Arch Lebensmittelhyg 62,
175–180 (2011)
DOI 10.2376/0003-925X-62-175

ISSN 0003-925X

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Detection, identification and typing methods for *Cronobacter* spp. – a review*

*Detektion, Identifizierung und (Sub-)typisierung von Cronobacter spp. – eine Literaturübersicht**

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** Dedicated to Prof. Dr. Karsten Fehlhaber on the occasion of his 65th birthday*
Herrn Prof. Dr. Karsten Fehlhaber zum 65sten Geburtstag gewidmet

Summary

Rapid and reliable identification of strains of the genus *Cronobacter* and its differentiation from phenotypically similar, apathogenic *Enterobacter turicensis, Enterobacter helveticus* and *Enterobacter pulveris* is important for surveillance, prevention and control of this food-borne pathogen. Moreover, for *Cronobacter* a species differentiation is relevant for epidemiological studies. This review summarizes methods for detection, identification and typing of *Cronobacter* spp.

Keywords: *Cronobacter* spp., detection, identification, typing

Zusammenfassung

Die zuverlässige Identifizierung von Stämmen der Gattung *Cronobacter,* welche eine Unterscheidung von den phänotypisch ähnlichen, jedoch apathogenen Arten *Enterobacter turicensis, Enterobacter helveticus* und *Enterobacter pulveris* ermöglicht, ist unerlässlich in Bezug auf die Überwachung, Prävention und Kontrolle dieses Lebensmittel-assoziierten pathogenen Organismus. Darüber hinaus stellt die Zuordnung von *Cronobacter* Isolaten auf Speziesebene die Voraussetzung zur Bearbeitung epidemiologischer Fragestellungen dar. Dieser Artikel gibt eine Übersicht über (etablierte) Methoden zur Detektion, Identifikation und (Sub-)typisierung von *Cronobacter* spp.

Schlüsselwörter: *Cronobacter* spp., Nachweis, Identifizierung, Typisierung

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Introduction

The genus *Cronobacter* (former *E. sakazakii*) comprises 6 species, *Cronobacter sakazakii, Cronobacter malonaticus, Cronobacter muytjensii, Cronobacter dublinensis, Cronobacter turicensis, Cronobacter* genomospecies 1, which have been recognized on the basis of a polyphasic approach using extensive geno- and phenotypic evaluations (Iversen et al., 2007a; Iversen et al., 2007b, Iversen et al., 2008b). The definition of six species within the genus *Cronobacter* is further supported by a recent study, where a multilocus sequence analysis (MLSA) approach was used (Kuhnert et al., 2009).

Cronobacter spp. are Gram-negative opportunistic foodborne pathogens and known as rare but important causes of live-threatening neonatal infections, which can lead to severe disease manifestations such as brain abscesses, meningitis, necrotizing enterocolitis and systemic sepsis (Lehner and Stephan, 2004). Neonates and infants under two months of age which were borne prematurely are at greater risk of *Cronobacter* infections from consuming *Cronobacter* contaminated powdered infant formulas (Hunter et al., 2008).

Rapid and reliable identification of strains of the genus *Cronobacter* and its differentiation from phenotypically similar, apathogenic *Enterobacter turicensis, Enterobacter helveticus* and *Enterobacter pulveris* is important for surveillance, prevention and control of food-borne diseases. Moreover, for *Cronobacter* spp. a species differentiation is relevant for epidemiological studies, and the different species show differences in sensitivity to chemical agents and antibiotics. The aim of this review is to summarize the knowledge on detection, identification and typing methods for *Cronobacter* spp.

Detection procedures

In 2002, the United States Food and Drug Administration (FDA) published a method for detection of *E. sakazakii* which included a pre-enrichment step in buffered peptone water (BPW), enrichment in *Enterobacteriaceae* Enrichment (EE) broth, plating on Violet Red Bile Glucose agar (VRBG) and picking of five typical colonies onto tryptone soy agar (TSA) plates (Anonymous, 2002). After incubation at 25 °C for 48–72 hours, yellow pigmented colonies on TSA plates are confirmed using the API 20E system. The main weak points of this procedure are the inability of some target strains to grow in the selective EE broth; the lack of discrimination between *Enterobacteriaceae* strains on VRBG; the variation in intensity of the pigmentation, with occasional observation of non-pigmented strains; and the weak reliability of the API 20E system (Iversen et al., 2007b). Gentil et al. (2005) published an alternative procedure based on selective enrichment in a modified lauryl sulphate tryptose broth (mLST), incorporating 0.5 M NaCl and 10 mg l^{-1} vancomycin hydrochloride. This method was further improved by the replacement of VRBG with a chromogenic agar and forms the basis of the ISO Technical Specification for detection of *E. sakazakii* in milk-based infant formula, ISO/TS 22964:2006 "Milk and milk products – detection of *Enterobacter sakazakii*".

Various chromogenic and fluorogenic agar media have been described in recent years for detection of *Cronobacter* spp. (Iversen et al., 2004; Oh and Kang, 2004; Restaino et al., 2006). These are based mainly on the enzyme α-glucosidase, which is constitutively expressed in *Cronobacter* spp., other organisms also produce presumptive colonies on these agars, notably the recently described species *E. helveticus, E. turicensis* and *E. pulveris* (Stephan et al., 2007; Stephan et al., 2008). These species can be found in the same ecological niches as *Cronobacter,* such as dried food products and factory environments and present a challenge to both culture-based as well as molecular isolation and identification methods.

Meanwhile, it has been established that some isolates of *Cronobacter* spp. do not grow well in mLST currently proposed for isolation of these organisms (Iversen and Forsythe, 2007). Therefore, Iversen et al. (2008a) developed a new *Cronobacter* Screening Broth (CSB). The broth is designed to circumvent the problems encountered with selective enrichment media for these organisms and to be complementary to current available chromogenic media in order to improve overall sensitivity and selectivity of *Cronobacter* spp. detection.

Moreover, modifications to the composition of BPW with a view to inhibition of competing Gram-positive background flora may potentially improve recovery of Gram-negative organisms from samples and will be a challenge for the future. Such an improvement may have useful application as a pre-enrichment step in other microbiological culture methods for recovery of Gram-negative pathogens, such as *Salmonella* spp. and *Cronobacter* spp., as well as indicator organisms.

An alternative method to avoid the difficulties of selective broths is the MATRIX PSAK50 Method (Matrix MicroScience Ltd, UK), which uses cationic paramagnetic particle capture to concentrate contaminating microorganism in a pre-enriched sample before plating directly onto isolation agar.

Other proposed rapid methods for the detection of *E. sakazakii* include two enzyme-linked immunoassays (EIAs): the Assurance for *Enterobacter sakazakii* (Bio-Control Systems, USA) and the TECRA HELIX *E. sakazakii* Method (TECRA International, Australia).

Phenotypic identification

To ensure the safety of infant formula and also to reduce unnecessary disposal of product, it is important to identify *Cronobacter* spp. as accurately as possible. The recently developed fluoro- and chromogenic media are useful tools in the phenotypic selection of presumptive *Cronobacter* spp. However *E. helveticus, E. pulveris, E. turicensis* and *E. cloacae*-complex species may also give characteristic colonies on some of these media and further confirmation is essential. The phenotypic identification of *Cronobacter* spp. with available commercial systems may be difficult (Iversen et al., 2007b). Molecular methods revealed that several strains identified as *E. sakazakii* by commercial biochemical kits belonged to *E. cloacae*-complex species (Iversen et al., 2007a).

Molecular based identification

Genus-specific detection and identification

A number of molecular approaches have been developed to specifically identify *Cronobacter* spp. strains. Conventio-

nal and real-time PCR methods enable (quantitative), sensitive, specific and rapid detection from enrichment broths and agar media. Targets for conventional PCR systems include the 16S rRNA gene (Lehner et al., 2004), the *omp*A gene (Mohan Nair and Ventkitanarayanan, 2006), the gene coding for the 1,6 α-glucosidase (Lehner et al., 2006a) and a gene encoding a zinc-containing metalloprotease (Kothary et al., 2007). The 'real-time'-assays that have been described so far, target the 16S rRNA gene (Kang et al., 2007), a region located between the 16S rRNA and the 23 rRNA genes (Liu et al., 2006), a region between the tRNA-glu and 23S rRNA genes (Derzelle and Dilasser, 2006) and the *dna*G gene (Seo and Brackett, 2005).

The U.S. Food and Drug Administration has also developed a revised method involving an enrichment step, centrifugation and a combination of two chromogenic agars and the *dna*G gene PCR for confirmation (Chen et al., 2009).

In the last few years, several real-time PCR based systems for detection of *Cronobacter* spp. are also commercially available. In a recent study a comparative evaluation of three diagnostic systems, namely the BAX® System PCR Assay *Enterobacter sakazakii* (DuPont, Qualicon, Wilmington, USA), the Assurance GDS™ *Enterobacter sakazakii* (BioControl, Bellvue, USA) and the foodproof® *Enterobacter sakazakii* Detection Kit (Biotecon Diagnostics, Potsdam, Germany) was done (Fricker-Feer et al., 2011). A specificity of 100 % was observed for two of the three real time PCR systems tested, namely the Assurance GDS™ *Enterobacter sakazakii* and the foodproof® *Enterobacter sakazakii* Detection Kit for pure cultures as well as artificially contaminated powdered infant formula (PIF) samples.

The VIT® (vermicon identification technology, Munich, Germany) represents an alternative to the DNA-targeted PCR-based detection and identification systems for *Cronobacter* spp. It is based on fluorescently labelled gene probes targeting specified regions on the ribosomal RNA of the bacteria, therefore only live cells are detected by the system. The test is performed on 1 ml of overnight culture of enrichment broth or rich media broth (e. g. BHI, LB) after inoculation with presumptive colony material according to the instructions of the manufacturer. The method has successfully been used in a comparative study evaluating cultural and molecular identification systems for *Cronobacter* spp. (Lehner et al., 2006b) Another hybridization based method using a peptide nucleic acid probe for the specific detection of *Cronobacter* genomospecies has been published (Almeida et al., 2008).

Species-specific identification

As identification of *Cronobacter* spp. isolates to the species level is required for epidemiological studies, a PCR system for the differentiation of the six proposed species was developed in a recent study (Stoop et al., 2009). The *rpo*B gene was chosen as target to develop different conventional PCR systems, which enable strains previously confirmed as belonging to the genus *Cronobacter* to be further discriminated to the species level. However, as the *rpo*B gene sequences for *C. sakazakii* and *C. malonaticus* are very closely related, a two-step procedure using the primers for the identification of *C. malonaticus* in a follow-up PCR on those strains positive for the *C. sakazakii* identification assay is necessary to reliably distinguish these species.

Identification by MALDI-TOF MS

The detection of protein mass patterns using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has become a convenient tool for the rapid analysis of bacteria. The method analyzes the profiles of proteins that are extracted from whole bacteria. In the study by Stephan et al. (2010) genus- and species-specific biomarker protein mass patterns were determined for the identification of the six *Cronobacter* species recognized so far. A reference MS database library including representative number of *Cronobacter* spp. target as well as non-target (e. g. phenotypically similar, non-pathogenic *Enterobacter helveticus, Enterobacter pulveris*) strains was established and included in the Spectral Archive and Microbial Identification System (SARAMIS) Super Spectrum database. Validation of the mass-spectrometry-based identification scheme yielded identical results as with PCR-based identification system demonstrating that MALDI-TOF MS is a reliable and powerful tool for the rapid identification of *Cronobacter* spp. strains to the genus and species level.

Subtyping methods

A number of methods have been developed that can be used to subtype strains of *Cronobacter* spp. including biotyping, serogrouping, plasmid profiling, ribotyping, random amplification of polymorphic DNA (RAPD), repetitive sequence primed PCR (REP-PCR), enterobacterial repetitive intergenic consensus (ERIC) PCR, amplified fragment length polymorphism (AFLP) and Pulsed-Field Gel Electrophoresis (PFGE). Techniques that generate a DNA fingerprint pattern are more discriminatory and reliable than classic techniques such as biotyping. PFGE is currently seen as the 'gold standard' for molecular subtyping of *Cronobacter* spp. and has been used to investigate outbreaks in neonatal intensive care units. It has also been successfully used to trace the dissemination of *Cronobacter* spp. strains within manufacturing facilities.

PFGE

Pulsed-Field Gel Electrophoresis (PFGE) is a non-amplified technique that separates long strands of DNA molecules (larger than 15–20 kb) previously digested with restriction enzyme(s). Periodical switching of the voltage in three directions results in the reorientation of DNA moving through the gel in a size dependent manner, which facilitates a finer resolution as it aligns and re-aligns to the applied electrical field.

In 1994 an outbreak occurred in France involving 17 neonates *Cronobacter* spp. isolates were obtained from various anatomical sites, prepared feeds and unused infant formula. PFGE analysis described four clusters, two of which contained isolates from neonates who ranged from asymptomatic colonised individuals to case fatalities. One other cluster contained isolates from the prepared feed, an asymptomatic neonate and a neonate with mild digestive problems. The fourth cluster comprised the isolates from the unused powdered formula. In this case no link could be made between the powdered formula and the outbreak and it is possible that the prepared feed became contaminated via an alternate source (Caubilla-Barron et al., 2007). A further outbreak occurred in France in 2004 involving 13

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infants, nine were asymptomatically colonised, two developed bacterial meningitis, one had conjunctivitis and one suffered haemorrhagic colitis. The outbreak was investigated using automated ribotyping (using restriction endonucleases *Eco*RI, *Pst*I and *Pvu*II) and by PFGE using the enzymes *Xba*I and *Spe*I. A total of nine isolates from eight neonates were found to have undistinguishable ribotypes and PFGE patterns from isolates obtained from four separate lots of infant formula, thus establishing a clear link between the outbreak and the product. Recently, factory surveillance studies have employed a PFGE method using *Xba*I for tracing *Cronobacter* spp. isolates within infant food manufacturing facilities (Mullane et al., 2008a; Iversen et al., 2009). Recently, in conjunction with the PulseNet Programme at the Centres for Disease Control and Prevention (CDC) in the US, a collaborative study was done in several international laboratories to develop a standard protocol for *Cronobacter* spp. PFGE typing. This protocol should be published soon.

REP-PCR

Although PFGE is considered the gold standard for the detection of clonality in disease outbreaks, PCR-based methods are cheaper, easier to perform, and provide faster results.

Recently the discriminative power of REP-PCR has been compared to PFGE for *Cronobacter* spp. isolates (Healy et al., 2008). Using Simpson's index of diversity, values of 0.974 and 0.998 were calculated for REP-PCR and PFGE respectively at a similarity cut-off of 95 % demonstrating good correlation with a high degree of genetic heterogeneity among the isolates.

RAPD

Random amplification of polymorphic DNA (RAPD) typing has been used to analyse clonal relationships between *Cronobacter* spp. strains in a number of independent studies (Drudy et al., 2006; Iversen et al., 2009). Nazarowec-White and Farber (1999) developed protocols for the molecular subtyping of *Cronobacter* spp. by ribotyping, RAPD and pulsed-field gel electrophoresis (PFGE). The authors showed that RAPD and PFGE were the most discriminatory sub-typing schemes for *Cronobacter* spp. followed by ribotyping and two microbiological typing methods – biotyping and antibiograms. However, the transferability of RAPD results to other labs can be a limitation of this technique. Moreover, in contrast to the PFGE method no databases of strain patterns exist for the RAPD technique.

Ribotyping

Ribotypes are generated by probing restriction fragments of genomic DNA for the highly conserved genes coding for the 16S and 23S rRNA. Small variations between strains occur in the less conserved, flanking genes and intergenic sections of the genome resulting in fragments of unequal size. These are separated using gel electrophoresis, transferred to a membrane and the individual strain fingerprints revealed by hybridization of chemiluminescent probes. Riboprint patterns are analyzed based on the number, size and signal intensity of the detected fragments. Comparison to existing entries in a riboprint database allows species- and subspecies-level identification.

The automated RiboPrinter™ Microbial Characterization System (Dupont Qualicon, USA) was used as part of a polyphasic taxonomic characterisation of *Cronobacter* spp. strains (Iversen et al., 2007a). Isolates were grown on TSA (18 h, 37 °C) and prepared according to standard procedures (Bruce, 1996) using the *Eco*RI restriction enzyme. The strains were divided into four distinct clusters, *C. dublinensis, C. turicensis and C. muytjensii* occupied individual clusters whereas *C. malonaticus* appeared as one subcluster among several subclusters of *C. sakazakii.* Nazarowec-White and Farber (1999) studying three *Cronobacter* spp. isolates obtained from one hospital over 11 years showed that they had indistinguishable ribotype patterns indicating possible persistence in the environment.

BOX-PCR

Proudy et al. (2008) examined the discriminative power of the 154 bp BOX element against the sequencing of the *fli*C gene and PFGE using 27 *Cronobacter* strains from clinical and environmental sources. The BOX-PCR results showed 92 % agreement with PFGE results indicating the potential of this typing method for epidemiological investigation, whereas *fli*C gene sequencing was poorly discriminative.

MLVA

Variable number tandem repeat (VNTR) motifs represent sources of genetic polymorphisms. These DNA sequence elements are often maintained within a bacterial species, with individual strains displaying different copy numbers. The length of a tandem repeat at a specific locus can vary as a consequence of DNA slippage during replication or unequal crossover elements. These differences can be analyzed by amplification of the region and sizing of the resulting amplicons. The high degree of polymorphism at these loci is particularly useful as a target for strain discrimination within bacterial species. Multi-locus VNTR analysis (MLVA) is a subtyping method that involves amplification and fragment size comparison of polymorphic VNTR regions. The availability of a complete *C. sakazakii* genome sequence (http://genome/wustl.edu/pub/organism/Microbes/Enteric_Bacteria/Enterobacter_sakazakii/assembly/Enterobacter_sakazakii-4.0/) enabled the identification of VNTR motifs within *C. sakazakii.* Subsequently an MLVA subtyping scheme was developed and applied on a genotypically and phenotypically diverse collection of *Cronobacter* spp. isolates (Mullane et al., 2008c).

AFLP

The amplified fragment length polymorphisms (AFLP) technique has been employed in plant and microbiological research to describe the molecular ecology of various niches and can be used to determine inter- and intra-species relatedness. This technique was included in a study by Iversen et al. (2007a) to clarify the taxonomic relationship of over 200 strains previously identified as *Cronobacter* spp. and gave discriminatory results comparable to DNA-DNA hybridization.

Molecular based serotyping

Serotyping assays based on PCR specific O-antigen genes were developed for the identification of *C. sakazakii* serotypes O1 and O2 using PCR primers specific to the O1 *weh*C gene and the O2 *weh*I gene (Mullane et al., 2008b). In a more recent study, six *Cronobacter wzw* genes were sequenced and analyzed leading to the development of three O-antigen cluster-specific PCR primer pairs, which identify five new *Cronobacter* serotypes (Jarvis et al., 2011). Genomic DNA from 231 *Cronobacter* spp. isolates

were screened with these primer pairs with *C. sakazakii* having the most diverse serotype profile of all species included in this study. However, twenty-one percent of the isolates in their collection were negative by the *Cronobacter* serotype-specific PCR assays developed to date, including all of the *C. dublinensis* and *C.* genomospecies 1 strains. Similarly, most of the *C. muytjensii* and *C. turicensis* strains tested in the study were negative by all of the serotype PCR assays, suggesting that there are additional serotypes within the *Cronobacter* genus.

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+++ Nachrichten aus Forschung und Industrie +++

(Die Verantwortlichkeit für die Texte liegt ausschließlich bei den Instituten und werbenden Unternehmen.)

Amselsterben in Süddeutschland: Tropisches Virus gefunden

Erfolg für Frühwarnsystem

Wissenschaftler des Bernhard-Nocht-Instituts für Tropenmedizin (BNI) in Hamburg haben gestern das tropische Usutu-Virus in mehreren Organen einer toten Amsel aus dem hessischen Birkenau nachgewiesen. Die Amsel wurde im Rahmen des Projekts „Vorkommen von Stechmücken in Deutschland" von Mitarbeitern der Kommunalen Aktionsgemeinschaft zur Bekämpfung der Stechmückenplage (KABS) aufgefunden und an das BNI weitergeleitet. „Dass das Massensterben der Vögel durch das Usutu-Virus bedingt ist, bleibt jedoch noch zu beweisen", sagt Dr. Jonas Schmidt-Chanasit, Leiter der virologischen Diagnostik am BNI. Bereits 2010 fand die Gruppe um Schmidt-Chanasit mit Partnern der KABS das ursprünglich aus Afrika stammende Virus erstmals in deutschen Stechmücken. „Unser bundesweites Frühwarnsystem funktioniert", erklärt Dr. Norbert Becker, Leiter der KABS. „Der Befund ist zwar alarmierend, da Usutu-Viren auch den Menschen infizieren können, jedoch sind in Deutschland bisher keine Infektionen von Menschen diagnostiziert worden", ergänzt Schmidt-Chanasit.

Seit etwa zwei Monaten beschäftigt ein rätselhaftes Amselsterben mit Tausenden von toten Tieren die Vogelexperten des Naturschutzbundes Deutschland (NABU) vor allem in Rheinland-Pfalz und Baden-Württemberg. In einigen Gebieten sind die Amseln fast vollständig verschwunden. Auch Mitarbeiter und Mitarbeiterinnen der KABS in Waldsee sammeln zurzeit tote Amseln und schickten erste Vögel zur Untersuchung ans BNI. „Wir vermuteten schon, dass Usutu-Viren die Ursache sein könnten", erklärt Becker. Mit einem im vergangenen Jahr entwickelten Schnelltest – basierend auf der RT-PCR-Methode – bestätigte die Gruppe um Schmidt-Chanasit über Nacht die Vermutungen der Experten aus Süddeutschland.

„Diese Diagnose ist der erste Erfolg für ein funktionierendes, verlässliches Frühwarnsystem, das wir mit dem Großprojekt ‚Vorkommen und Vektorkompetenz von Stechmücken in Deutschland' etablieren wollten", erklärt Schmidt-Chanasit. Es sei wichtig, rechtzeitig vorhersagen zu können, welche durch Stechmücken übertragenen Viren sich in Deutschland ausbreiten und möglicherweise Menschen und Tiere bedrohen könnten. Anfang des Jahres bewilligte die Leibniz-Gemeinschaft die Förderung eines interdisziplinären Forschungsprojekts mit rund einer dreiviertel Million Euro. Neben dem BNI als Projektkoordinator sind das Senckenberg Deutsches Entomologische Institut (SDEI) in Müncheberg und die KABS als Projektpartner beteiligt.

Usutu-Virus auch auf Menschen übertragbar

Das Usutu-Virus ist kein reines Vogelvirus, sondern kann über einen Mückenstich auch auf den Menschen übertragen werden. Es gibt zurzeit keine Hinweise darauf, dass das Usutu-Virus in Deutschland auf Menschen übertragen wird oder gar eine Epidemie auslöst", beruhigt Schmidt-Chanasit. Es sei jetzt wichtig, die medizinische Bedeutung der Ergebnisse für die Bevölkerung in Deutschland näher zu untersuchen. Im Herbst 2009 wurde erstmals Usutu-Fieber bei Patienten in Italien diagnostiziert. Schwere Verläufe wurden bei immungeschwächten und älteren Menschen beobachtet. Die Infektion geht mit Fieber, Kopfschmerzen und Hautausschlägen einher und kann im schlimmsten Fall eine Gehirnentzündung (Enzephalitis) auslösen. Nach dem gestrigen Befund informierte die KABS umgehend das Gesundheitsministerium in Baden-Württemberg.

Weitere Informationen (Quelle):
Bernhard-Nocht-Institut für Tropenmedizin (BNI)
www.bnitm.de