Inability of *Haemophilus haemolyticus* to invade respiratory epithelial cells *in vitro*

Non-typeable *Haemophilus influenzae* (NTHi) is a commensal of the upper respiratory tract and an important opportunistic pathogen, causing both acute and chronic respiratory infections (Murphy et al., 2004; Clementi & Murphy, 2011; King, 2012). Many isolates of NTHi have been shown to be able to invade respiratory epithelial cells *in vitro*, and it has been suggested that this may be a virulence attribute, particularly in persistent infections where the intracellular environment would protect the bacterial cells from both the host immune response and antibiotic exposure (Ketterer et al., 1999; Swords et al., 2000; Hotomi et al., 2010; Clementi & Murphy, 2011; King, 2012). *Haemophilus haemolyticus* is very closely related to NTHi and shares the same upper respiratory tract habitat but is not an opportunistic respiratory pathogen (Mukundan et al., 2007; Hinz et al., 2015; Pickering et al., 2016). Little information is available on the ability of *H. haemolyticus* to invade respiratory epithelial cells *in vitro*.

If *in vitro* invasion is an indicator of ability for *in vivo* invasion and is important in the pathogenesis of NTHi infection, then we hypothesise that isolates of *H. haemolyticus* will be comparatively non-invasive. To test this hypothesis we established a collection of 20 *H. haemolyticus* isolates recovered from normal throat flora of healthy individuals. Isolates were identified as *H. haemolyticus* based on positive PCR for sodC and negative PCR for *hylD* and *fucK* using the methods and algorithm as described previously (Witherden & Tristram, 2013).

The nature of penicillin binding protein 3 (PBP3) in the isolates was investigated using PCR to identify single nucleotide *fisl* polymorphisms responsible for altered PBP3 with substitution of asparagine to lysine at position 526 (N526K) as described previously (Witherden et al., 2013). This was performed because previous studies have shown an association between altered PBP3 and increased invasion in NTHi. In our 20 isolates, 12 were detected to have altered PBP3.

All isolates were then tested for *in vitro* invasion using the gentamicin survival assay in BEAS-2B cells as described previously, using a known invasive NTHi isolate as a control (Okabe et al., 2010; Atkins et al., 2014). Results are expressed as the percentage of bacterial cells from the challenge inoculum that were recovered from the BEAS-2B intracellular space after 4 h co-incubation. All 20 isolates had invasion rates of <0.01%. To determine if this ‘non-invasiveness’ extended to other cell lines, we similarly tested five selected isolates (two normal PBP3 and three altered PBP3) for invasion using NHBE, A549 and NCI-H292 cell lines. All isolates had invasion rates of <0.01% in NHBE and A549 cells, but had invasion rates ranging from 0.03 to 0.67% for NCI-H292 cells, although it is worth noting that this is still less than the >1% that Okabe et al. (2010) used as criteria for invasion. In summary, we have shown that our population of *H. haemolyticus* isolates are non-invasive.

These results provide some additional insight into what is already known about *in vitro* invasion in NTHi. Although previous work by Atkins et al. (2014) showed that *in vitro* invasion was not an essential attribute for pathogenesis, as many of the clinical isolates in that study were non-invasive, the observation that none of the tested *H. haemolyticus* isolates are invasive supports the notion that *in vitro* invasion may be associated with some aspect of *in vivo* pathogenicity. Similarly, Atkins et al. (2014) showed that although altered PBP3 was associated with increased *in vitro* invasion, altered PBP3 alone was unable to enhance *in vitro* invasion in non-invasive NTHi isolates. Our demonstration that altered PBP3 did not have any effect on invasiveness in *H. haemolyticus* supports the conclusion of Atkins et al. (2014) that PBP3 is not directly associated with invasion.

The molecular mechanism of invasion in NTHi has not been fully elucidated (Clementi & Murphy, 2011; Ikeda et al., 2015) and our demonstration of non-invasiveness in *H. haemolyticus* may provide a novel avenue to study the genetic determinants of invasion. As *H. haemolyticus* and NTHi are phylogenetically very closely related (Witherden et al., 2013; Price et al., 2015), a genome-wide comparison between *H. haemolyticus* and both invasive and non-invasive NTHi may reveal key genomic regions associated with invasion. A similar approach has been used previously to identify the genomic regions associated with NTHi that cause otitis media, by comparing isolates from cases of otitis media with isolates from the throat of non-diseased individuals (Xie et al., 2006).

In conclusion, we have shown that our collection of *H. haemolyticus* isolates are non-invasive using an *in vitro* model and suggest that this is consistent with their inability to cause opportunistic respiratory tract infections.

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