Microbial update

Enterobacter sakazakii

Produced as a service to the food industry by Oxoid Ltd.

Enterobacter sakazakii is a Gram negative rod shaped bacterium within the family Enterobacteriaceae. The organism was previously called 'yellow pigmented Enterobacter cloacae' until 1980 when it was renamed in honour of the Japanese microbiologist, Riichi Sakazaki, as Enterobacter sakazakii. Further classification of the bacterium has recently been investigated and it has been proposed that the bacterium be renamed as the type species of a new genus, Cronobacter.

E. sakazakii is a rare but life threatening cause of neonatal meningitis, septicaemia and necrotising enterocolitis. Low birth weight and premature infants, and those with underlying medical conditions, are most at risk of infection. The fatality rate among newborns with severe infection can be as high as 80% and those that survive are often left with neurological disorders.

Source of infection

There is a strong association between E. sakazakii infection in neonates and the use of powdered infant formulae. The FDA and the FAO/WHO stress that, although ready-to-use liquid infant formulae have been sterilised, powdered infant formulae are not sold as sterile. They are not processed at high enough temperatures, or for sufficient time, to achieve sterility and therefore may remain contaminated and a threat to susceptible infants. In a study that tested various powdered infant formulae from a number of different countries, E. sakazakii was recovered from 14% of samples.

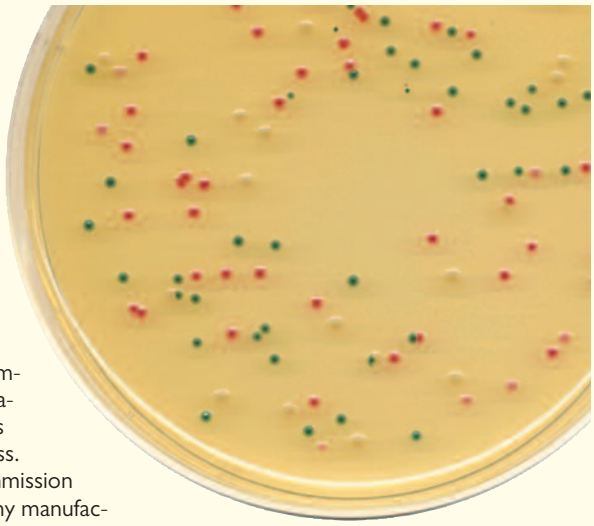
In addition to milk based powdered formulae, human milk fortifiers (for pre-term breast milk) and soy based powders are also a risk and even low levels of contamination can lead to infection. Furthermore, since E. sakazakii has been isolated from both hospital and processing plant environments, the risk of contamination is present during preparation of infant formulae as well as during the manufacturing process.

With the introduction of Commission Regulation EC 2073:20058, many manufacturers have increased their testing of the production environment, raw material and end product. These regulations state that dried infant formulae and dried dietary food for special medical purposes must have an absence of enterobacteriaceae in 10g.

If enterobacteriaceae are detected then the sample must be further tested for E. sakazakii and salmonella. Even with the resulting increase in surveillance, the full scale of the problem is difficult to assess since there is still a lack of reporting systems for E. sakazakii in many countries.

The World Health Organisation estimates that the rate of invasive infection among infants of less than one year, is one case per 100,000.

Oxoid's BAX system for the detection of E. sakazakii.



E. sakazakii colonies (blue-green) growing on Oxoid chromogenic Enterobacter sakazakii agar (DFI formulation).

Detection dilemmas

The established conventional method for detecting E. sakazakii was pioneered by Muytjens et al. in 1988, and was subsequently recommended for use by the FDA. This method involves pre-enrichment of a sample in sterile water and enrichment in EE broth, followed by plating on to violet red bile glucose agar (VRBGA) for presumptive identification of enterobacteriaceae. Five presumptive enterobacteriaceae colonies are picked and inoculated on to tryptone soya agar (TSA) where yellow pigmented colonies are considered presumptive E. sakazakii.

Following concerns of poor sensitivity due to the potential for other enterobacteriaceae to outgrow E. sakazakii during the pre-enrichment and enrichment stages of the FDA method above, an interim draft ISO method was established in 2006; ISO/TS 22964.

This method stipulates the testing of nine replicates incubated overnight in buffered peptone water (BPW)

Each of these enrichments is further incubated in modified lauryl sulphate tryptose broth (mLST) for 24 hours after which they are streaked onto selective chromogenic agar. Up to five typical colonies from each of the nine chromogenic agar plates are then subcultured onto TSA where yellow pigmentation is once again used for identification.

This can result in as many as 45 purified colonies for full confirmation using either a commercially available miniaturised biochemical ID kit or a selection of: oxidase, L-lysine decarboxylase, L-ornithine decarboxylase, L-arginine dihydrolase, fermentation of sugars, or utilisation of citrate.

In both these methods, incubation at a raised temperature (~44°C) is used to inhibit competing bacteria. However, some experts consider this too high as not all strains of E. sakazakii grow at this temperature.

In addition, not all E. sakazakii strains produce the yellow pigment which is used in both methods as an initial diagnostic feature of the organism.

Both methods take a minimum of five days to a positive result. This delay can result in either product being released before results

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become available, thus taking a risk that contaminated product could reach the end user, or delaying dispatch and holding stock until results are made available.

The future of detection

A new horizontal method which is intended to form a full ISO standard is currently under development. It is intended that this method will incorporate a reduced incubation temperature and will remove the reliance on yellow pigmentation, thus increasing both sensitivity and specificity over the FDA and ISO TS methods.

Diagnostic companies are further striving to reduce the total time to result.

Oxoid has developed a chromogenic medium, chromogenic Enterobacter sakazakii agar – Druggan-Forsythe-Iverson (DFI) formulation, which has reduced the recovery and detection time for E. sakazakii to just three days, with equivalent performance to the interim ISO method.

This medium should be used following pre-enrichment and selective enrichment.

It contains the chromogenic substrate, 5-bromo-4-chloro-3-indolyl-α D-glucopyranoside, which is cleaved by the enzyme α-glucosidase (present in E. sakazakii) to form easily distinguishable blue-green colonies (see photograph).

The increased specificity of this method removes the necessity for VRBGA and TSA plates and the reliance on yellow pigmentation. Furthermore, it can be performed easily in any microbiology laboratory, without the need for specialist equipment.

In a comparative evaluation, all clinical and food strains of E. sakazakii (n=95) were detected on the Oxoid chromogenic Enterobacter sakazakii agar (DFI formulation), at least two days sooner than by the FDA method.

An alternative method, the Enterobacter sakazakii kit for use with the DuPont Qualicon BAX system, replaces the post enrichment steps above with a PCR test allowing the positive release of product in less than 32 hours from sample receipt.

Following enrichment in mLST broth for 22-24 hours, sample is diluted and grown back in brain heart infusion broth for three hours. The cells are then lysed, using a protease enzyme and heat, and then undergo amplification, detection and analysis within the automated BAX system.

This end point detection method, which uses the polymerase chain reaction (PCR), is the first rapid end point detection method developed solely for the detection of E. sakazakii in dehydrated infant formula.

Conclusion

The devastating consequences of E. sakazakii demand the utmost care and attention to ensure that newborn and immunocompromised infants are protected from this opportunistic pathogen.

By harnessing the very latest technology for microbial identification, the methods described above allow E. sakazakii to be isolated and identified quickly and reliably.

Between them, there is a product for every application, whether it is fast and reliable diagnosis of infection, rapid release of end products, pinpointing sources of contamination or proactive environmental monitoring. n

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References

1Farmer, J. J., III, M. A. Asbury, F. W. Hickman, D. J. Brenner and the Enterobacteriaceae study group. (1980). Enterobacter sakazakii: a new species of "Enterobacteriaceae" isolated from clinical specimens. Int. J. Syst. Bacteriol. **30**: 569-584.

1Iversen et al. (2007). The taxonomy of Enterobacter sakazakii: proposal of a new genus Cronobacter. BMC Evol. Biol. **7**:64.

1National Association of Pharmacy Regulatory Authorities (Canada) (2002). Enterobacter sakazakii Infection and Powdered Infant Formulas. Advisory. September 20, 2002.

1US FDA (2002) Center for Food Safety and Applied Nutrition. Isolation and enumeration of Enterobacter sakazakii from dehydrated powdered infant formula.

1WHO (2004) Joint FAO/WHO workshop on Enterobacter sakazakii and other micro-organisms in powdered infant formula. Background, Executive Summary and Questions and Answers.

1US FDA (2002) Letter to Health Professionals on Enterobacter sakazakii Infections associated with the use of Powdered (Dry) Infant Formulas in Neonatal Intensive Care Units.

1Muytjens, H.L., Roelofs-Willemse, H. and Jaspar G.H.J. (1988). Quality of powdered substitutes for breast milk with regards to members of the family Enterobacteriaceae. J. Clin. Microbiol. **26**: 743-746.

1Commission Regulation (EC) No 2073:2005 on microbiological criteria for foodstuffs 15th November (2005).

1Nazarowec-White, M. and Farber, J. (1997). Incidence, survival and growth of Enterobacter sakazakii in infant formula. J. Food Protect. **60**:226-230.

1Iversen, C., Lane, M. and Forsythe, S. (2004). The growth profile, thermotolerance and biofilm formation of Enterobacter sakazakii grown in infant formula milk. Lett. Appl. Microbiol. **38**:378-382.