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Short communication

Rapid detection of *Cronobacter* spp. with a method combining impedance technology and rRNA based lateral flow assay

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ABSTRACT

Cronobacter spp. is an important release test parameter for powered infant formula (PIF). An impedance method is proposed for the rapid detection of this pathogen in PIF. An impedance based method (BacTrac 4300 Microbiological Analyzer) combined with a RNA hybridisation assay (RiboFlow™) was evaluated using 23 strains in PIF samples and compared to a culture based reference method (ISO/TS 22964). The influences of competitive flora, heat and dry stress on the reliability of the impedance method were investigated. Seven different *Cronobacter* species were included in the evaluation, among them are strains with high susceptibility to low pH and high temperatures. Compared to the reference method, a higher sensitivity (85%) and specificity (100%) was observed using the impedance method, combined with the commercial rRNA based lateral flow test kit as a confirmation tool. The detection time was substantially shortened by using the impedance technique and RiboFlow™. *Cronobacter* could be detected within maximally 29 h, while the reference method takes up to five days when including confirmation of positive results.

1. Introduction

The genus *Cronobacter* comprises seven species: *C. sakazakii*, *C. malonaticus*, *C. muytjensii*, *C. dublinensis*, *C. turicensis*, *C. uiversalis* and *C. condimenti* (Iversen et al., 2007a; Joseph et al., 2011). *Cronobacter* spp. is regarded as a causative agent of meningitis, septicemia or necrotizing enterocolitis in infants with a mortality of 20 to 50% (Lehner and Stephan, 2004). Contaminated powdered infant formulas (PIF) have been identified as the most likely vehicle of transmission of *Cronobacter* associated with hospital outbreaks (van Acker et al., 2001). A rapid and accurate detection of *Cronobacter* spp. is of particular importance for the risk management of *Cronobacter* related food borne diseases.

The International Standards Organization (ISO) (Anonymous, 2006) and the US Food and Drug Administration (FDA) (Anonymous, 2002) have published standardised methods for detection of *Cronobacter* in PIF. For rapid detection and identification of the micro-organism, a number of conventional PCR as well as real-time PCR systems based on different target regions have been developed (Lehner et al., 2006; Nair and Ventkitanarayanan, 2006; Seo and Brackett, 2005).

Another alternative to conventional microbiological method is the impedance method. The concept of using impedance for bacterial growth detection by measuring the change of electrical conductivity caused by bacterial metabolism has been described as early as 1899 (Steward, 1899). For the detection and enumeration of foodborne pathogens such as *Clostridia* and *Salmonella*, the impedance measurement has been assessed as a valid method (Dromigny et al., 1997; Joosten et al., 1994). In the recent years, the impedance technology has been optimised in its specificity by the development of the specific media and the combination with immunological or molecular biological confirmation tools.

The aim of the present study is to evaluate the performance of an impedance method combined with a commercially available RNA hybridisation assay for *Cronobacter* detection in PIF.

2. Materials and methods

2.1. Bacterial strains

The following strains were used in this study: *C. sakazakii* (2688), *C. malonaticus* (2689), *C. malonaticus* (248), *C. muytjensii* (2682), *C. dublinensis* (2687), *C. turicensis* (2683), *C. universalis* (2684), *Enterobacter pulveris* (DSM 19144), *Enterobacter helveticus* (DSM 18306), *Enterobacter turicensis* (DSM 18307), *Enterobacter cloacae* (ATCC 13047), *Escherichia hermanni* (330), *Citrobacter freundii* (ATCC 8454), *Klebsiella pneumoniae* (1947), *Serratia ficaria* (1955), *Pseudomonas aeruginosa* (ATCC 9027), *Leclercia adecarboxylata* (770), *Acenitobacter baumanni* (ATCC 15308), *Bacillus cereus* (ATCC 11778), *Pantoea agglomerans* (ATCC 27155), *Staphylococcus aureus* (ATCC 27733), *Enterococcus casseliflavus* (ATCC 27284) and *Enterococcus faecalis* (DSM 20380).

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2.2. Specificity test

Specificity was determined by analysing seven *Cronobacter* strains and 16 non-*Cronobacter* strains. Strains frozen at −80 °C on biobeads (Transia, Germany) were first grown overnight in Buffered Peptone Water (BPW) (Merck, Germany) at 37 °C and diluted in bacteriological peptone (Oxoid, UK). For specificity analysis, PIF was first sterilised with 25 kGray gamma irradiation. 100 g sterilised PIF was spiked with respective target and non-target strains at 1–10 CFU/100 g. For each sample the impedance analyses were performed in six replicates.

2.3. Cronobacter detection with competitive flora

Sterilised PIF was inoculated with fresh overnight cultures of *C. sakazakii* or *C. universalis* in 1–10 CFU/100 g respectively and with competing micro-organisms at levels of 1, 100, 1000 CFU/100 g. The impedance analyses were performed in six replicates for *C. sakazakii* and duplicates for *C. universalis*. In an extended experiment focusing on acid- and heat-sensitive *Cronobacter* strains, PIF was inoculated with *C. malonaticus* 248 and *C. universalis* at 10 and 100 CFU/100 g respectively and simultaneously with *C. feundii* at 1 CFU/100 g. There were five replicates per target strain at each inoculation level.

2.4. Detection of heat and dry stressed Cronobacter strains

100 g sterilised PIF was spiked with *C. sakazakii* in 8×10^4 CFU/g and desiccated at 50 °C for 4 h and kept dry at 20° for 96 h. This resulted in a reduction of the *Cronobacter* load by 2 to 3 log units.

2.5. Cronobacter detection in commercial PIF samples

Ten commercially manufactured PIF samples and 50 skim milk powder samples of various manufacturers were randomly collected. Each sample was tested in 100 g with the impedance–RiboFlow™ method and the reference method (Section 2.6.2) in parallel.

2.6. Method performance comparison

2.6.1. Impedance measurement combined with RiboFlow™ assay

100 g spiked or commercial PIF samples were diluted in 900 ml BPW. After incubation at 37 °C for 16 h 0.1 ml of the pre-enriched samples was transferred into the measuring cells filled with 10 ml BiMedia 144A (Sylab, Austria) supplemented with 10 mg/l vancomycin. The impedance measurement was performed at 42 °C for 24 h in the BacTrac 4300 Microbiological Analyzer (Sylab, Austria). The medium impedance (M-value) detects the change of the impedance caused by the changed conductivity of the growth medium. A detection time (DT) is defined as incubation time required from analysis start to reach a certain change percentage in the impedance which is selected as threshold value. For the current evaluation a 5% threshold for the M-value was defined. As soon as the threshold value was achieved, samples were subjected to further confirmation using the RiboFlow™ *Cronobacter* kit (Sylab, Austria). 0.5 ml of the cultured BiMedia 144A was used and processed according to the manufacturer's instructions (briefly: spin at 13,000 g and reconstitution with specific kit buffer, application of 0.1 ml to the lateral flow assay, incubation for 0.25 h at 37 °C).

2.6.2. Conventional reference procedure

After pre-enrichment of 100 g samples in 900 ml BPW for 16 h at 37 °C, 0.1 ml of pre-enriched culture medium was transferred into 10 ml modified lauryl sulphate tryptose (mLST) broth (Merck, Germany) with 0.5 M NaCl and 10 mg/l vancomycin for 24 h at 44 °C and streaked on Brilliance™ *E. sakazakii* chromogenic agar Druggan–Forsythe–Iversen (DFI) formulation (Oxoid, UK) and incubated for 24 h at 37 °C. For each sample in this study, the reference method was performed in parallel to the impedance method.

3. Results and discussion

3.1. Determination of the cut-off time and specificity test for cronobacter screening with impedance

The slowest-growing *Cronobacter* species *C. universalis* was used to establish the cut off time (incubation time in BacTrac required to exceed the preset threshold of M-value) for the impedance-based screening step on *Cronobacter* in PIF. The average DT of 4.24 h with a standard deviation of 1.09 was used to calculate a tolerance interval of $m\pm2$ s (2.06 h to 6.42 h) and the upper limit was rounded to 7 h. Samples with DTs longer than 7 h would presumptively be free of *Cronobacter* with a confidence of at least 95%. Therefore the cut-off time to discriminate presumptively positive samples from negative ones was set at 7 h. Totally 138 impedance analyses were performed on 23 PIF samples spiked with *Cronobacter* or non-*Cronobacter* species as pure inoculum respectively (Table 1). All seven samples spiked with *Cronobacter* were detected within 7 h, with the maximum DT of 4.24 h observed in *C. universalis*. 56 of the 96 analyses spiked with non-*Cronobacter* strains were detected within 7 h in the impedance assay. These 56 positive results included eight samples containing non-*Cronobacter Enterobacteriaceae* strains, with the longest impedance response time of 5.60 h obtained in *S. ficaria*. Of the samples with non-*Enterobacteriaceae* strains, only one inoculated with *P. aeruginosa* was detected within 7 h. The RiboFlow™ assay detected all samples containing *Cronobacter* correctly and confirmed all non-target strains as negative. The reference method provided presumptive positive results on DFI for samples spiked with *E. pulveris*, *E. helveticus* and *E. turicensis*.

3.2. Cronobacter analyses with competitive flora in spiked and plain commercial PIF samples

In an earlier investigation, isolates of *C. universalis* 2684 and *C. malonaticus* 248 were found to be sensitive to low pH (inhibited growth at pH 5.0) and sensitive to a higher temperature (no growth at 45 °C), while the strain *C. sakazakii* 2688 grew well up to 46 °C and at pH 5.0 (data not shown). The recovery of the acid- and heat-resistant strain of *C. sakazakii* and the sensitive strain of *C. universalis* with competitive flora in spiked PIF is shown in Table 2. All mixed culture samples spiked with *C. sakazakii* were detected within 7 h and were positively identified in all cases using RiboFlow™, fully comparable with the results obtained from the reference method. *C. universalis* provided delayed DT responses except when co-contaminated with *E. cloacae* or *C. freundii* in amounts of 100 and 1000 CFU/100 g respectively and in the latter cases DTs were around 3 h. While *C. sakazakii* in co-cultures was always detectable with both methods, *C. universalis* was out-competed by *E. cloacae* or *C. freundii* and therefore not recovered in the RiboFlow™ assay or with the reference method. DTs exceeding 7 h were found in six samples with *S. aureus*, *E. faecalis* and *P. aeruginosa* although the target strains were detected in all cases with both methods. This has been investigated in more detailed experiments involving two sensitive *Cronobacter* strains and *C. freundi*. These results revealed for samples inoculated with critical target strains at levels of 10 CFU/100 g a recovery rate of 60% (6/10) and 40% (4/10) for the impedance–RiboFlow™ and the reference method respectively. Samples spiked with *Cronobacter* at higher levels of 100 CFU/100 g were correctly identified in 9 of 10 (90%) cases with the impedance–RiboFlow™ and in 5 of 10 (50%) cases with the reference method.

The detection of bacteria in the impedance devices requires that the micro-organisms grow to a cell density of 10^6–10^7 CFU/ml (Firstenberg-Eden, 1984). After the pre-enrichment in BPW for 16 h, a bacterial count of 10^8 CFU/ml was obtained for *C. sakazakii* 2688 (data not shown), indicating that the bacterial count is still in the detectable level of approximately 10^6 CFU/ml or would reach this level within a short incubation period when 0.1 ml aliquot is transferred from pre-enrichment into the impedance measuring