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PFGE-typing of *Enterobacter cloacae, Klebsiella pneumoniae* and *Leclercia adecarboxylata* isolates from an infant formula processing plant

PFGE-Typisierung von Enterobacter cloacae, Klebsiella pneumoniae und Leclercia adecarboxylata Isolaten aus einem Verarbeitungsbetrieb für Säuglingsanfangsnahrung

Alexandra Popp[1], Claudia Fricker-Feer[2], Karl Gschwend[2], Roger Stephan[1]

Summary

The family of *Enterobacteriaceae* is a useful indicator for hygiene conditions in food production facilities and food products. For powdered infant formula (PIF) the absence of *Enterobacteriaceae* in 100 g is required. Nevertheless, occasionally *Enterobacteriaceae* can be detected. A recent study concentrated on the occurrence of a specific foodborne pathogen out of the *Enterobacteriaceae* family, *Cronobacter* spp., in PIF manufacturing facilities in order to investigate clonal persistence and identify possible transmission routes. The aim of this study was to genotype isolates from frequently found other species within the family of *Enterobateriaceae* in order to elucidate and trace back transmission routes not only limited on *Cronobacter* spp.

In total 216 isolates from three different species (*Enterobacter (E.) cloacae, Klebsiella (K.) pneumoniae* and *Leclercia (L.) adecarboxylata*) were genotyped. The isolates originated from raw ingredients, environment and products of an infant formula processing plant. Restriction digest with *Xba*I revealed discriminative PFGE patterns consisting of 10–20 bands for all three species. Heat sensitive additives could be traced back as contamination source for products. Furthermore, the production environment was found as a reservoir for persisting strains. Showing analogy to the situation described for *Cronobacter* spp., especially *E. cloacae* that can be found in the same niches as *Cronobacter* spp. but more frequently might therefore be used for hygiene monitoring along the processing chain. Certain genotypes of *E. cloacae,* that are able to persist within the factory environment, might possess special properties as e. g. enhanced desiccation tolerance enabling them to survive the harsh environmental conditions.

Keywords: PFGE, genotyping, *Enterobacteriaceae,* infant food

Zusammenfassung

Für Säuglingsanfangsnahrung in Pulverform (PIF) ist der Parameter *Enterobacteriaceae* als Prozesshygienekriterium (nicht nachweisbar in 100 g) definiert. Dennoch können aber gelegentlich *Enterobacteriaceae* in solchen Produkten nachgewiesen werden.

Eine kürzlich publizierte Studie konzentrierte sich auf das Vorkommen von *Cronobacter* spp., einen pathogenen Vertreter aus der Familie der *Enterobacteriaceae,* um mögliche Kontaminationswege zu untersuchen.

Das Ziel der aktuellen Studie war es, andere häufig gefundene Spezies aus der Familie der *Enterobacteriaceae* aus Rohstoffen, dem Produktionsumfeld und von Endprodukten eines PIF Verarbeitungsbetriebes mittels PFGE zu genotypisieren, um mögliche Kontaminationswege aufzuzeigen und die Eignung dieser Spezies für betriebsepidemiologische Studien zu evaluieren. Insgesamt wurden 216 Isolate von drei verschiedenen Spezies (*Enterobacter (E.) cloacae, Klebsiella (K.) pneumoniae* und *Leclercia (L.) adecarboxylata*) in die Untersuchung miteinbezogen. Der Restriktionsverdau mittes *Xba*I ergab für alle drei Spezies aussagekräftige PFGE Muster. Endproduktkontaminationen konnten zum einen auf hitzeempfindliche Zusatzstoffe, aber vor allem auch auf das Produktionsumfeld zurückgeführt werden. Dabei hat sich vor allem die Spezies *E. cloacae,* die in den gleichen Nischen wie *Cronobacter* spp. gefunden werden kann, als ein guter Parameter für betriebsepidemiologische Untersuchungen erwiesen. Bestimmte Genotypen von *E. cloacae* scheinen fähig, im Produktionsumfeld zu persistieren, was möglicherweise auf besondere Eigenschaften, wie z. B. verstärkte Austrocknungstoleranz zurückzuführen ist.

Schlüsselwörter: PFGE, Genotypisierung, *Enterobacteriaceae,* Säuglingsnahrung

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Introduction

The family of *Enterobacteriaceae* is a useful indicator for a hygiene monitoring system in food production facilities and food products. For powdered infant formula the absence of *Enterobacteriaceae* in 100 g (n=10; M=absence in 10 g) is required (Anonymous, 2005). Nevertheless, occasionally *Enterobacteriaceae* can be detected in infant formula and production environment (Muytjens et al., 1988; Iversen and Forsythe, 2004; Estuningsih et al., 2006; Popp et al., 2009). On one hand there are supplements that need to be added after the drying process since they are not heat stable, and on the other hand during the filling process contamination is possible while the system is not completely close.

Up to now, few studies have focused on the presence and the diversity of *Enterobacteriaceae* in infant formula and the related production environment (Muytjens et al., 1988; Iversen and Forsythe, 2004; Estuningsih et al., 2006). Two recent surveys concentrated on the occurrence of a specific foodborne pathogen out of the *Enterobacteriaceae* family, *Cronobacter* spp., in powdered infant formula (PIF) manufacturing facilities in order to investigate clonal persistence and identify possible dissemination routes along the processing chain by the use of PFGE (Mullane et al., 2007; Iversen et al., 2009). Identical pulso-types of strains isolated from different locations indicated that the production environment is an important source for product contamination.

In a previous study (Popp et al., 2009) 470 *Enterobacteriaceae* isolates from different sample types of a Swiss infant formula processing plant were identified. Frequently isolated species were *Enterobacter cloacae* (34 %), *Klebsiella pneumoniae* (8 %) and *Leclercia adecarboxylata* (6 %). The aim of this study was to genotype these isolates in order to elucidate and trace back transmission routes not only limited on *Cronobacter* spp.

Materials and Methods

Manufacturing facility

The firm is one of the leading milk processing companies in Switzerland and has two milk-associated production sites (site A, site B) about 100 km apart. It handles about 350.000 tons of milk per year and fabricates various products like milk powder, powdered infant formula and follow on and growing up formula that are sold in over 60 countries. Customers include the food industry, the retail industry, bakeries and the catering trade.

Isolates

Isolates were collected within a previous study (Popp et al., 2009) as different sample types from the two different production sites. Sample types were raw ingredients (for example milk, flours, starches, sugars, vitamins and flavorings), environmental samples (for example filling equipment, walls, floors, cleaning equipment, dust, sinks, elevators and personnel) and product samples. Product samples were taken from different steps of manufacturing and can be differentiated between semifinished products (before adding supplements) and finished products. Finished products were powdered infant formula and follow on formula from different brands that can be bulk goods or shelf-ready products.

A total of 216 isolates from three different species were selected for genotyping. From the 155 *Enterobacter cloacae* isolates 25 were from raw ingredients, 16 from the environment and 94 from products. From the 37 *Klebsiella pneumoniae* isolates 4 were from raw ingredients, 6 from the environment and 27 from products. From the 24 *Leclercia adecarboxylata* isolates 11 were from raw ingredients, 7 from the environment and 6 from products.

Pulsed-Field Gel Electrophoresis

Isolates were grown on blood agar overnight at 37 °C. Bacteria were harvested from the plate using a cotton swab and transferred into 2 ml cell suspension buffer (100 mM Tris, pH 8.0; 100 mM EDTA, pH 8.0). The suspension was adjusted spectrophotometrically to an optical density of 1.0 at 600 nm. 400 µl of this suspension was mixed with 20 µl of Proteinase K solution (20 mg/ml) and 400 µl of 1.4 % (w/v) pulsed-field certified agarose (Bio-Rad Laboratories AG, Reinach BL, Switzerland). Agarose plugs were prepared in plug moulds (Bio-Rad) and allowed to solidify. Then plugs were placed into tubes containing 5 ml lysis buffer (50 mM Tris, pH 8.0; 50 mM EDTA, pH 8.0; 1 % (w/v) N-Laurylsarcosine) with addition of 0.1 mg/ml Proteinase K immediately prior to use. After incubation at 55 °C overnight the plugs were washed with distilled water twice and with TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0) four times with incubation time of 30 min and 60 min respectively at 50 °C. The washed plugs were stored in TE buffer at 4 °C. For restriction endonuclease digestion, agarose plugs were cut in 2 mm slices and equilibrated in 300 µl restriction buffer H (Roche) for 20 min. The restriction digest was performed in 300 ml fresh restriction buffer H containing 80 U of enzyme *Xba*I (Roche) for 12–14 h. Restriction fragments were separated in a 1 % (w:v) pulsed-field certified agarose gel (Bio-Rad) in 0.5 TBE running buffer with a CHEF-DR III system (Bio-Rad). The running buffer was supplemented with 50 mM Thiourea (Sigma); this overcomes the problem of non-typeable strains due to the nucleolytic activity of a peracid derivative of Tris, which can form at the anode during electrophoresis (Ray et al., 1995). *Salmonella* Braenderup strain H9812 digested with *Xba*I was used as a molecular size standard (Hunter et al., 2005). The following conditions were used for the separation of the digested fragments, pulse time: 5–50 sec, linear ramping for 20 h at 14 °C, 120 ° included angle. Following electrophoresis, gels were stained for 30 min with ethidium bromide (5 mg/l) and destained in distilled water for 1 minute. Fingerprint patterns were visualized and captured using a CCD photography system (Bio-Rad, Hercules, CA) from which tagged image file format (TIFF) files were imported into GelCompar II software version 5.1 (Applied-Maths, Sint-Martens-Latem, Belgium). Clustering of the PFGE fingerprint patterns was performed using the DICE coefficient and the unweighted pair group method with arithmetic mean (UPGMA), with an optimization of 3 % and band position tolerance of 1.5 %. Cophenetic correlations ranged from 61–100 % and the relatedness of the fingerprint patterns was compared at 95 % similarity.

Isolates with identical band patterns were considered as a group.

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Results and Discussion

Restriction digest with *Xba*I revealed discriminative PFGE patterns for all three species. *E. cloacae* showed patterns consisting of 10–20 bands, *K. pneumoniae* had patterns with 11–20 bands and the patterns for *L. adecarboxylata* contained 11–19 bands (fig. 1). In summary no difference in discriminative power could be observed between the three species, indicating that *Xba*I was appropriate for all of them.

The 155 *E. cloacae* isolates showed 92 different pulso-types, allocated in 24 groups with 2–11 isolates each. 17 groups contained only isolates from product samples, 3 groups only environmental samples. The other 4 groups contained isolates from different sample types. For *K. pneumoniae* there were 37 isolates resulting in 25 different pulso-types. 5 groups containing 2-6 isolates were formed, all containing product samples only. The 25 *L. adecarboxylata* isolates resulted in 21 different pulso-types with 2 groups. One of them contained 2 isolates from raw ingredients, the other contained 3 isolates from product and environmental samples.

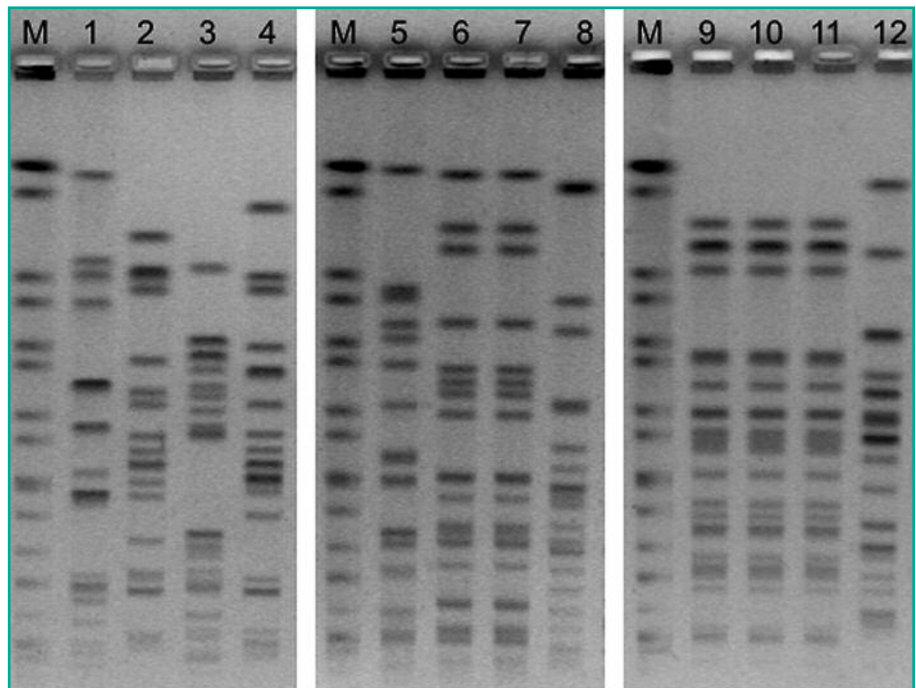

FIGURE 1: *PFGE patterns of chromosomal DNA restriction fragments digested with XbaI resolved in 1 % pulsed-field certified agarose gel (Bio-Rad) in 0.5 TBE running buffer. M, molecular size standard (Salmonella Braenderup strain H9812 digested with XbaI); lines 1-4 isolates of E. cloacae; lines 5–8, isolates of L. adecarboxylata; lines 9–12 isolates of K. pneumoniae.*

Various groups, especially for *E. cloacae,* were formed by isolates from different kind of finished products. A closer look at these products revealed that they were either produced on the same spray dryer, blended in the same mixer or packed in the same filling line. In other cases groups contained isolates from the same kind of finished product but manufactured on different dates. This possibly indicates a persistent contamination of the facilities. Since only dry cleaning is practicable in this kind of production environment, disinfectants are not efficient. Furthermore dust formation can not totally be avoided and therefore air flow management is very important.

An other possible way of contamination is shown by the following example. One group (fig. 2) showed the same genotype of *E. cloacae* isolates from products that were manufactured in the two different production sites, 100 km apart, so the environment as possible source of contamination could be excluded. Checking the ingredients of those various products made evident that they all contained lactose, which is a carrier for heat sensitive additives, and therefore added after the drying process, from the same supplier. This indicates heat sensitive

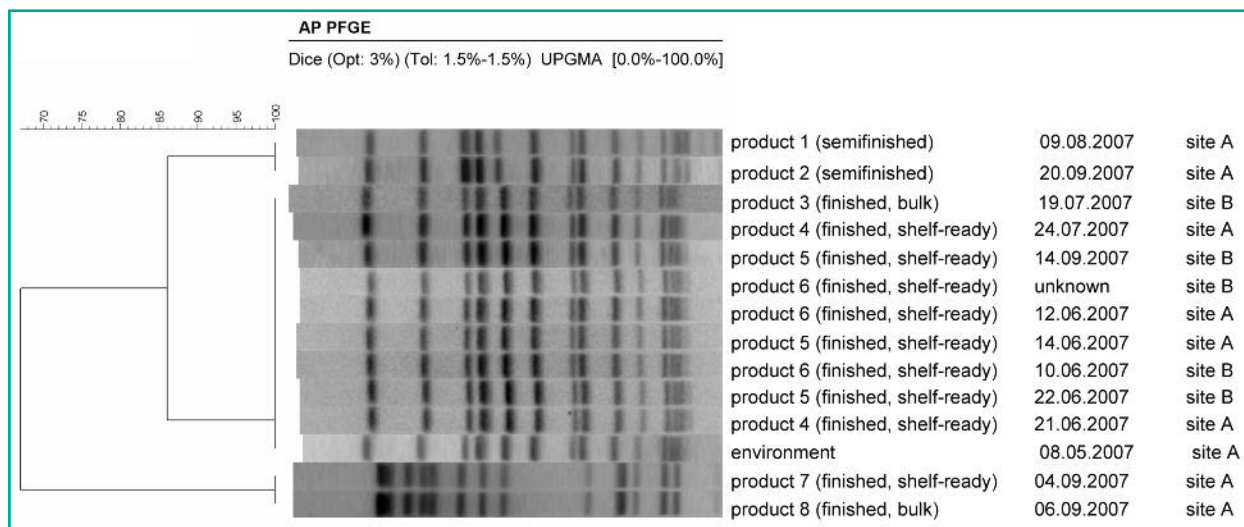

FIGURE 2: *Comparison of band patterns of three groups of E. cloacae isolates calculated with the GelCompar II software. Different product number means different kind of products, which means different brand names. Identical product numbers means the same product brand, which means an identical product.*

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additives as an important source of entry into the production facilities.

To summarize, the findings are comparable with the situation described for *Cronobacter* spp. (Mullane et al., 2007; Iversen et al., 2009). Especially *E. cloacae* that can be found in the same niches as *Cronobacter* spp. but more frequently might therefore be used for a hygiene monitoring program along the processing chain in order to trace back contamination routes. Certain genotypes of *E. cloacae,* that are able to persist within the factory environment, might possess special properties as e. g. enhanced desiccation tolerance and therefore demonstrate the need for further evaluation of stress response mechanisms in this species.

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