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SHORT COMMUNICATION

Report on Overcoming the Poor Quality of *Apa*I Pulsotypes with a Short Review on PFGE for *Listeria monocytogenes*

DARJA KUŠAR*, MAJA KAVALIČ, MATJAŽ OCEPEK and IRENA ZDOVC

Institute of Microbiology and Parasitology, Veterinary Faculty, University of Ljubljana, Ljubljana, Slovenia

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Abstract

Since listeriosis, caused by *Listeria monocytogenes*, is one of the important concerns of public health in Europe related to foodborne zoonoses, an efficient protocol for isolate typing is necessary when performing epidemiological studies. Three standardized PFGE protocols available for *L. monocytogenes* were briefly reviewed. Since observing a poor-quality of *Apa*I pulsotypes in our laboratory, enzymes from three different manufacturers were compared. The obtained pulsotypes showed that restriction digestion with *Apa*I from New England BioLabs should be complemented with a subsequent overnight incubation of PFGE plugs in TE buffer for better performance.

Key words: *Listeria monocytogenes*, *Apa*I restriction, quality of pulsotypes, PFGE, standardized protocol

Listeriosis is caused by bacteria of the genus *Listeria*, mostly by *Listeria monocytogenes*, and is affecting both animals and humans (Fenlon, 1999). A substantial proportion of herd animals may shed significant numbers of *L. monocytogenes* in faeces and milk (European Food Safety Authority, 2010; Wesley, 2007). Transmission to humans most commonly occurs by consumption of raw animal products (Wesley, 2007), especially by foods contaminated during manufacturing and post-processing (Boerlin and Piffaretti, 1991; Hitchins and Whiting, 2001; Tompkin, 2002). In humans, foodborne listeriosis usually occurs in pregnant women, neonates or immunocompromised adults (Painter and Slutsker, 2007). *L. monocytogenes* is the most important cause of death from foodborne infections in industrialized countries and though the human infections are rare, they are very important given the associated high mortality rate (European Food Safety Authority, 2010).

Pulsed-field gel electrophoresis (PFGE) is currently considered the gold standard for epidemiological studies of bacterial foodborne pathogens, including *L. monocytogenes* (Giovannacci *et al*., 1999; Graves *et al*., 2009). Following a standardized protocol generating good-quality results, the obtained PFGE profiles (pulsotypes) can be subjected to a computer-based processing and compared at the international level. For *L. monocytogenes*, the original standardized protocol [A] was launched in 1998 (Graves and Swaminathan, 2001) by the PulseNet, today known as the PulseNet International uniting seven laboratory networks (*e.g.* PulseNet USA and PulseNet Europe). Later, the original protocol A was optimized and dispatched in 2009 by the European Union Reference Laboratory (EURL) to the participant European laboratories as the EURL in-house method [B1]. It was similar to the updated PulseNet USA PFGE standardized protocol [B2] published soon after (Halpin *et al*., 2010). Major differences between the two were presented previously (Félix *et al*., 2012). In brief, the B2 protocol, in comparison to B1, recommends lower cell density per PFGE plug, the preparation of lysozyme solution in TE buffer (vs. water) and incubation at 56°C (vs. 37°C), and higher amount of the restriction enzymes and the total volume of restriction mixtures (200 µl vs. 100 µl). In comparison to A, both B1 and B2 protocols recommend a much lower amount of *Apa*I in the restriction digests, namely 10 U (B1) and 50 U (B2) in comparison to the original 160–200 U (A) per sample, and the addition of bovine serum albumin (BSA) to the restriction mixtures even when not recommended by the manufacturer.

The aim of the present paper was to present how the selection of different suppliers/protocols *Apa*I restriction can affect the quality of *L. monocytogenes* *Apa*I pulsotypes, exerting higher complexity (14–17 bands expected) than *Asc*I profiles (6–12; Carriere *et al*., 1991). In our study, PFGE employing restriction enzyme *Apa*I was performed as recommended by the original protocol A. As a molecular size-marker,

* Corresponding author: D. Kušar, Institute of Microbiology and Parasitology, Veterinary Faculty, University of Ljubljana, Gerbičeva 60, SI-1115 Ljubljana, Slovenia; phone: +386 1 4779 158; fax: +386 1 4779 352; **e-mail: darja.kusar@vf.uni-lj.si**

Salmonella enterica serovar Braenderup (strain H9812, digested with *Xba*I) was used (Hunter *et al*., 2005). Electrophoresis was performed on CHEF-DR II apparatus (BioRad, Hercules, CA, USA). Three different *Apa*I enzymes were compared. First, *Apa*I from Roche Diagnostics [RD] (Mannheim, Germany) with 40 U/µl stock concentration and the optimal incubation temperature of 30°C, since recommended in A and B1 (and in B2 as the protocol refers to A regarding the restriction enzymes). Second, *Apa*I from Thermo Fischer Scientific [TS] (Waltham, MA, USA; formerly purchased under a trademark of Fermentas, Vilnius, Lithuania) with 10 U/µl concentration and 37°C incubation temperature, since manufactured by an established company in the field of restriction enzymes. Third, *Apa*I from New England BioLabs [NEB] (Ipswich, MA, USA) with 50 U/µl concentration and 25°C incubation temperature, since recommended for *Asc*I restriction in A and B1. No separately purchased BSA was included in any of the *Apa*I restriction mixtures as not yet recommended in A; BSA is, however, already present in the restriction buffer from TS and supplied separately with *Apa*I (but not with *Asc*I) enzyme from NEB. Since given as a potential stopping point, incubation of PFGE plugs in TE buffer (0.01 M Tris-EDTA, pH 8.0) was tested after restriction using *Apa*I from NEB, namely the restriction mixture was removed and replaced by equal volume of TE for an overnight (one-day) incubation of plugs at 4°C (Cooper, 2010).

Selection of *Apa*I restriction enzymes purchased from different manufacturers can affect the quality of *L. monocytogenes* pulsotypes. *Apa*I from RD has been employed for several years in our laboratory, namely since 2006 when we started the PFGE typing of *L. monocytogenes* following the protocol A, and we have been obtaining good-quality results (Fig. 1a). Later, *Apa*I from TS was tested and it also generated pulsotypes of satisfactory quality (Fig. 1b, lanes 3 and 4); however, the volume of enzyme solution needed for the preparation of restriction mixture was higher in comparison to *Apa*I from RD due to a lower concentration of the enzyme available from TS. This can be acknowledged as a drawback due to two aspects. First, since glycerol is present in the storage buffer of enzymes, larger consumption of enzyme solution leads to a higher glycerol concentration in the restriction mixture, possibly conditioning the star activity of restriction enzymes (Freeman, 2011). Second, larger consumption demands for larger enzyme stocks. Therefore, an enzyme with higher concentration was tested, namely *Apa*I from NEB. In comparison to RD and TS, NEB enzyme generated poor-quality pulsotypes (Fig. 1b, lanes 1 and 2; Fig. 1c). However, when an overnight TE-incubation of plugs after restriction was tested, the obtained pulsotypes markedly improved (Fig. 1d).

It had been shown before that enzyme digestion was not optimal when *e.g.* BSA was not included in the restriction mixture, not enough or too many units of restriction enzyme were used, and incubation time,

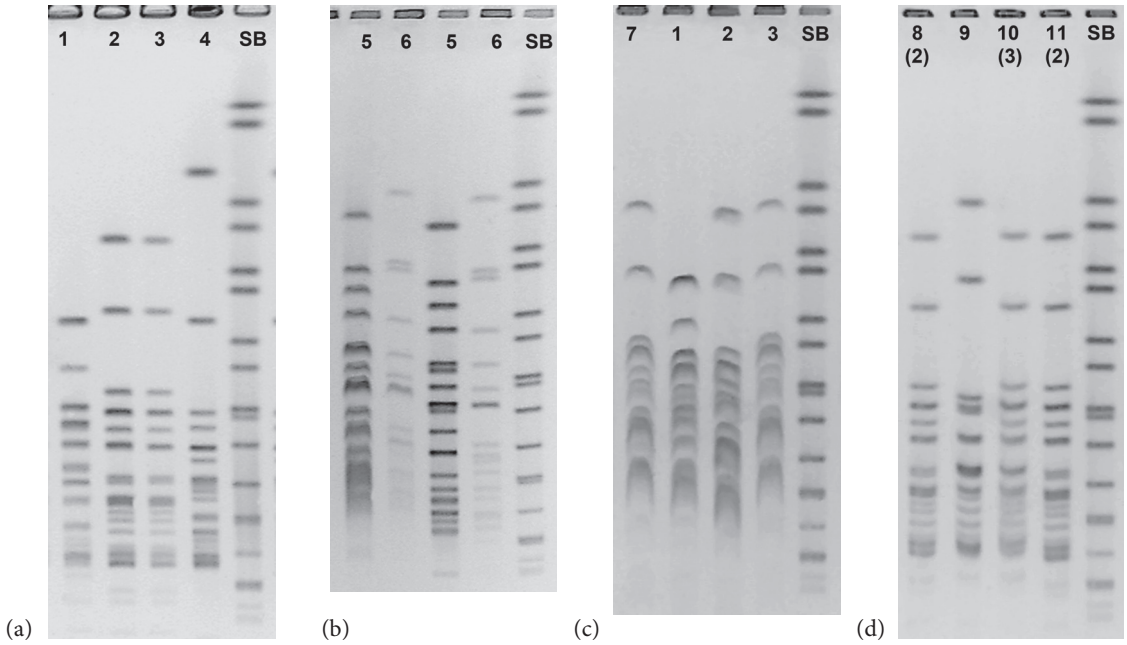

Fig. 1. PFGE separation of *Apa*I fragments from *L. monocytogenes* isolates. *Salmonella* Braenderup strain H9812 (SB) was employed as size standard.

(a) *Apa*I from Roche Diagnostics, isolates 1–4;
(b) *Apa*I from New England BioLabs (NEB; lanes 1 and 2) and from Thermo Fischer Scientific (lanes 3 and 4), isolates 5 and 6;
(c) *Apa*I from NEB, isolates 1–3 and 7;
(d) *Apa*I from NEB followed by an overnight incubation in TE buffer, isolates 8–11 (8 and 11 showed an identical pulsotype as isolate 2, and 10 identical as isolate 3, as indicated in the parentheses)

temperature or buffer were not correct (Freeman, 2011). However, in the case of *Apa*I from NEB in our study, digestion did not seem to be compromised in such manner as the number of bands, appearance of ghost/shadow bands, resolution or high background could not be discussed as a main problem. Band distortion was a much more serious issue impairing the pulsotype quality and interpretation. Wavy bands, as seen in Figs. 1b and 1c, were observed before in plugs digested with *Apa*I from NEB, in the presence of BSA and with different laboratory personnel (Freeman, 2012). It was concluded that band distortion could be directly correlated with the units of restriction enzyme added to the master mix, namely an increasing distortion was observed when 100 or more U of *Apa*I were employed (in our study, 200 U were added according to the original protocol A). Moreover, the anomaly was observed with new enzyme lots in January 2012 (Freeman, 2012). In our study, *Apa*I with a lot number higher from the ones tested by Freeman (2012) was used, assuming the enzyme was also released in January 2012 or later.

It can be concluded that variations in the standardized PFGE protocols can, among other reasons discussed elsewhere like the quality of water, in-house prepared buffers, laboratory personnel or others, appear due to the lot-to-lot variations of restriction enzymes from the same manufacturer or between enzymes from different manufacturers. The EURL in-house method for the PFGE typing of *L. monocytogenes* (protocol B1) recommends the use of *Asc*I from NEB, but *Apa*I from RD, and comments that the use of materials and reagents of other suppliers could generate deviation in the results. It was also recommended (protocols B1 and B2) that the amount of restriction enzymes, especially of *Apa*I, should not be in excess when aiming for pulsotypes of high quality. In other studies, an overnight (or longer) incubation in TE buffer was not suggested as a curative step in the protocol of *Apa*I restriction; however, improved the quality of pulsotypes obtained in our study when testing *Apa*I from NEB.

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