Characterization of an extended-spectrum beta-lactamase *Enterobacter hormaechei* nosocomial outbreak, and other *Enterobacter hormaechei* misidentified as *Cronobacter* (*Enterobacter*) *sakazakii*

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Enterobacter hormaechei is a Gram-negative bacterium within the *Enterobacter cloacae* complex, and has been shown to be of clinical significance by causing nosocomial infections, including sepsis. *Ent. hormaechei* is spread via horizontal transfer and is often associated with extended-spectrum beta-lactamase production, which increases the challenges associated with treatment by limiting therapeutic options. This report considers 10 strains of *Ent. hormaechei* (identified by 16S rDNA sequencing) that had originally been identified by phenotyping as *Cronobacter* (*Enterobacter*) *sakazakii*. Seven strains were from different neonates during a nosocomial outbreak in a California hospital. PFGE analysis revealed a clonal relationship among six of the seven isolates and therefore a previously unrecognized *Ent. hormaechei* outbreak had occurred over a three-month period. Antibiotic-resistance profiles were determined and extended-spectrum beta-lactamase activity was detected. The association of the organism with powdered infant formula, neonatal hosts and *Cr. sakazakii* suggested that the virulence of these organisms may be similar. Virulence traits were tested and all strains were shown to invade both gut epithelial (Caco-2) and blood–brain barrier endothelial cells (rBCEC4), and to persist in macrophages (U937). Due to misidentification we suggest that *Ent. hormaechei* may be an under-reported cause of bacterial infection, especially in neonates. Also, its isolation from various sources, including powdered infant milk formula, makes it a cause for concern and merits further investigation.

Received 7 July 2008
Revised 11 September 2008
Accepted 19 September 2008

INTRODUCTION

Enterobacter hormaechei is a Gram-negative rod within the *Enterobacter cloacae* complex, and is most frequently isolated from clinical sources. The species was originally defined by O'Hara *et al.* (1989) when a large hybridization group of enteric organisms was isolated and found to be associated with bloodstream infections. A recent report has further defined species in the *Ent. cloacae* complex via DNA cross-hybridization, specifically naming three new subspecies of *Ent. hormaechei*: *Ent. hormaechei* subsp. *steigerwalti*, *Ent. hormaechei* subsp. *hormaechei* and *Ent. hormaechei* subsp. *oharae* (Hoffmann *et al.*, 2005). *Ent. hormaechei* has been shown to be of clinical significance by the report of several outbreaks of sepsis in neonatal intensive care units

in Brazil and the USA (Campos *et al.*, 2007; Wenger *et al.*, 1997). One of these outbreaks was suggested to have originated from contaminated parenteral nutrition, indicating a similar route of infection to that of *Enterobacter sakazakii* (Campos *et al.*, 2007). This organism has recently been reclassified into four species in the newly designated genus *Cronobacter*, following detailed DNA analysis (Iversen *et al.* 2004b, 2008).

Recent concern over the presence of *Cronobacter* (*Enterobacter*) *sakazakii* in powdered infant formula and its association with neonatal disease (Biering *et al.*, 1989; Coignard *et al.*, 2006; Himelright *et al.*, 2002; Jarvis, 2005; Muyltjens *et al.*, 1983; da Silva *et al.*, 2002; van Acker *et al.*, 2001) has prompted government agencies to carefully examine regulations concerning the presence of bacteria in powdered infant formula (FAO-WHO, 2006). Therefore, misidentification of *Ent. hormaechei* as *Cr. sakazakii* can have significant repercussions. False-negative screening results may result in manufacturers' releasing contami-

Abbreviations: BBB, blood–brain barrier; ESBL, extended-spectrum beta-lactamase; HPS, human plasma serum.

The GenBank/EMBL/DDBJ accession numbers for the *Ent. hormaechei* strains sequenced in this study are AM947037–AM947046.

nated material, while false-positive results could cause manufacturers unnecessary financial losses. This is exacerbated by specific virulence traits and the intrinsic immunodeficiency of infants (Euler *et al.*, 1977). Further, misidentification as *Cr. sakazakii* during clinical infection may result in inappropriate treatment of the disease and under-reporting of the infections caused by *Ent. hormaechei*.

Ent. hormaechei infections have been frequently associated with extended-spectrum beta-lactamase (ESBL) production, thus further complicating clinical therapeutics. Studies indicate that CTX-M-, SHV- and TEM-related enzymes often facilitate *Ent. hormaechei* ESBL activity (Ho *et al.*, 2005). Frequently in *Ent. hormaechei*, ESBL activity is attributed to *bla*_{CTX-M} genes. CTX-M (unlike TEM and SHV ESBL enzymes) preferentially hydrolyses cefotaxime and ceftriaxone compared with ceftazidime and was discovered in 1990 (Bauernfeind *et al.*, 1990). A recent study found that over a two-year period, 17.9% of *Ent. hormaechei* (*n*=39) isolates from bloodstream infections tested positive for ESBL (Ho *et al.*, 2005). Further, the only *Cr. sakazakii* isolated in this study was ESBL-positive (Ho *et al.*, 2005). Two *Cr. sakazakii* ESBL strains were also reported in a recent report of the largest *Cr. sakazakii* outbreak that occurred in France in 1994 (Caubilla-Barron *et al.*, 2007).

During 16S rRNA gene sequencing of our culture collection, we found seven strains of *Ent. hormaechei* that had been identified by phenotyping as *Cr. sakazakii*. These seven strains were from different neonates suffering bacterial sepsis in 1997, in California. At that time, the cases were not linked, and were not attributed to an outbreak. Further analysis of our strain collection revealed six other *Ent. hormaechei* isolates from different countries that had also been misidentified as *Cr. sakazakii*. Three are included in this study and three other clinical isolates, recently identified in the UK, are considered in a separate case study (results not shown; S. Soo and others, personal communication).

In this study, a previously unreported neonatal outbreak is attributed to *Ent. hormaechei*. These strains were also shown to produce ESBL activity, further complicating clinical treatment of the infection. Like *Cr. sakazakii*, *Ent. hormaechei* is isolated from enteral feeding products and powdered infant formula (Aldová *et al.*, 1983; Muytjens *et al.*, 1988; Campos *et al.*, 2007), and this study considers various virulence traits by investigating the ability to invade blood-brain barrier (BBB) cells and gut epithelial cells, and to persist in human macrophages. This organism is similar to *Cr. sakazakii* with respect to potential source of infection and virulence (Caubilla-Barron *et al.*, 2007; Pagotto *et al.*, 2007, 2003; Townsend *et al.*, 2007a, b). The specific problem of misidentifying *Ent. hormaechei* as *Cr. sakazakii* is the need for species-specific information, as it relates to isolation of contaminants and sources of infection. Thus, masked by misidentification as *Cr. sakazakii*, *Ent. hormaechei* may be an unrecognized contaminant of powdered infant formula that merits further examination.

METHODS

Bacterial strains and identification. Ten *Ent. hormaechei* strains used in this study had previously been identified as *Cr. sakazakii* using phenotyping methods (Table 1). Seven were from Diane Citron at Los Angeles County/University of Southern California Medical Center, Los Angeles, CA. Two other strains (505 and 550) had been kindly provided by Harry Muytjens from the Aldová strain collection (Aldová *et al.*, 1983) and the 1988 survey of powdered infant formula (Muytjens *et al.*, 1988), respectively. The species type strain was obtained from the Belgium culture collection (CCUG 27126^T). Identification of the strains based on phenotyping was determined using a number of commercially available systems: API 20E (bioMérieux), ID32E (bioMérieux) and Microbact GNB 24E (Oxoid). Partial and full 16S rDNA gene sequence analysis was used as the reference method for strain identification by Accugenix using the MicroSeq500 16S rDNA Bacterial Sequencing kit (Applied Biosystems), as previously described (Iversen *et al.* 2004a, 2006). DNA was prepared for PCR by quick-heat lysis by removing one colony into a tube of PrepMan Ultra reagent (Applied Biosystems) and incubated at 99 °C for 10 min. Two microlitres of genomic DNA was amplified in 50 µl of a master mixture consisting of 0.4 µM of TGGAGAGTTTGATCCTGGCTCAG and TACCGCGGCTGCTGGCAC primers, 200 mM deoxynucleoside triphosphates, PCR buffer, 0.3 U AmpliTaq DNA polymerase, and 10% (v/v) glycerol. PCR conditions were 95 °C for 10 min; 30 cycles each of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s; and a final step at 72 °C for 10 min. Purification of the PCR product to remove excess primers and nucleotides was performed using Montage SEQ96 filter plates (Millipore). Cycle sequencing was performed with the sequencing module, and after removal of excess dyes using Montage SEQ96 filter plates (Millipore), the labelled extension products were separated on an ABI 3100 16 capillary genetic analyser (Applied Biosystems). Partial sequencing was performed for all strains; the length of the partial rDNA was 528 nt.

Non-pathogenic *Escherichia coli* K-12 HB101, *E. coli* K1 neonatal meningitic cerebrospinal fluid (CSF) isolate RS218 (O18:K1:H7), *Citrobacter koseri* SMT319 (isolated from the CSF of an infant with *Cit. koseri* meningitis), *Cit. freundii* and *Salmonella enterica* serovar Enteritidis (NCTC 3046) were included as control organisms for the *in vitro* virulence assays, as appropriate. *Cr. sakazakii* strains NCTC 11467^T and ATCC 12868 were used as positive controls for PCR detection methods. *E. coli* NCTC 10418 was used as the control strain for antibiotic-sensitivity testing. Bacterial isolates were subcultured onto TSA agar (Oxoid) prior to analysis.

DNA isolation and PCR methods for *Cr. sakazakii* identification. Genomic DNA was prepared using the GenElute bacterial genomic DNA kit (Sigma) from 1.5 ml of overnight culture grown in LB broth according to the manufacturer's instructions. The methods described by Keyser *et al.* (2003) and Mohan Nair & Venkitanarayanan (2006) for *Cr. sakazakii* identification were followed.

PFGE. PFGE was performed as previously described (Caubilla-Barron *et al.*, 2007) following the Pulse Net USA protocol for molecular subtyping of *E. coli* O157:H7, non-typhoidal *Salmonella* serotypes, and *Shigella sonnei* (Gerner-Smidt *et al.*, 2006).

Antimicrobial susceptibility. The disc-diffusion method on Iso-sensitest-agar (CM0471, Oxoid) according to the British Society for Antimicrobial Chemotherapy (2007) protocol was used, as previously described (Caubilla-Barron *et al.*, 2007). The combination disc method as described in Health Protection Agency QSOP 51 (Health Protection Agency, 2006) was used to detect ESBL activity.

Serum resistance. Qualitative assessment of serum sensitivity tolerance was performed using Labsystems Bioscreen analysis (Life

Table 1. Identification of *Ent. hormaechei* strains according to biochemical profiling and 16S rRNA gene sequencing

ND, Not done.

<i>Ent. hormaechei</i> strain	Source	Origin	Isolation date (day.month.year)	Strain identification by:				
				Vitek	API 20E	ID32E	GNB 24E	16S rRNA sequence identity
611	California	Blood	9.10.96	<i>Cr. sakazakii</i>	<i>Cr. sakazakii</i> 98.4 %*	<i>Ent. cloacae</i> 99.9 %*	<i>Ent. cloacae</i> 99.9 %*	<i>Ent. hormaechei-hormaechei</i> 0.47 %†
612	California	Blood	28.01.97	<i>Cr. sakazakii</i>	<i>Cr. sakazakii</i> 98.4 %	<i>Ent. cloacae</i> 99.9 %	<i>Ent. cloacae</i> 99.9 %	<i>Ent. hormaechei-hormaechei</i> 0.09 %
613	California	Catheter	15.02.97	<i>Cr. sakazakii</i>	<i>Cr. sakazakii</i> 98.4 %	<i>Ent. cloacae</i> 99.9 %	<i>Ent. cloacae</i> 99.9 %	<i>Ent. hormaechei-hormaechei</i> 0.47 %
614	California	Blood	19.02.97	<i>Cr. sakazakii</i>	<i>Cr. sakazakii</i> 98.4 %	<i>Ent. cloacae</i> 99.9 %	<i>Ent. cloacae</i> 99.9 %	<i>Ent. hormaechei-hormaechei</i> 0.09 %
615	California	Peritoneal fluid	24.02.97	<i>Cr. sakazakii</i>	<i>Cr. sakazakii</i> 98.4 %	<i>Ent. cloacae</i> 99.9 %	<i>Ent. cloacae</i> 99.9 %	<i>Ent. hormaechei-hormaechei</i> 0.57 %
616	California	Blood	26.03.97	<i>Cr. sakazakii</i>	<i>Cr. sakazakii</i> 98.4 %	<i>Ent. cloacae</i> 99.9 %	<i>Ent. cloacae</i> 99.9 %	<i>Ent. hormaechei-hormaechei</i> 0.47 %
617	California	Blood	05.06.97	<i>Cr. sakazakii</i>	<i>Cr. sakazakii</i> 51.1 %	<i>Ent. cloacae</i> ND	<i>Ent. cloacae</i> 45.9 %	<i>Ent. hormaechei-steigerwaltii</i> 0.76 %
161	England	Herb	2003	ND	<i>Ent. cloacae</i> 95.1 %	<i>Ent. cloacae</i> 55.9 %	<i>Ent. cloacae</i> 99.9 %	<i>Ent. hormaechei-steigerwaltii</i> 0 %
505	Czech Republic	Infant formula	1983	ND	<i>Cr. sakazakii</i> 51.1 %	<i>Enterobacter cancerogenus</i> 53.0 %	<i>Ent. cloacae</i> 99.9 %	<i>Ent. hormaechei-hormaechei</i> 0.57 %
550	Holland	Infant formula	1988	ND	<i>Ent. cloacae</i> 95.1 %	<i>Ent. cancerogenus</i> 96.8 %	<i>Ent. cloacae</i> 99.9 %	<i>Ent. hormaechei-steigerwaltii</i> 0.66 %
727	Hawaii	Blood	1989	ND	ND	ND	ND	<i>Ent. hormaechei</i> CCUG 27126 ^T

*Percentage match.

†Percentage difference from type strain.

Sciences International). Overnight cultures (50 µl) were inoculated in duplicate into Bioscreen plates and treated with 50 µl of human pooled serum. The mixture was incubated in the Bioscreen at 37 °C. Bioscreen readings (OD₆₀₀) were taken at 15 min intervals. Serum studies were carried out over a 2 h period. Percentage survival was determined as (number of bacteria that survived 3 h treatment/inoculum size) × 100. The percentage survival values were compared between human plasma serum (HPS) and heat-inactivated HPS. Strains able to survive in HPS at levels similar to that observed in heat-inactivated HPS were considered resistant. *E. coli* K-12 and *Cr. sakazakii* ATCC 12868 were used as negative and positive controls, respectively.

Macrophage persistence studies. As previously described (Townsend *et al.*, 2007b), U937 macrophages were seeded into 75 ml tissue-culture flasks (Sundstrom & Nilsson, 1976). The gentamicin protection assay was performed with *E. coli* K-12 and *Cit. koseri* as negative and positive controls, respectively. Results are presented as invasion efficiency expressed as the percentage of inoculum that was intracellular.

Epithelial cell studies. The attachment and invasion assays of Caco-2 cells were primarily based on the method of Szymanski *et al.* (1995), as described by Townsend *et al.* (2008). *E. coli* K-12 and *S. Enteritidis* were used as negative and positive controls, respectively. Data are presented as the average percentage invasion: [100 × (number of bacteria recovered/number of bacteria inoculated)]. The level of detection was 100 c.f.u. per well. Values were compared using the *t* test (*P* = 0.05).

Endothelial cell studies. The rat brain capillary endothelial cell line 4 (rBCEC4) was a kind gift from I. E. Blasig (Leibniz-Institut für Molekulare Pharmakologie, Berlin) and was used to evaluate invasion of BBB cells, as described by Townsend *et al.* (2008). *E. coli* K-12 and *Cit. koseri* were used as negative and positive controls, respectively. Data are presented as the percentage invasion as determined by 100 × (number of bacteria recovered/number of bacteria inoculated). Values were statistically evaluated using the *t* test.

RESULTS

Californian *Ent. hormaechei* outbreak description

The outbreak in California involved six neonates with *Ent. hormaechei-hormaechei*, and one neonate with *Ent. hormaechei-steigerwaltii* (Table 1). It began with the isolation of *Ent. hormaechei-hormaechei* (strain 611) from neonate 1 blood on 10 September 1996. Three months later (28 January 1997), strain 612 was isolated from neonate 2 blood, and 18 days later strain 613 was isolated from a catheter of neonate 3. Four days later (19 February 1997), strain 614 was isolated from a blood sample of neonate 4. After a further 5 days (24 February 1997) strain 615 was isolated from the peritoneal fluid of neonate 5. The last *Ent. hormaechei-hormaechei* isolate (strain 616) was one month later (26 March 1997) from the blood of neonate 6. *Ent. hormaechei-steigerwaltii* (strain 617) was isolated two months later from a blood sample of a seventh neonate.

General description of *Ent. hormaechei* isolates

All isolates had been sent to Nottingham as *Cr. sakazakii* following their identification by the source laboratory using standard biochemical profiling methods. This misidentification

was confirmed at Nottingham using a range of phenotyping methods, including API 20E, GNB 24E and ID32, as shown in Table 1. For phenotypic tests such as API 20E, a high percentage indicates a better match for identification. The 16S identification values indicate percentage divergence from the type strain, and thus a lower value indicates the best match. API 20E biochemical profiling identified the California isolates as *Cr. sakazakii* (51.1–98.4 %). However, both GNB 24E and ID32 identified these isolates as *Ent. cloacae* (99.9 %). All *Ent. hormaechei* strains (except 161) were white on DFI agar, a chromogenic selective agar for *Cr. sakazakii*, which forms blue-green colonies on this agar (Iversen *et al.*, 2004b). In addition, identification of these strains (including non-outbreak-associated strains) using the OmpA (Mohan Nair & Venkitanarayanan, 2006) and Keyser *et al.* (2003) PCR identification methods for *Cr. sakazakii* gave negative results.

Most *Ent. hormaechei* strains were serum-resistant. Only *Ent. hormaechei-hormaechei* strain 613 showed significant reductions in viability following HPS treatment and was considered serum-sensitive.

PFGE

PFGE of the California isolates (strains 611–616) was clonal for *Ent. hormaechei-hormaechei*, and was separate from *Ent. hormaechei-steigerwaltii* strain 617 (Fig. 1). There was a three bands or less difference among strains 611–616. Isolates 613 and 616 were identical; they presented one band difference with isolates 611 and 615, two bands difference with isolate 614, and three bands difference with 612. Strain 617, which was isolated in June 1997, did not show any band commonality to any of the other California outbreak strains. The strains that presented fewer than three band difference were closely related following the Tenover *et al.* (1995) band interpretation guidelines, and probably formed part of the same outbreak. Those strains were digested with a second enzyme, *SpeI*, to confirm whether they were clonal strains. Strains 611–616 gave identical *SpeI* restriction profiles, and therefore it is likely that they were multiple isolates of the same strain. As expected, none of the remaining strains (161, 505 and 550) showed any relatedness based on PFGE banding patterns (Fig. 1).

Antimicrobial susceptibility

The antimicrobial susceptibility of each strain to several classes of antibiotics was tested via the disc diffusion method. As recommended by Health Protection Agency (2006), *E. coli* NCTC 10418 was used as the control strain and was sensitive to all antibiotics tested. All *Ent. hormaechei* strains were sensitive to amikacin and imipenem (Table 2). Most of the isolates from the California outbreak (strains 611–617) showed resistance to most of the antibiotics tested. The exceptions were: strain 614, which was sensitive to ciprofloxacin; 611, which was

Fig. 1. PFGE profiles of *Ent. hormaechei* strains. Dendrogram obtained from cluster analysis, using Dice coefficient and unweighted pair group method with arithmetic mean (UPGMA). The tolerance in the band was 1.5 %, with an optimization of 1.5 %.

sensitive to co-amoxiclav; strains 611, 613 and 614, which were sensitive to cefotetan; and strains 611 and 616, which were both sensitive to gentamicin (Table 2). Due to sensitivity to gentamicin, these two strains were used to evaluate mammalian cell invasion via the gentamicin protection assay (see below).

The combination disc method, as described in the Health Protection Agency (2006) QSOP 51 was used to detect ESBL production. All *Ent. hormaechei-hormaechei* strains from the California outbreak were shown to produce ESBL (Table 2). *Ent. hormaechei-steigerwaltii* strain 617 did not produce ESBL and had been isolated two months after the

Table 2. Antibiotic resistance profiles of *Ent. hormaechei* isolates

R, resistant; I, intermediate; s, sensitive.

Antibiotic	<i>Ent. hormaechei</i> strain										
	611	612	613	614	615	616	617	161	505	550	727*
Amikacin	s	s	s	s	s	s	s	s	s	s	s
Ampicillin	R	R	R	R	R	R	I	R	R	R	s
Cefotaxime	R	R	R	R	R	R	s	s	s	s	s
Cefuroxime	R	R	R	R	R	R	s	s	s	R	s
Ciprofloxacin	R	R	R	s	R	R	s	s	s	s	s
Co-amoxiclav	s	R	R	R	R	I	s	I	s	I	s
Doxycycline	R	R	R	R	R	R	R	R	R	R	R
Gentamicin	s	R	R	R	R	s	s	s	s	s	s
Imipenem	s	s	s	s	s	s	s	s	s	s	s
Piperacillin	R	R	R	R	R	R	s	s	s	s	s
Trimethoprim	R	R	R	R	R	R	s	s	s	s	s
Streptomycin	R	R	R	R	R	R	s	s	s	s	s
Chloramphenicol	R	R	R	R	R	R	R	s	s	R	s
Cephalothin	R	R	R	R	R	R	R	R	R	R	R
Ceftazidime	R	R	R	R	R	R	s	s	s	s	s
Cefoperazone	R	R	R	R	R	R	s	s	s	s	s
Cefotetan	s	R	s	s	s	R	s	s	s	s	s
Ceftriaxone	R	R	R	R	R	R	s	S	s	s	s
ESBL production	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No

**Ent. hormaechei* CCUG 27126^T.

last isolation of *Ent. hormaechei-hormaechei*. The non-outbreak *Ent. hormaechei* strains (161, 505 and 550) did not have ESBL activity (Table 2).

Caco-2 invasion

Gentamicin does not penetrate mammalian cells and can be used to determine the number of intracellular bacterial cells in mammalian tissue cultures as opposed to those that are attached to the surface and susceptible to gentamicin. To investigate the ability of these strains to invade the intestinal epithelial layer *in vitro*, the Caco-2 cell line was utilized. This cell line is the most common and well-characterized model for intestinal epithelial studies. The gentamicin protection assay was performed with *E. coli* K-12 and *S. Enteritidis* as negative and positive controls, respectively. The *Ent. hormaechei* strains tested were significantly more invasive than *E. coli* K-12 ($P \leq 0.05$) and significantly less than *S. Enteritidis* ($P \leq 0.01$) (Fig. 2). Further, the strains isolated in the California outbreak were more invasive, with values not significantly different from the *Ent. hormaechei* type strain (727) or *Cit. koseri*. The non-Californian strains 161, 505 and 550 were significantly less invasive ($P \leq 0.005$) than all other strains tested.

Macrophage persistence

To investigate the ability of these strains to persist in human macrophage cells *in vitro*, the U937 cell line was utilized (Sundstrom & Nilsson, 1976). The gentamicin protection assay was performed with *E. coli* K-12 and *Cit. koseri* as negative and positive controls, respectively. All bacterial strains were shown to be taken up by the macrophages following the initial 45 min incubation. However, only *Cit. koseri* and *Ent. hormaechei-hormaechei* strains 611 and 616 from the California outbreak were shown to persist (and replicate) in macrophages after 24 h (Fig. 3). The other strains showed low levels of persistence after 24 h, including the *Ent. hormaechei* type strain (727).

rBCEC4 invasion

To investigate the ability of these strains to invade brain capillary endothelial cells *in vitro*, the rBCEC4 cell line was utilized. This cell line has been used previously in similar studies and shows levels of invasion comparable to those obtained with human brain microvascular endothelial cells (HBMEC) (Townsend *et al.*, 2007b). The gentamicin protection assay was performed using *E. coli* K-12 as a negative control that was unable to invade this cell line. *Cit. koseri* and *Cit. freundii* were used as positive controls. *Cit. koseri* was significantly more invasive than all strains tested ($P \leq 0.006$). *Ent. hormaechei* strains 161, 611 and 616 were not significantly different from *Cit. freundii* (Fig. 4), whereas strains 505, 550 and 617 were not significantly different from the *Ent. hormaechei* type strain (727).

DISCUSSION

Following 16S rRNA gene sequencing of our *Cr. sakazakii* strain collection, a subgroup of *Ent. hormaechei* strains from various sources was identified. Seven were from the same hospital in California (strains 611–617), three from the same hospital in the UK (not included in this study), a herb isolate (strain 161) from the UK, and two infant formula isolates (strains 505 and 550) from the Czech Republic, and another from Holland (Table 1). This study reports the clonal relationship of the Californian outbreak strains and focuses on their virulence, with comparison to other *Ent. hormaechei* strains from three different countries.

The Californian strains, originally identified as *Cr. sakazakii* by Vitek, had been collected from neonates (five isolated from blood) over a three-month period. PFGE showed that they were clonal *Ent. hormaechei-hormaechei*, apart from strain 617, which was *Ent. hormaechei-steigerwaltii* (Fig. 1). There was three bands or less difference among strains 611–616 when they were digested with the enzyme *Xba*I and no differences when they were digested with *Spe*I. Consequently, using the criteria of Tenover *et al.* (1995), it is probable that the isolates were part of an outbreak.

Fig. 2. Invasion of Caco-2 epithelial cells. Bacterial invasion of Caco-2 epithelial cells was determined by gentamicin protection assay at 3 h. Results are presented as the percentage of the inoculum that was intracellular. Data are means $\pm$ SEM of two independent experiments performed in triplicate. *E. coli* K-12 and *S. Enteritidis* were used as negative and positive controls, respectively.

Fig. 3. *Ent. hormaechei* survival within macrophages. The gentamicin protection assay was performed on human U937 macrophages using mid-exponential-phase bacteria at 0 h (T0) and 24 h (T24). For each time point indicated, results are presented as the percentage of intracellular cells of the initial inoculum. Data are means $\pm$ SEM from a representative assay performed in triplicate. *E. coli* K1 (persists), *E. coli* K-12 (killed) and *Cit. koseri* (replicates) were used as appropriate controls.

The *Ent. hormaechei* outbreak occurred from November 1996 to March 1997. During this period, six isolates were recovered from different neonates. There was a peak in February 1997, when three isolates were recovered. By April 1997, no further isolates with the same PFGE were recovered. Isolates were identified as *Cr. sakazakii* by the hospital, and had not been linked together as an outbreak. In June 1997, *Ent. hormaechei-steigerwaltii* (strain 617) was recovered and was not related to the previous outbreak strains. This strain was also misidentified by the hospital as *Cr. sakazakii*. Strain 617 did not form part of an outbreak, as no further clonal strains were isolated. All strains showed multiple resistances to a broad spectrum of antibiotics, and the *Ent. hormaechei-hormaechei* isolates were ESBL-positive (Table 2). Despite six strains not being distinguishable by PFGE with *Xba*I and *Spe*I restriction enzymes, there were physiological differences between the six outbreak strains with respect to antibiotic sensitivity and virulence traits. This has previously been reported in *Cr. sakazakii* isolates (Caubilla-Barron *et al.*, 2007; Townsend *et al.*, 2008), and reveals that genomic variation can occur which is not detected using rare-cutting restriction enzymes.

A commonality between the Californian outbreak and the other strains collected from the UK, Czech Republic and The Netherlands is the misidentification of strains as *Cr. sakazakii*. This organism is also associated with neonatal infections, and reconstituted powdered infant formula is a recognized source of infection (FAO-WHO, 2006). *Ent. hormaechei-steigerwaltii* strain 505 from the Czech Republic was reported to be *Cr. sakazakii* (Aldová *et al.*, 1983) and was isolated from powdered infant formula following a neonatal outbreak. *Ent. hormaechei-hormaechei* (strain 550) from Holland was isolated and reported as *Cr. sakazakii* during a survey of powdered infant formula in 1988 (Muytjens *et al.*, 1988). These two strains illustrate that in the past *Ent. hormaechei* has been isolated from powdered infant formula and mistaken for *Cr. sakazakii*. Recently, it was reported that Vitek GNI had identified *Ent. hormaechei* strains as *Ent. cloacae* (Ho *et al.*, 2005). The distinction was made via the D-glucose oxidation test. This fault is common to several different bacterial identification methods (Table 1), suggesting that the *Ent. hormaechei* species phenotype is complex and not distinguishable with current testing strategies that rely on phenotypic information. Fortunately, *Cr. sakazakii* detection and identification

Fig. 4. *Ent. hormaechei* invasion of rat brain capillary endothelial cells. The gentamicin protection assay was performed using rat rBCEC4 cells to model BBB invasion. Bacteria were inoculated and incubated for 1.5 h, then treated with gentamicin for 30 min. No bacteria were recovered at T0. Results for T2 are presented as the percentage of intracellular cells of the initial population inoculated after 2 h. *E. coli* K-12 (non-invasive), *Cit. koseri*, *Cit. freundii* and *E. coli* K1 (invasive) were used as controls. Data are means $\pm$ SEM from two independent assays performed in triplicate.

methods have greatly improved in their sensitivity and specificity (Fanning & Forsythe, 2007). Consequently, these strains would not have been misidentified as *Cr. sakazakii* using current selective, chromogenic agars such as DFI, or using DNA probes.

It might be expected that a bacterium associated with sepsis would be capable of invading gut epithelial cells, or even of persisting in human macrophages. Indeed, the ability for invasion of brain endothelial cells is a concern, especially if *Ent. hormaechei* is found to be a common contaminant of powdered infant formula. Therefore, virulence studies were performed to better understand the pathogenic potential of *Ent. hormaechei*. This study demonstrated the differing ability of *Ent. hormaechei* strains to invade intestinal epithelial cells. Specifically, *Ent. hormaechei-steigerwaltii* strains were less able to invade the intestinal epithelium than *Ent. hormaechei-hormaechei* strains (Fig. 2). This trend was also observed in macrophage persistence experiments, which showed that virulent *Ent. hormaechei-hormaechei* outbreak strains were able to persist and replicate within human macrophages, whereas other *Ent. hormaechei* strains (including the type strain 727) only persisted at lower levels within the macrophages after 24 h (Fig. 3). Further invasion studies investigated the potential of *Ent. hormaechei* to invade the BBB. This study suggests that *Ent. hormaechei-steigerwaltii* shows a trend of being less invasive of brain endothelial cells than *Ent. hormaechei-hormaechei* strains. *Ent. hormaechei* invasion is lower than that of *Cr. sakazakii* (Townsend *et al.*, 2007b, 2008). This may indicate why *Ent. hormaechei* is frequently isolated from blood instead of the central nervous system. A 22-year study found that *Enterobacter* spp. (identified via API 20E) are increasingly isolated from neonates and that the infections are commonly nosocomially acquired (Hervas *et al.*, 2001). Sepsis and meningitis were associated with *Enterobacter* spp., and hence there is a possibility that *Ent. hormaechei* may be associated with meningitis (Hervas *et al.*, 2001). Therefore, the ability of *Ent. hormaechei* to invade BBB cells and its isolation from powdered infant formula may be a cause for concern.

Ent. hormaechei misidentification is problematic because it prevents the full disclosure of sources, infections, and the disease potential attributed to this ESBL-producing organism. We have shown that this is a widespread problem spanning several phenotypic identification methods and several countries. Misidentification of *Ent. hormaechei* as *Cr. sakazakii* further complicates a sensitive issue that spans industrial, academic, government and clinical institutions. It is therefore important that the databases that support phenotyping kits are improved in the future, and are more reliant upon DNA sequence analysis as the gold standard. The misidentification of *Ent. hormaechei* also reveals the inaccuracy of three databases for *Cr. sakazakii* (Table 1). This could have major implications for manufacturers and regulatory authorities with respect to meeting international microbiological criteria for powdered infant formulae. The virulence traits

associated with these strains, particularly *Ent. hormaechei-hormaechei*, suggest that they are capable of invading deep tissues and may be under-reported in the clinical setting. Further investigation of this organism is warranted, given its virulence and isolation from various sources, including powdered infant formula.

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Edited by: S. D. Bentley