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Cronobacter sakazakii clinical isolates overcome host barriers and evade the immune response

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ABSTRACT

Cronobacter sakazakii is the most frequently clinically isolated species of the *Cronobacter* genus. However the virulence factors of *C. sakazakii* including their ability to overcome host barriers remains poorly studied. In this study, ten clinical isolates of *C. sakazakii* were assessed for their ability to invade and translocate through human colonic carcinoma epithelial cells (Caco-2) and human brain microvascular endothelial cells (HBMEC). Their ability to avoid phagocytosis in human macrophages U937 and human brain microglial cells was investigated. Additionally, they were tested for serum sensitivity and the presence of the *Cronobacter* plasminogen activation gene (*cpa)* gene, which is reported to confer serum resistance.

Our data showed that the clinical *C. sakazakii* strains invaded and translocated through Caco-2 and HBMEC cell lines and some strains showed significantly higher levels of invasion and translocation. Moreover, *C. sakazakii* was able to persist and even multiply in phagocytic macrophage and microglial cells. All strains, except one, were able to withstand human serum exposure, the single serum sensitive strain was also the only one which did not encode for the *cpa* gene. These results demonstrate that *C. sakazakii* clinical isolates are able to overcome host barriers and evade the host immune response indicating their capacity to cause diseases such as necrotizing enterocolitis (NEC) and meningitis. Our data showed for the first time the ability of *C. sakazakii* clinical isolates to survive and multiply within human microglial cells. Additionally, it was shown that *C. sakazakii* clinical strains have the capacity to translocate through the Caco-2 and HBMEC cell lines paracellularly.

1. Introduction

The *Cronobacter* genus is a member of the *Enterobacteriaceae* family. It comprises a distinct group of Gram-negative bacilli that are catalase-positive, oxidase-negative, non-spore forming, facultatively anaerobic, and motile via peritrichous flagella [1–3]. The *Cronobacter* genus contains 7 different species including *Cronobacter condimenti*, *Cronobacter dublinensis*, *Cronobacter malonaticus*, *Cronobacter muytjensii*, *Cronobacter sakazakii*, *Cronobacter turicensis*, and *Cronobacter universalis* [4–6]. *C. sakazakii* isolates represent 72.1% (n = 1400) of the total *Cronobacter* genus isolates in the open access *Cronobacter* PubMLST database (http://www.pubmlst.org/cronobacter/), and this species has been linked to several fatal NEC and meningitis cases around the world [7–10]. *C. sakazakii* have been isolated from prepared infant feeds associated with neonatal intensive care unit (NICU) infections. Cases of necrotising enterocolitis (NEC), bacteraemia, and meningitis have a 40–80% mortality rate, and 20% of the survivors develop serious neurological disorders [11–14]. *C. sakazakii* distinct pathovars which are clonal lineages, of particular clinical significance being clonal complex 4 (CC4) that contains sequence type 4 (ST4), as well as ST12. These are strongly associated with invasive meningitis and NEC cases, respectively [15]. One of the most studied NICU outbreaks was in 1994 when 3 infants died from infections by *C. sakazakii* ST4 strains [14].

For organisms to establish a systemic infection they must adhere to the host cell, translocate to the underlying tissues, and then disseminate throughout the body. Therefore, the intestinal epithelium has an important role in protecting the body against bacterial invasion. Once this layer loses its integrity, the invading organism can infect the tissue beneath [16]. The ability of *C. sakazakii* to invade the intestinal epithelium and brain

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endothelium is therefore a crucial step for its pathogenesis. It was shown previously that *C. sakazakii* has the ability to adhere to epithelial and endothelial cells *in vitro* [13,17]. A study by Townsend et al. [18] used isolates from the French outbreak in 1994, and showed that the *C. sakazakii* strains were able to adhere and invade Caco-2 and rat brain capillary endothelial cells (rBCEC4) cell lines. Moreover, the organism was able to persist and multiply within the human macrophage U937 cell line [19]. Another study by Giri et al. [12] showed that food and environmental strains of *C. sakazakii* have the ability to invade the HeLa subline INT407 (human embryonic intestinal cells) and human brain microvascular endothelial cells (HBMEC).

The translocation process of the organism follows the initial attachment and invasion phases. It is the step that initiates the pathogenesis at the next tissue level after passing through the epithelial layer. Townsend et al. [20] reported that the presence of lipopolysaccharide (LPS) in infant formula increased the permeability of the intestinal epithelium leading to the translocation of *C. sakazakii*. Giri et al. [12] showed that the invasive food and environmental *C. sakazakii* strains were able to translocate intracellularly through the intact monolayers of the Caco-2 and HBMEC cell lines. This suggests that the bacterium is able to overcome the physical host barriers in intestines and CNS.

A number of virulence traits have been identified in *Cronobacter*, which may facilitate the invasion and dissemination of the organism in the host. Franco et al. [21] reported that the plasmid-borne *Cronobacter* plasminogen activator (Cpa) may provide resistance to bactericidal activity of serum through cleaving complement components C3 and C4b, and the activation of plasminogen and inactivation of α2-AP. In a study of over 100 *Cronobacter* genomes, *cpa* was found in *C. sakazakii* and not *C. malonaticus* [23; http://pubmlst.org/cronobacter/], and therefore may contribute to the higher clinical incidence of this species. It has also been reported that the outer membrane protein A (OmpA) of *Cronobacter* spp. has a role in the colonisation of the gastrointestinal tract (GIT) [21,23]. Also, it was demonstrated that the outer membrane proteins OmpA and OmpX were required for the basolateral invasion of enterocyte-like human epithelial cells by *C. sakazakii* [23]. Singamsetty et al. [24] demonstrated that the entry of *Cronobacter* spp. into HBMEC requires *ompA* expression and depends on microtubule condensation in these cells. This might help in the invasion of human intestinal cells and invasion of the brain endothelial cells to cause meningitis [25]. Moreover, it was recently shown that *C. sakazakii* ST4 strain 767 was able to produce outer membrane vesicles (OMVs) that have the capacity to increase the host's cell proliferation and stimulate a pro-inflammatory innate immune response [26].

This study used clinical isolates of *C. sakazakii* which had been previously genotyped by multilocus sequence typing (MLST), and many of which had been whole genome sequenced [23; http://pubMLST.org/cronobacter/]. The research aim was to study the virulence potential and pathogenicity of well characterised *C. sakazakii* clinical isolates and their ability to overcome host physical barriers and evade host immune response.

2. Results

2.1. Invasion efficiencies of C. sakazakii clinical isolates to Caco-2 and HBMEC cell lines

The invasion assay, using gentamicin protection to kill the extracellular bacteria, was used to assess the ability of 10 *C. sakazakii* clinical isolates to invade the Caco-2 and HBMEC cell lines. With regard to the Caco-2 cell line, different invasion levels were noted among these isolates, and strain 695 was the most significant ($P < 0.05$). The level of invasion by 695 was as high as *S.* Enteritidis, which was used as positive control strain for the assay. Strains 20, 767, 1221, and 696 were moderate in invasion, whereas strains 1240, 1242, 1249, 658 and 680 were low (Fig. 1). Regarding the HBMEC cell line invasion, strain 767 was the most significant ($P < 0.01$) being as high as *Cit. koseri*, the positive control. The other strains were moderate except for strains 658 and 680, which were the lowest (Fig. 1).

2.2. Translocation of C. sakazakii clinical through Caco-2 and HBMEC polarised monolayers

The aforementioned results showed that *C. sakazakii* isolates were able to invade Caco-2 and HBMEC monolayers. Therefore, these isolates were tested for their ability to translocate through the polarised monolayers of the Caco-2 and HBMEC cell lines. *C. sakazakii* strain 695 was the highest in translocating through the Caco-2 cell line over 5 h of infection ($P < 0.01$). The other strains including 767, 1221, 1240, 1242, 1249, 658, and 696 were moderate, while strains 20 and 680 were the lowest (Fig. 2). With regard to the HBMEC cell line, strains 20, 695, 1221, 1240, and 696 were high in translocation ($P < 0.01$), and strain 767 was the most significant over 5 h of incubation ($P < 0.001$). *C. sakazakii* strains 1242 and 1249 were moderate, whereas 658 and 680 were the lowest (Fig. 2). It

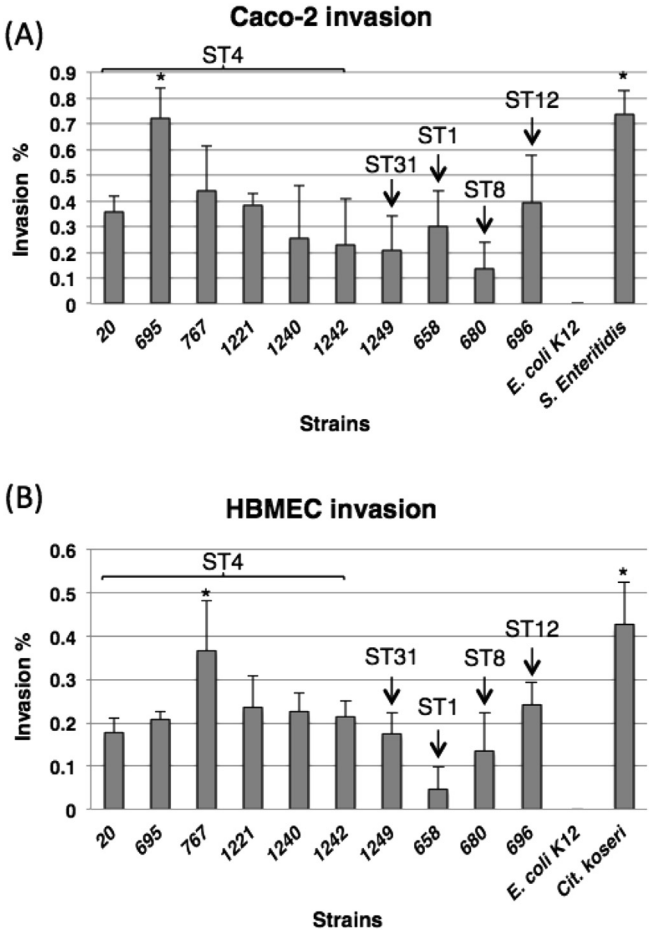

Fig. 1. *C. sakazakii* invasion assay using Caco-2 (A) and HBMEC (B) cell lines over 3 h of incubation showing the differences in invasion levels among strains. The displayed data are the mean ± standard deviation of invasion efficiency % of the initial inoculum (10^6 cfu/ml) of two independent experiments in triplicate. The asterisks above the bars indicate statistically significant differences (*$P < 0.05$; Kruskal–Wallis).