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Short communication

Population diversity of *Listeria monocytogenes* in quargel (acid curd cheese) lots recalled during the multinational listeriosis outbreak 2009/2010

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ABSTRACT

It has been possible to determine the genotype diversity of *Listeria monocytogenes* in the actual cheese lots of acid curd cheese that caused a multinational outbreak between 2009 and 2010. Following product recall in January 2010 all lots were investigated. A total of 422 *L. monocytogenes* isolates were characterized by genotyping. In a first approach the PCR serogroups were defined by multiplex-PCR assays. Subsequently, the isolates were subtyped by pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST). Sequence types were assigned by submitting the DNA sequences to the *Listeria* MLST database at the Institute Pasteur. The serogroup PCR resulted in a homogeneous 1/2a – 3a (genetic linage II) cluster. The generated PFGE patterns divided the strains into two clusters (type 1 and 2) diverging at a homogeneity level of 74%. PFGE-type 2 was predominant, accounting for 98.3% ($n = 415/422$) of the isolates and was isolated during the whole period of acid curd cheese processing (01.12.2009–13.01.2010). 1.7% of all tested *L. monocytogenes* isolates ($n = 7/422$) belonged to PFGE-type 1 and were isolated from 28% of all cheese lots ($n = 5/18$) produced between the time span of 08.12.2009 to 13.01.2010. Furthermore, PFGE-type 1 and 2 showed the same PFGE patterns as the human outbreak strains (clone 1 and clone 2).

1. Introduction

Over the past 27 years *Listeria monocytogenes* has become increasingly important as a food-associated pathogen, and dairy products are seen as one of the main vehicles (Gaulin et al., 2012; Jackson et al., 2011; Lomonaco et al., 2009). Recently, an invasive multinational listeriosis outbreak was linked to cheese. Clinical cases were reported between June 2009 and February 2010. Thereof, 25 persons were affected in Austria (seven fatal cases), eight in Germany (one fatal), and one in the Czech Republic. An epidemiological investigation revealed the source of infection to be quargel cheese produced by an Austrian manufacturer which was contaminated with two different *L. monocytogenes* serotype 1/2a clones (Fretz et al., 2010).

On the 23rd January 2010 implicated quargel cheese lots were withdrawn from the market. On the 27th January the Institute of Milk Hygiene in Vienna was assigned to clarify the *L. monocytogenes* contamination patterns in the Austrian cheese production chain by the manufacturer. On the same day it was possible to inspect the company and seize all recalled lots.

Food products are frequently contaminated by *L. monocytogenes* due to recontamination processes (Hoelzer et al., 2012; Reij et al., 2004). Although contamination within a food facility has often been traced to a particular step during processing, the initial source of *L. monocytogenes* in the plant is often unknown (Lianou and Sofos, 2007). Identification of the pathogen genotype thereby facilitates sourcing, and the combination of different typing methods leads to a more precise discrimination of bacterial strains (Sabat et al., 2013; Yde et al., 2012).

Pulsed-field gel electrophoresis (PFGE) is still the 'gold standard' among molecular typing methods for a variety of clinically important bacteria with a high discriminatory power compared with other typing methods (e.g. multi-locus variable number tandem repeat analysis (MLVA), multilocus sequence typing (MLST) (Chenal-Francisque et al., 2013; Ward, 2013). MLST of *L. monocytogenes* housekeeping genes facilitates the rapid inter-laboratory and -disciplinary comparison on an open accessible database (available from: Institute Pasteur; http://www.pasteur.fr/mlst; accessed: 06.04.2013).

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Consequently, the aim of this study was not only to determine the contamination level, but also to define the genotype diversity of the *L. monocytogenes* isolates identified in all recalled lots, and to compare the PFGE patterns and sequence types (ST) with those of the human outbreak strains.

2. Material and methods

2.1. Recalled cheese lots

The Austrian company manufactured two different acid curd cheese types: (i) red smear ripened (*Brevibacterium linens*; BLO, Cargill France SAS, La Ferte sous Jouarre, France) and (ii) mould coated/white veined (*P. candidum*; PC TAM5, Cargill France SAS, La Ferte sous Jouarre, France). Both cheeses were trademarked and marketed under the company's brand name in different package sizes of 125 g, 150 g or 200 g with a shelf-life of 50 days after ripening. Of the two types, mould coated quargel had a smaller market size and was exclusively manufactured for German consumers (mould coated cheeses comprised 11% of production). In total the company produced approximately 16 tonnes of quargel cheese each week. Almost 50% of the product was exported to Germany and small amounts to the Czech Republic and Slovakia.

Recall began on the 23rd January 2010 and more than a hundred kilogram of cheese (six kilogram cheese per lot) was sent to the Institute of Milk Hygiene, Milk Technology and Food Science, Vienna, for analysis.

2.2. Cheese sample preparation, isolation, quantification and confirmation of L. monocytogenes

Pooled samples of each cheese lot were prepared. Depending on the weight of the cheeses (125, 150 or 200 g) 25, 30 or 40 g subsamples were randomly selected according to ISO 707 (Anonymous, 2008) to comprise 600 g pooled samples. All experiments were performed in triplicate (Schoder et al., 2012).

The standard ISO 11290-1 method was used for qualitative examination of the samples (Anonymous, 1996). This comprised addition of 25 g samples to 225 ml Half-Fraser (HF) broth (Merck, Darmstadt, Germany), followed by homogenization for three minutes in a stomacher (Lab Blender 400 Seward, UK) and incubated for 24 h at 30 °C. Thereafter 0.1 ml aliquots of the HF broth were transferred to tubes containing 10 ml of Full-Fraser broth (FF, Merck) prior to incubation at 37 °C for 48 h. Both, HF and FF enrichments were streaked onto Palcam (Biokar Diagnostics, Beauvais Cedex, France) and Oxoid Chromogenic Listeria agar (OCLA; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom). After an incubation period of 24–48 h at 37 °C, the selective agar plates were investigated for the presence of *Listeria* spp. colonies. Subsequent quantitative examination was carried out in accordance with ISO 11290-2 (Anonymous, 1998). *Listeria* spp. colonies were counted on Palcam and OCLA agar using serial dilutions on all samples. A lowest detection limit of 1.0 *log* CFU/g cheese was achieved by plating additionally 1 ml initial suspensions in triplicate onto Palcam and OCLA agar (Anonymous, 2007).

Initially five suspicious *L. monocytogenes* colonies were selected from OCLA and Palcam agar for further genotyping. Subsequently, the whole Palcam and OCLA agar surface was swabbed and transferred to 1 ml 0.01 M Tris-Hydrochloride buffer (Sigma Aldrich, St. Louis, MO, USA). Subsequently, a short Chelex-based DNA isolation method (Walsh et al., 1991) was applied, followed by a PCR assay targeting the 16S rRNA gene specific for all *Listeria* spp., and the *hly* gene specific for *L. monocytogenes* (Border et al., 1990). Additionally, a multiplex-PCR for *Listeria* species or species group differentiation (*iap* gene) was performed (Bubert et al., 1999).

Finally, the cheese samples were investigated applying a combined *L. monocytogenes* enrichment/real-time PCR detection published by Rossmanith et al. (2006).

Therefore, nine ml HF enrichment (acc. ISO 11290-1) were transferred to sterile polypropylene tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) and centrifuged at 50 *g* for two minutes. The resulting pellet was discarded and the supernatant was centrifuged at 3220 *g* for ten minutes. This pellet was subjected to bacterial target DNA extraction using the NucleoSpin® tissue kit (Macherey–Nagel, Düren, Germany), followed by real-time PCR detection targeting the *prfA* gene (274 bp amplicon) of *L. monocytogenes*.

2.3. Molecular biological subtyping methods

The *L. monocytogenes* isolates included in the current publication were cryo-conserved (−80 °C, Microbank Pro-Lab Diagnostics, Round Rock, TX, U.S.A) and stored in the *Listeria* collection of the Institute of Milk Hygiene, Milk Technology and Food Science (Vetmeduni Vienna, Austria).

L. monocytogenes serogroups were defined using a multiplex PCR targeting the specific target genes *lmo0737, lmo1118*, ORF2819, ORF2110 and *Listeria* spp. specific *prs* published by Doumith et al. (2004) and amended by Leclercq et al. (2011) for PCR IVb-VI.

DNA isolation and PFGE were performed following the most up-to-date International Standard PulseNet protocol with the following modifications: applying 40U *Asc*I and 50U *Apa*I restriction enzymes incubated for three hours at 37 °C and 25 °C, respectively (Pulsenet International, 2013; http://www.pulsenetinternational.org/assets/PulseNet/uploads/pfge/PNL04_ListeriaPFGEProtocol.pdf; accessed on: 21.10.2013). Restricted samples were electrophoresed on 1% SeaKem Gold agarose in 0.5 × TBE at 6 V/cm on a Chef DR III system (Bio-Rad, Hercules, CA, USA). A linear ramping factor with pulse times from 4.0 to 40.0 s at 14 °C, and included angle of 120° were applied for 22.5 h. The gels were stained with ethidium bromide and digitally photographed with Gel Doc 2000 (Bio-Rad, Hercules, CA, USA). The TIFF images were compared using the Fingerprinting II Cluster Analysis (Bio-Rad), and normalized using the PFGE global standard *Salmonella* ser. Braenderup H98124. Pattern clustering was performed using algorithms within Fingerprinting II Cluster Analysis (Bio-Rad). Specifically, the unweighted pair group method using arithmetic averages (UPGMA) and the Dice correlation coefficient with a position tolerance of 1.0% were applied. In order to identify identical PFGE types, a Dice coefficient similarity of 100% was used. When any differences in PFGE patterns were observable, the patterns were reported as different (Barrett et al., 2006).

MLST based on the seven housekeeping loci *acbZ* (ABC transporter), *bglA* (beta glucosidase), *cat* (catalase), *dapE* (succinyl diaminopimelate desuccinylase), *dat* (D-amino acid aminotransferase), *ldh* (L-lactate dehydrogenase), and *lhkA* (histidine kinase) was performed according to Ragon et al. (2008). For each housekeeping gene an allele number was assigned and sequence types (ST) were determined and compared using the Institute Pasteur *L. monocytogenes* MLST database (accessed: 12.04.2013; available from: http://www.pasteur.fr/recherche/genopole/PF8/mlst/Lmono.html).

To define the relationships among strains at the micro evolutionary level, an allelic profile-based comparison applying a minimum spanning tree (MST) was performed applying the Institute Pasteur online tool (available on: http://www.pasteur.fr/recherche/genopole/PF8/mlst/). Clonal complexes (CC) were defined as groups of STs differing by only one housekeeping gene from another member of the group (Ragon et al., 2008).