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Desiccation survival of *Acinetobacter* spp. in infant formula

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ABSTRACT

Acinetobacter spp. are included under category B in the FAO-WHO list of organisms of concern for neonatal health following the consumption of powdered infant formula. However, the ability of *Acinetobacter* spp. to maintain their viability in desiccated infant formula over a storage period consistent with the shelf-life of commercially available powdered infant formula (2 years) has not been demonstrated. In this study, 9 clinical and food isolates of *Acinetobacter baumannii*, *A. calcoaceticus*, and *Acinetobacter* genomosp. 3 were desiccated in infant formula and then reconstituted at designated time points. Bacterial viability was followed for a maximum period of 24 months or until the strain became undetectable ($<5 \times 10^2$ cfu/ml). For comparative purposes, one *Enterobacter hormaechei* and two *Enterobacter cloacae* strains were also monitored for their desiccation survival. The seven clinical and food strains remained cultivable for the whole duration of the study and showed biphasic survival curves. The initial drop in viable count was up to 3.5 $\log_{10}$ cfu/ml within 18 h of desiccation exposure. By the end of the study, the reduction in viability was between 3.6 and 4.8 $\log_{10}$ cfu/ml. In contrast the *A. baumanii* and *A. calcoaceticus* species type strains only persisted for 6 and 9 months, respectively, possibly due to laboratory adaptation. The *E. cloacae* and *E. hormaechei* strains were undetectable after 12 and 17 months, respectively. The persistence of *Acinetobacter* spp. strains in desiccated powdered infant formula, supports the FAO-WHO designation of this organism as a risk to neonatal health.

1. Introduction

Considerable attention has been given to the rare, though often fatal infection of neonates through the ingestion of reconstituted powdered infant formula (PIF) contaminated with *Cronobacter sakazakii* (Holy & Forsythe, 2014). However, the FAO-WHO microbiological risk assessments of PIF also highlighted the potential for infant infections due to other Enterobacteriaceae (FAO-WHO, 2004), and later also included *Acinetobacter* spp. as organisms of concern (FAO-WHO, 2006). *Acinetobacter* spp. are of particular concern as they frequently carry multiple antibiotic resistance factors. Infant formula intrinsically contaminated with *Acinetobacter baumannii* and *Acinetobacter johnsonii* has been reported by Cawthorn, Botha, and Witthuhn (2008) and Miled et al. (2010). Marino, Goddard, Whitelaw, and Workman (2007) isolated *Acinetobacter* spp. from 37/82 samples of reconstituted PIFs. These samples were taken from feeding bottles before distribution to hospitalized infants. The organism can also form biofilms on the surfaces of neonatal enteral feeding tubes which are in-place for up to 2 weeks (Hurrell, Kucerova, Loughlin, Caubilla-Barron, & Forsythe, 2009; Hurrell et al., 2009). The ability of *Cronobacter* species to survive under desiccated conditions similar to those found in PIF has already been demonstrated (Caubilla-Barron & Forsythe, 2007). However, no similar studies have been undertaken for the survival of *Acinetobacter* spp. in PIF. This is despite the known ability of *Acinetobacter* to persist in dry state on inanimate surfaces in the hospital environment (Manian, Griesnauer, & Senkel, 2013; Zenati, Touati, Bakour, Sahli, & Rolain, 2016). Accordingly, the work presented here assesses the extent to which *A. baumanni, A. calcoaceticus*, and *Acinetobacter* genomosp. 3 can survive in desiccated infant formula.

2. Material and methods

2.1. Bacterial strains

A total number of nine *Acinetobacter* strains were evaluated in this study. Four *A. baumannii* isolates (1095, 1096, 1098, and 1099) were clinical isolates from the Queen's Medical Centre in

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Nottingham, UK. *Acinetobacter* genomosp. 3 (415), and *A. calcoaceticus* (418) were food isolates. *A. calcoaceticus* 1097 (NCTC 7844), *A. calcoaceticus* 1103 (ATCC 23055^T), and *A. baumannii* 1102 (ATCC 19606^T) were used as the species type strains. *Enterobacter cloacae* 50 (gift from Oxoid; UK) and 597 (PIF isolate) as well as *Enterobacter hormaechei* 790 (neonatal feeding tube isolate; Hurrell et al., 2009) were included for comparative purposes.

2.2. Preparation of bacterial cultures prior to the desiccation assay

Acinetobacter strains were grown on skimmed milk-tryptic soy agar (SM-TSA) prepared by adding 200 ml TSA (Oxoid Thermo Fischer, CM0131) to 50 ml of 10% sterilized skimmed milk (w/v) (LAB M, MC027). Each strain was streaked onto five SM-TSA plates and incubated at 37 °C for 48 h.

2.3. Examination of capsule

Acinetobacter strains were examined by microscopy for the presence of a capsule using the India ink stain. A small portion of colonies was emulsified in a drop of sterile saline on a glass slide using a sterile straight wire. Two drops of India ink were then added to the smear. A coverslip was placed over the smear and pressed gently to avoid any air bubbles before observation using light microscopy.

2.4. Long-term desiccation survival assay

2.4.1. Desiccation procedure

The desiccation assay followed the previously described procedure of Caubilla-Barron and Forsythe (2007) for *Cronobacter* spp. Bacterial cultures were harvested from overnight SM-TSA plates, and then resuspended in 7 ml of liquid infant formula. Bacterial suspensions were then decimally diluted to 10^{-8}. An aliquot (12.5 μl) of each dilution were transferred into 96-well microtitre plates. Two plates containing 16 replicates per dilution were assigned for each sampling time point and resulted in the total preparation of forty 96-well microtitre plates. Plate counts were performed using the Miles and Misra method and incubated at 30 °C for 24 h in order to determine the initial concentration of the bacterial suspension before desiccation. These suspensions were allowed to air-dry overnight (18 h) in a class 2 safety cabinet. The plates were then sealed and stored in the dark at room temperature (21 °C).

2.4.2. Reconstitution of the desiccated strains

At each timed interval, the bacterial cells in two 96-microtitre trays were rehydrated with 200 μl of sterile liquid infant formula, and then incubated at 37 °C for 48 h. Reconstitution time points were 18 h, 3, 7, 14, and 20 days, followed by 1, 2, 3, 4, 5, and 6 months, then every two months for up 24 months.

2.4.3. Determination of cell viability

Following the 48-h incubation of the reconstituted cells, two TSA plates were inoculated with aliquots from each rehydrated well. After overnight incubation, the plates were examined for the presence or absence of growth of sixteen replicates per dilution. The viability of each strain was determined using the most probable number (MPN) technique. The recovery of the bacterial strains was followed for a maximum period of 24 months or until the strain became undetectable ($<5 \times 10^2$ cfu/ml).

3. Results

3.1. Capsule formation

All *Acinetobacter* strains appeared to be capsuled when stained with India ink and examined by light microscopy. The presence of the encapsulated cells was indicated by clear halos surrounding the cells.

3.2. Desiccation persistence of Acinetobacter spp.

The initial cell density of the overnight cultures was 10^8–10^{10} cfu/ml. After 18 h of desiccation in infant formula, the viability of all *Acinetobacter* strains decreased by 2.4–3.5 $\log_{10}$ cfu/ml (Fig. 1), and continued to do so over the next 19 days by up to 0.98 $\log_{10}$ cfu/ml (Fig. 1). Thereafter, the viable counts of *A. baumannii* 1095, 1096, 1098, and 1099, as well as *A. calcoaceticus* 1097 were stable for 5 months before starting to further decline (Fig. 2). Bacterial counts were in the order of 10^3–10^4 cfu/ml, when a plateau was reached by 12 months of desiccation, showing from 3.1 to 3.7 $\log_{10}$ cfu/ml reduction compared to the first desiccation time point.

The food isolates *Acinetobacter* genomosp. 3 (415), and *A. calcoaceticus* 418 exhibited similar desiccation survival trends to those of the clinical strains (Fig. 2). However, differences in the loss of viability was apparent after 3 months of desiccation. Unlike that of the clinical strains (at 12 months), the decline in the recovery of strains 415 and 418 continued only for up to 8 months before the persistence phase was reached. At this time point (8 months), the number of detected cells was 1.6×10^3 cfu/ml, giving a total decrease in viability of 3.5 and 3.7 $\log_{10}$ for strains 418 and 415, respectively).

Overall, the 7 *Acinetobacter* clinical and food isolates persisted for 24 months while desiccated in infant formula (Fig. 2). In contrast, the species type strains *A. baumannii* 1102 (ATCC 19606^T) and *A. calcoaceticus* 1103 (ATCC 23055^T) both exhibited reduced desiccation tolerance. The initial drop in viable count (2.4 and 3.1 $\log_{10}$ cfu/ml respectively) was followed by a persistent phase for 3 months at *ca.* 10^5 cfu/ml. After which, both strains continued to lose their viability until they were no longer detectable after 6 and 9 months' storage respectively.

Based on the comparison of their survival curves (Fig. 2), the desiccated strains were divided into 3 groups. Strains in the first category were the most sensitive to desiccation, and consisted of the species type strains *A. calcoaceticus* ATCC 23055^T and *A. baumannii* ATCC 19606^T. The food isolates *A. calcoaceticus* (415), and *Acinetobacter* genomosp. 3 (418) formed the second cluster, being more persistent that the type strains. The final group comprised the clinical isolates *A. baumannii* 1095, 1096, 1097, 1098, and 1099 which showed the least loss in viability during desiccation.

3.3. Desiccation persistence of bacterial species belonging to the Enterobacteriaceae family

Desiccating *E. cloacae* strains 50 and 597, and *E. hormaechei* 790 resulted in an initial decrease in viability of 4.2, 4.3, and 3.4 $\log_{10}$ cfu/ml, whereas the loss in viability in the following 19 days was considerably less (0.1, 0.4, and 0.0 $\log_{10}$ cfu/ml, respectively) (Fig. 3). Afterwards, the cell count declined to the limit of detection ($<5 \times 10^2$ cfu/ml) (Fig. 4). *E. hormaechei* 790 persisted the longest and remained recoverable up to 17 months, with an overall decrease of 5.8 $\log_{10}$ cfu/ml. In contrast, *E. cloacae* 597 was undetectable by 10 months of desiccation, followed by *E. cloacae* 50 which was no longer recoverable after 12 months. The total