Title: Desiccation survival of *Acinetobacter* spp. in infant formula

Author names and affiliations: Noha A. Juma[a*1], Georgina Manning[a], Stephen J. Forsythe[a]

[a]Pathogen Research Group, School of Science and Technology, Nottingham Trent University, Clifton Lane, Nottingham, United Kingdom, NG11 8NS.

[*]**Corresponding author:** Noha A. Juma, jumanoha@gmail.com.

[1]**Present address:** Noha A. Juma, Department of Medical Microbiology and Parasitology, Faculty of Medicine, King Abdul-Aziz University, Kingdom of Saudi Arabia.

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 *A. 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 (< $5x10^2$ 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.

Keywords: *Acinetobacter*; Desiccation; Powdered infant formula; Neonates.

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 *A. baumannii* and *A. 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 *et al*., 2009ab). 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 *et al*., 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 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 *E. hormaechei* 790 (neonatal feeding tube isolate; Hurrell *et al.*, 2009b~~a~~) 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 37ºC for 48h.

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 7ml of liquid infant formula. Bacterial suspensions were then decimally diluted to 10^{-8}. An aliquot (12.5µl) 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 30ºC 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).

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2.4.2 Reconstitution of the desiccated strains

At each timed interval, the bacterial cells in two 96-microtitre trays were rehydrated with 200µl of sterile liquid infant formula, and then incubated at 37ºC for 48h. 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-hour 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 (< $5x10^2$ 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 (Figure 1), and continued to do so over the next 19 days by up to 0.98 $\log_{10}$ cfu/ml (Figure 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 (Figure 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 (Figure 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 $1.6x10^3$ 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 (Figure 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 (Figure 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) (Figure 3). Afterwards, the cell count declined to the limit of detection (< $5x10^2$ cfu/ml) (Figure 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 viability loss of these strains was 6.4 and 6.2 $\log_{10}$ cfu/ml, respectively.

4. Discussion

The persistence of opportunistic bacterial pathogens in powdered infant formula is of concern due to the risk of infant infection following ingestion, especially neonates due to their reduced immunocompetence (FAO-WHO, 2004; FAO-WHO, 2006). Reconstitution of infant feeds with water >70°C to reduce the number of viable bacteria present has been recommended by FAO-WHO (2004), FAO-WHO (2006), and WHO (2007). However, this guidance has not been adopted by all countries and is not feasible for small volumes feeds as required for premature infants (Holy & Forsythe, 2014). Subsequently, the presence and persistence of organisms of concern needs to better understood. Despite the considerable improvements in the detection and control of *Cronobacter* spp. in PIF (Cetinkaya, Joseph, Ayhan, & Forsythe, 2012; Turck, 2012; Holy & Forsythe, 2014), there has not been equivalent consideration of other opportunistic pathogens in PIF such as *Acinetobacter* spp. which were recognised by the revised FAO-WHO risk assessment of organisms found in PIF as of concern for neonatal health (FAO-WHO, 2006).

To our knowledge, this work is the first to describe the persistence of desiccated *Acinetobacter* in infant formula. The findings demonstrate the ability of this organism to maintain its viability during long-term desiccation in infant formula and to efficiently recover after reconstitution. The food and the clinical *Acinetobacter* isolates were recoverable after two years of storage, and showed biphasic reductions in their viability. The clinical strains had a smaller proportion of desiccation sensitive

cells in its populations than the food isolates. This could explain the sharper decline in viability of the second group. The higher sensitivity of the type strain of *A. baumannii* (ATCC 19606^{T}) compared to the remaining strains is not unusual and is in agreement with previous studies that monitored the desiccation persistence of clinical and type cultures and laboratory adaptation (Jawad *et al.*, 1996; Wendt, Dietze, Dietz, & Ruden, 1997; Jawad *et al.*, 1998). Milk-based infant formula products contain milk fat, proteins, and lactose. These components would provide a degree of protection to desiccated *Acinetobacter* strains through their protective osmotic effects (Gardiner *et al.*, 2000; Lian, Hsiao, & Chou, 2002; Wang, Yu, & Chou, 2004). Another factor contributing to the protection of bacterial cells against desiccation is likely to be the presence of the capsule which retains water molecules (Roberson & Firestone, 1992; Ophir & Gutnick, 1994). All the *Acinetobacter* strains tested in this study were capsulated and these capsules may have consequently enhanced their persistence under desiccated conditions.

E. cloacae and *E. hormaechei* were more sensitive to desiccation than the *Acinetobacter* strains. These two species cause neonatal infections and are found in PIF as well as neonatal enteral feeding tubes (Hurrell *et al.*, 2009ba; Jackson, Parra, Fernandez-Escartin, & Forsythe, 2015). *E. cloacae* was no longer recoverable after 10 and 12 months, which is comparable with the 8-month survival period for *E. cloacae* ATCC 13047^{T} reported by Caubilla-Barron and Forsythe (2007). Of interest is that *Acinetobacter* strains studied here survived longer than 20 months for *Cronobacter* and *Salmonella* strains reported by Caubilla-Barron and Forsythe (2007). This observation supports the potential risk of neonatal infection by *Acinetobacter* through the consumption of contaminated feed as originally proposed by FAO-WHO (2006).

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The ingested dose of *Acinetobacter* cells required for the initiation of a neonatal infection is unknown. However, it is reasonable to propose that it could be relatively low given the state of the immune system and the deficiency in the competing gut flora of the neonate. In addition, the organism can form biofilms in enteral feeding tubes which are in place for up to 2 weeks (Hurrell *et al*., 2009b) and consequently the neonate would ingest clumps of bacterial cells as the biofilm disperses. Therefore, it is important to reduce the risk of exposure to *Acinetobacter* through improved microbiological quality of PIF, or the use of water >70°C to reduce the number of viable organisms in reconstituted feeds as recommended by FAO-WHO (2004) and FAO-WHO (2006).

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