar at 37 °C in 5% CO_2 for 24 h before use in invasion assays.

2.2. Epithelial cell lines and cell culture

Immortalised BEAS-2B (Sigma-Aldrich), NHBE (Lonza), A549 (Public Health England) and NCI-H292 (ATCC) epithelial cell lines were grown and maintained in LHC8 (Gibco), BEGM (Lonza), DMEM growth medium (Sigma-Aldrich) supplemented with 2 Mm Glutamine and 10% Fetal Bovine Serum and RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), respectively at 37 °C in 5% CO_2.

2.3. Invasion assay

The invasive ability of the different NTHi strains was determined for each epithelial cell line using the gentamicin survival assay as described previously (Okabe et al., 2010; Atkins et al., 2014). Briefly, bacterial isolates grown overnight on chocolate agar were used to prepare bacterial suspensions in corresponding growth medium at a concentration of approximately 10^6 bacteria ml^{-1}. BEAS-2B, NHBE, A549 and NCI-H292 epithelial cells were seeded in a 24-well collagen coated cell culture plates (ThermoFisher Scientific) at a density recommended by supplier per well and were incubated for approximately 48 h until >90% confluence was achieved. Monolayers were washed twice with their respective pre-warmed culture medium before exposure to NTHi strains. Monolayers were inoculated with the NTHi strains in triplicate at 10^6 bacteria per well and incubated for 4 h after which the monolayers were washed three times with Hank's Buffered Salt Solution (HBSS). Pre-warmed cell culture medium containing 200 μg gentamicin ml^{-1} was added to all 24 wells and incubated for 2 h to kill extracellular bacteria. Monolayers were then washed three times with HBSS after which intracellular bacteria were released by lysing the cells with 1% Saponin in HBSS for 15 min, and collected by scraping and vigorous vortexing for 1 min. The lysates were serially diluted and spread on chocolate agar in duplicate. The invasion rate was calculated by counting the c.f.u. and calculating a % invasion in relation to the original inoculum. The assay for each strain was conducted in triplicate on three different days.

2.4. Statistical analysis

All data analysis was conducted using GraphPad Prism software (version 6.0d). A Shapiro-Wilks test was performed to assess the normality or otherwise of the distribution of invasion rates across the isolates. Analysis of the differential invasion rate of all strains in the four different cell types (BEAS-2B, NHBE, A549 and NCI-H292) was conducted using a Kruskal-Wallis test and the analysis of the invasion rate of each individual strain over the four cell types was conducted using a two-way ANOVA. A Mann-Whitney test was performed to analyse the difference in invasion rate between strains with normal and altered PBP3 for each cell type. P values < 0.05 were considered as statistically significant.

3. Results

The invasion rates were not normally distributed, so non-parametric tests were selected for subsequent analysis. The invasion rates for all 16 isolates with all 4 epithelial cell types are given in Table S1 and shown in Fig. S1 (available in the online Supplementary Material). The range and median invasion rates for all isolates across all cell types was <0.01 to 43.45% and 1.10%, and individually for BEAS-2B cells was 0.03 to 43.45% and 3.17%, for NHBE cells was 0.10 to 13.99% and 2.31%, for A549 cells was <0.01 to 8.98% and 0.11%, and for NCI-H292 cells was 0.23 to 12.72% and 1.52%. The median invasion with BEAS-2B cells was significantly higher compared to A549 cells ($P = 0.015$), however, there was no significant difference in comparison to NHBE or NCI-H292 cells, and the median invasion with A549 cells was significantly lower than NCI-H292 (P = 0.015) cells but did not exhibit any statistical difference with NHBE cell types (Fig.1). There were marked differences in invasion rates for some isolates depending on the cell type used, where for example, isolate L341 gave 43.45% invasion in BEAS-2B cells but only 3.63% in A549 cells, L267 ranged from 22.12% (BEAS-2B) to 2.87% (A549) and Ci51 from 18.01% (BEAS-2B) to 1.68% (A549).

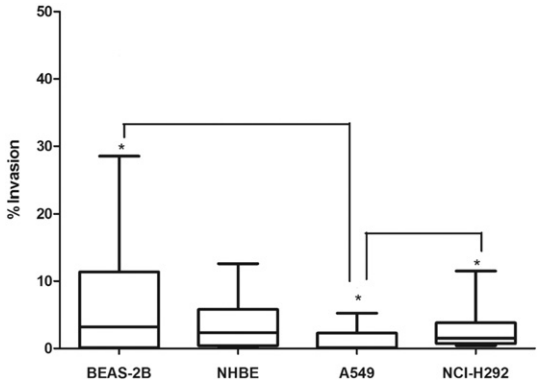

Fig. 1. Box-plot of invasion and epithelial cell type Box lines represent median, 25th and 75th percentile and entire data range. * Represents significant difference ($p < 0.05$).