CRONOBACTER SAKAZAKII GROWTH ON DIFFERENT TYPES OF NASO-GASTRIC TUBING

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Abstract

Cronobacter sakazakii (C. sakazakii) is a ubiquitous environmental contaminant most notable for being associated with the deaths of neonates fed infant formula via naso-gastric tubing. Sixteen isolates from different origins were screened for their potential to form bioflim using a microtitre plate assay. All clinical isolates formed strong biofilm while environmental isolates formed biofilms of mixed strength. Growth on three different types (silicone, polyvinyl chloride (PVC) and polyurethane) of naso-gastric tubing was monitored over 24 h. Clinical, dairy and environmental isolates were able to form biofilm on the three different materials of infant feeding tubes but initial attachment and growth was slow on the silicone tubing compared with the other two types of tubing. Silicone tubing appeared to be the best choice for premature babies that need feeding using feeding tubes, as it was slow to become colonised compared with the PVC and polyurethane tubing.

Keywords: *Cronobacter sakazakii,* biofilm, naso-gastric feeding tubes.

1. Introduction

Cronobacter sakazakii is recognised as an emerging opportunistic pathogen that has caused much concern in the food industry and also in the medical field (Forsythe, 2005; Fanning and Forsythe, 2008 and Iversen et al. 2008). This bacterium is unique in that it is ubiquitous in the environment (Skladal et al., 1993 and Kandhai et al., 2004) and for the most part affects only a small subset of the population, such as premature infants and infants under 1 year of age (Biering et al., 1989; Gurtler et al., 2005; Iversen and Forsythe, 2003 and Iversen and Forsythe, 2004).

The intestinal tract of newborn infants is microbiologically sterile and the neonate rapidly acquires its microbial flora during passage through the vagina at birth, through contact with the environment and through feeding. Originally contamination through the birth canal was thought to be the source of *C. sakazakii* infections in newborn infants but this has now been refuted with infant feeding believed to be the primary source of infection (Gurtler et al., 2005). The stomach of newborns, especially the premature babies, is less acidic than adults so this may be a possible factor in the survival of an infection with *C. sakazakii* in infants (Iversen and Forsythe., 2004). Infection is likely to be exacerbated by the weak immune system and lack of mature competing intestinal microflora in neonates (Townsend and Forsythe., 2008)

Powdered infant formula is used as an alternative to human breast milk in providing newborns with nutritional needs, either in addition to breast milk, or on its own when breast feeding is not possible. Powdered infant formulas have been implicated as the cause of a number of infectious *C. sakazakii* outbreaks presenting as meningitis, necrotizing enterocolitis (NEC) and sepsis (Forsythe, 2005, Maytjens et al., 1983 and Van Acker et al., 2001). *C. sakazakii* is ubiquitous in nature; however only powdered infant formula and preparation equipment have been linked to *C. sakazakii* outbreaks among infants. *C. sakazakii* also survives the milk powder drying

process very well and can be found in dry food products such as skimmed milk powder, lactose, starch, lecithin, banana powder, all ingredients that can be added to powdered infant formula (Iversen and Forsythe., 2004). Since *C. sakazakii* affects the youngest and most vulnerable segment of our population, the issue has been drawn the attention of the public, infant formula manufacturers and food safety authorities.

The origin of the contamination of infant formula with *C. sakazakii* is often associated with the dried infant formula, whereas the origin could well be the environment in which the formula is prepared for feeding. *C. sakazakii* is reported to be capable of forming biofilms on most surfaces (Kim et al, 2008). Bacterial biofilms colonise a wide variety of substances including medical devices, wastewater treatment systems and tissues (Characklis and Marshall, 1990). Biofilm formation on the naso-gastric tubing is likely to be responsible for the numbers of *C. sakazakii* reaching levels that threaten infant health. Biofilm formation is influenced by the surrounding environmental conditions. Growth on medical implants including gastrostomy tubes has been shown to lead to adverse conditions associated with patient health and tube deterioration and eventually leads to device removal (Habash and Reid, 1999).

The focus of this study was to investigate the ability of *C. sakazakii* to attach and form biofilm on different types of naso-gastric tubing.

2. Methodology

2.1 Source of isolates

The cultures used in this study originated from clinical, dairy and environment isolates (Table 1).

2.2 Maintenance of *C. sakazakii* culture

Stock cultures were received on agar slants or freeze dried. These were grown to active cultures as described below. Where cultures were going to be needed for a significant period of time (some months) active cultures were stored in glycerol at -80°C. For temporary storage, C. *sakazakii* was maintained on tryptic-soy agar slants at 4^{o}C. A new series of cultures was initiated from the frozen stock on a biweekly basis.

2.3 Culture preparation

For the routine preparation of cultures, all isolates were cultured in Tryptic soy broth (TSB) (Becton, Dickinson & Company, USA) and on Tryptic soy agar (TSA) (Merck, Germany). Cultures were grown aerobically at 37°C for 18 - 24 h.

2.4 API 20E

The identification of the isolates was confirmed using the API 20E biochemical identification kit (bioMerieux, Chemin de I'Orme, France) according to the manufacturer's instructions. The API 20E biochemical profiles were calculated using the API 20E V4.1 (Biomereux, https://apiweb.biomerieux.com)

2.5 Microtitre plate assay

A microtitre plate assay based on (Oh and Kang, 2005; Oh et al., 2007) study was used to screen the different strains of *C. sakazakii* for their ability to attach to surfaces and form biofilm. A 96-well flat-bottomed polystyrene microplate (Microtest™ 96, 35 3072, Becton, Dickinson & Company, USA) was filled with 230 µL of 10% reconstituted infant formula milk. Eight wells were filled with milk only as negative

Table 1. Source of isolates of *C. sakazakii*

Nomenclature	Strain	Source
F1	4.10 C	Dried infant formula, S. Edelson-Mammel, FDA
F2	SK 90	Clinical J. M. Farber, Health Canada
F3	3465-4 A	Environment isolate
F4	3465-6 B	Environment isolate
F5	4648-4 C	Environment isolate
F6	576736-3 D	Environment isolate
F7	578349-7 E	Environment isolate
F8	607	Clinical, FDA
F9	LCDC-648	Clinical J. M. Farber, Health Canada
F10	EWFAKRS 11 NN 1493	Clinical J. M. Farber, Health Canada
A1	3427 4767 251	Environment isolate
A2	3427 4767 250	Environment isolate
A3	3427 6763 250	Environment isolate
A4	3407 4763 050	Environment isolate
A5	3407 4767 050	Environment isolate
A6	3427 4763 251	Environment isolate
A7	3427 6767 051	Environment isolate
E1	NCTC 8155	Dried milk
E2	CDC 4562/70	Clinical, Child's throat

controls. Overnight *C. sakazakii* culture (20 µL) was added into the test wells and the plates were incubated aerobically for 24 h at 30°C. The contents of the plates were removed by inverting the plates, and then the wells washed three times with 300 µl of sterile distilled water. The remaining attached *C. sakazakii* were fixed with 250 µl of methanol per well for fifteen minutes. The liquid was discarded and the plates air-dried. The microplate wells were stained with 250 µl of 0.05% (w/v) crystal violet for five minutes.The excess stain was rinsed off by placing the microplate under running distilled water. After the microplates air-dried, the dye bound to the adherent cells was resolubilized with 250 µl of 33% (v/v) glacial acetic acid per well. The OD of each well was measured at 550 nm using an automatic 96-well microplate reader (ELx 808-

Ultramicroplate reader, Bio-tek Instruments, INC, Winooski, VT, USA) (Appendix 1). Each strain was tested in 8 replicate wells.

2.6 Enumeration of *C. sakazakii,* using impedance detection with the BacTrac® 4000

The BacTrac® 4000 (Minitrac, Sy-Lab, Purkers dorf, Austria) was used to estimate the number of viable bacteria on surfaces, based on a previous publication (Flint and Brooks 2001). A calibration was prepared in Tryptic Soy Broth (TSB) at 30^{o}C for 24 h. Cell numbers were determined from this calibration.

2.7 Plate counts

Plate counts were carried out by the spread plate method using Plate Count Agar.

2.8 Biofilm development on naso-gastric tubing

Three different types of new infant feeding tube composed of different materials; silicone, polyurethane and PVC, each with an internal diameter of 5-French (1.67 mm) were used in this study. To assay for biofilm formation on the tubing surfaces, feeding tube segments were cut to give identical surface area (2 cm²) based on the outer diameter measurements specified by manufacturers. The tubing segments were sliced longitudinally to ensure ready access to all surfaces of the tubing. The tubing segments were cleaned and sterilized by immersing into 95% ethanol for 10 min, rinsed three times with 10 mL volumes of phosphate-buffered saline (PBS, pH 7.15) and autoclaved (121.1°C 15 min).

2.9 Inoculum preparation

One loop of selected strain was inoculated into 10 mL TSB and incubated at 37°C for 18 - 24 h prior to experimental work on the next day. The cultures were centrifuged at 4000 x *g* for 15 minutes to collect the cell pellets from the solution and washed by reconstituting in phosphate buffer solution (PBS). Centrifugation and resuspension in PBS was repeated three more times to thoroughly remove all culture media. The initial inocula were approximately 8 log CFU mL^{-1}.

2.10 Inoculation media – 10% reconstituted infant formula milk

The inoculation media used for the trial, comprised 10% reconstituted infant formula milk, prepared by adding 10 g of infant formula milk powder into 90 mL of distilled water and mixed to reconstitute. The pH of the milk sample was adjusted if required to achieve pH 7.0 ±0.1 using 1M KOH or 1M HCl, then autoclaved at 121.1°C for 15 min. The *C. sakazakii* cell pellets were transferred into the sterile 10% reconstituted infant formula milk sample and mixed using a vortex mixer for one minute.

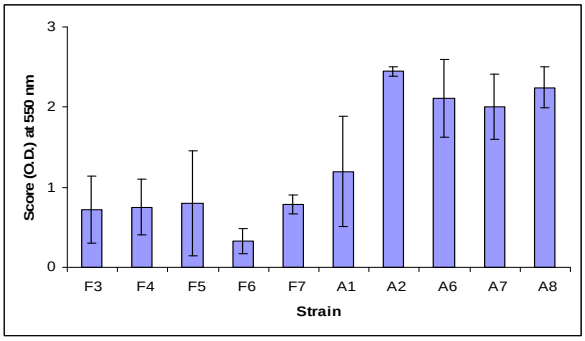
2.11 Incubation

A 5 mL volume of the inoculated 10% reconstituted infant formula milk was pipetted into each sterile screw-capped bottle containing the tube samples (PVC, polyurethane and silicone) and incubated in an aerobic shaker at 25°C and speed at 50 rpm for 24 h. Each test was done in duplicate. The samples of milk (containing planktonic cells) and tubing (containing biofilm cells) were taken every 4, 12 and 24 hours. One mL of each milk sample was pipetted into a BacTrac® 4000 tube containing 9 mL of TSB and incubated at 30°C 24 h for planktonic cells counts. Each sample of tube was washed five times with sterile distilled water and inserted into a BacTrac® 4000 tube containing 10 mL of TSB and incubated in the BacTrac® 4000 microbiological growth analyser at

30°C for 24 h. and the number of viable cells recorded using the BacTrac® 4000 algorithm.

3. Results and discussions

Biofilm development in the microtitre plate screening assay, varied with the origin of the *C. sakazakii* strains (Figure 1). Clinical isolates all produced good biofilms while the strains from environmental and dairy origin varied in the amount of biofilm formed.



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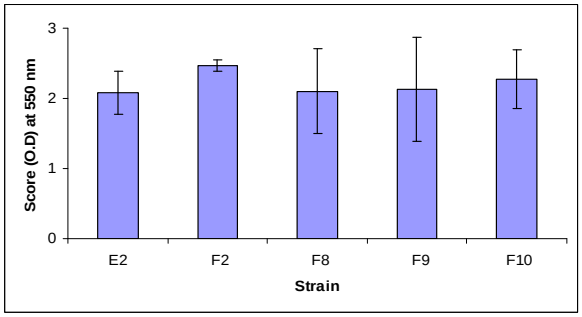

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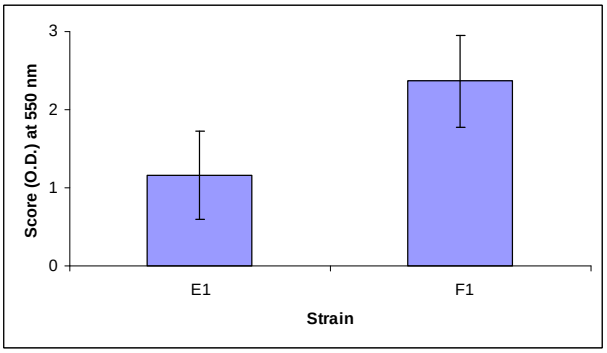


(c)

Figure 1. Biofilm development, determined by the microtitre plate assay (24 h 30ºC) from strains of different origin (a = clinical, b = environmental, c = dairy). Results expressed as mean and standard deviation of 8 replicates.

All the clinical strains attached and formed strong biofilms (OD @ 560 nm > 2.0) when inoculated in infant formula milk and this may be a reflection of their potential pathogenicity. Strain variation in biofilm formation has been observed by others (Hurrel *et al.* 2009) but the

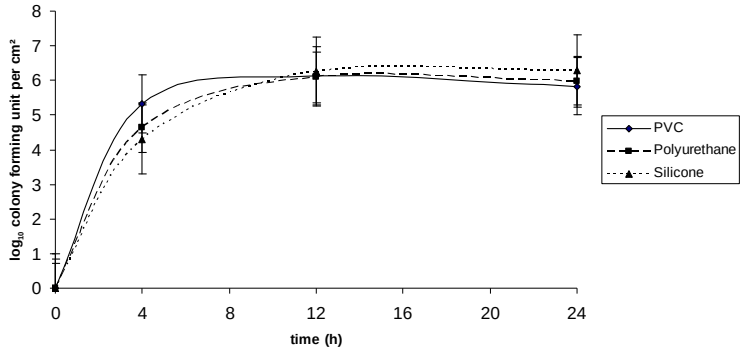
role of clinical isolates in producing a consistently strong biofilm has not been reported. It seems likely that there is a specific sub group of *C. sakazakii* that are best adapted to causing clinical disease, reflected in their ability to grow as biofilm. While only two isolates of dairy origin were available to us, these both showed variable ability to form bioflm, indicating that dairy isolates are likely to similar to general environmental isolates in their ability to form biofilm. If biofilm growth and clinical significance are linked, then dairy isolates are no more likely to cause disease than *C. sakazakii* from any other environmental source.

Differentiating and identifying the sub types of *C. sakazakii* of clinical significance may be useful in targeting control measures to prevent contamination of infant formula. However, contamination is likely to come from variable sources, therefore is unlikely this will ever be totally eliminated from powdered infant formula (Kandhai *et al.* 2004). In a recent study by Chap *et al.* (2009), 12 % (21/182) of infant foods contained *C. sakazakii.* The detection of *C. sakazakii* was through enrichment of samples, implying that numbers within the milk powder are low (< 1/g). Low numbers, however, are likely to be sufficient to attach to surfaces and form a biofilm in naso-gastric tubing while it remains in the infant for several days. Several different types of naso-gastric tubing are available for neonate feeding. Choosing the tubing that is less likely to support biofilm growth would be an added protection against neonate infections.

Three strains (A6, F1 and F8), one environmental (A6), one dairy (F1) and one clinical (F8) isolate were chosen to investigate the formation of biofilm on PVC, polyurathane and silicone tubing (Figure 2). The inocula were higher than would be expected in infant formula, in order to obtain a result in a short time frame. No flow was used in these experiments but it is likely that the slow flow of liquid through a naso-gatric feeding tube would not significantly affect biofilm formation. Incubation in a shaker incubator was used to produce some liquid movement, as would be expected in the tubing during normal use. In addition, we concluded that most of the growth is likely to occur between feedings as the tubing remains inserted in warm conditions suitable for biofilm growth. Biofilm growth on enteral feeding tubes is dependent on temperature and nutrient availability (Kim *et al.* 2006) irrespective of the feeding regime (Hurrel *et al.* 2009) and nutrient depletion, as could be expected between feedings, does not appear to decrease the biofilm population (Kim *et al.* 2006).

All three strains tested for biofilm growth on new naso-gastric feeding tubing produced good biofilm. The dairy strain, F1 grew more slowly than the other two strains and the biofilm at 24 h contained fewer cells than the other two strains after 24 h incubation. Variation in biofilm formation between different strains has been noted previously, however the trend for clinical isolates to produce strong biofilms, confirming the link between biofilm formation and disease. This may relate to the greater likelihood of these strains forming biofilm in feeding tubes producing a high inoculum and high chance of infection.

In all tests, the growth on silicone tubing was slower than on the tubing made from PVC or polyurathane. While the difference in growth rate in these trials is not large, it is consistent and likely to be important in the infant feeding environment where the number of *C. sakazakii* is likely to be very low, requiring the establishment of a bioflim over time to produce a life threatening situation. The colonisation of PVC and polyurethane tubing is likely to reach levels that threaten infant health earlier than on silicone tubing. The silicone tubing is slower to colonise than PVC or polyurethane tubing and is likely to be the best tubing for neonate feeding to avoid *C. sakazakii* infection from biofilm growth.



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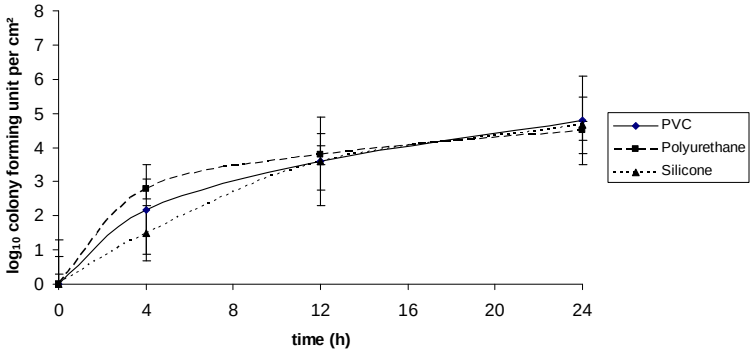

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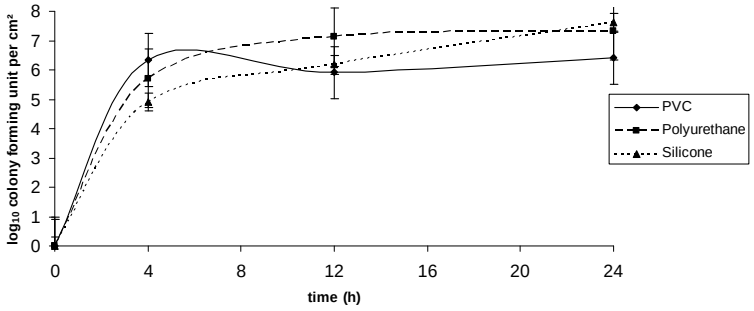

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Figure 2. Biofilm growth on three different types of naso-gastric feeding tubing in infant formula milk over 24 h incubation at 30ºC. (a = A6, b = F1 and c = F8)

4. Conclusion

The effect of temperature on the growth of *C. sakazakii* biofilm growth observed during these trials may be able to be manipulated in infant feeding to reduce the likelihood of biofilm growth and neonatal infection.

Further improvements in delaying the establishment of a biofilm in naso-gastric tubing may be possible by the incorporation of antimicrobial compounds such as bacteriocins, in the silicone

tubing or by forming antimicrobial coatings on the tubing. However, tubing impregnated with silver ions was not successful in preventing biofilm development (Hurrell, *et al.* 2009).

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