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Article *in* Asia-Pacific Journal of Molecular Biology and Biotechnology · January 2001

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Asia Pacific Journal of Molecular Biology and Biotechnology, 2001
Vol. 9 (2) : 147 – 150

RESEARCH NOTE

Optimised conditions for Pulsed Field Gel Electrophoresis (PFGE) separations of *Pasteurella multocida* B:2 DNA

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Received 2 August 2001/Accepted 5 September 2001

Abstract. Pulsed field conditions specific for *Pasteurella multocida* B:2 have been optimised to obtain a good separation resolution. Restriction enzyme, *Not*I, was the most suitable as it generates discernible number of bands. Other optimised electrophoretic conditions were: 1.5% agarose gel concentration, 200 V, ramped pulsed time of 1-25 s and a run time of 26 h. This research is important for fast and accurate characterisation of this bacterial strain following an outbreak in Malaysia.

Key Words: *Pasteurella multocida*, PFGE, restriction enzymes

Pulsed field gel electrophoresis (PFGE) has successfully shown greater discrimination in separating DNA restriction fragments ranging from less than 10 kilobase pairs (kb) to large chromosomal DNA with more than 4 million base pairs (Mb) (Smith *et al.*, 1987; Leblond *et al.*, 1990; Townsend *et al.*, 1992; Gunawardena *et al.*, 2000). PFGE is able to separate large DNA molecules by forcing them to change their direction of migration periodically (Schwartz and Cantor, 1984). Several modifications of this original technique can be observed in the literature to improve the resolution of results obtained (Hunt *et al.*, 2000). Preliminary experiments were carried out to determine the optimal pulsed times, gel concentration, voltage gradient and run time for maximum separation of DNA fragments.

When combined PFGE with restriction enzyme digestion of genomic DNA, a study of genetic variability amongst individuals is possible (Hunt *et al.*, 2000). A 'rare-cutting' enzyme is desirable as it would generate large fragments that can be separated on a PFGE ideal for analysis.

Haemorrhagic septicaemia (HS), a fatal disease of cattle and buffaloes, is caused by *Pasteurella multocida* (serotype B:2) in the tropics. There is the need to study the molecular characterisation of this organism for the development of better vaccines. Therefore the aim of this analysis was to optimise gel concentration, voltage gradient, run time and pulsed time together with restriction enzyme analysis digestions to enable a detailed molecular investigation of this bacteria.

P. multocida serotype B:2 used in this study was provided by the Veterinary Diagnostic Laboratory (VDL), Petaling Jaya and Veterinary Research Institute (VRI), Ipoh. Suspect colonies were identified by standard bacteriological techniques (Rimler and Rhoades, 1989) and confirmed as *P. multocida* serotype B:2. For DNA preparation, the cell pellet was harvested by centrifugation at 4°C for 10 min at 6000 rpm. The cell pellet was washed twice in 1 ml cold SB (10 mM Tris, pH 7.5, 1 mM NaCl) buffer and standardised to 1 X 10^7 cell/ml. Equal volume of the cell suspension and low melt agarose (1.5%) were mixed and allowed to set in a mould . The agarose plugs were then incubated in 10 ml of LB (10 mM Tris pH 7.5, 100 mM NaCl, 0.5 mM EDTA pH 8.0, 0.2% sodium deoxycholate, 0.5% Sarcosyl, 0.5% Brij-58) buffer in a 50 ml conical tube containing 0.01 g lysozyme and 10 ml RNAse (10 mg/ml) incubated overnight at 37°C with gentle agitation, followed by deproteination with 1mg/ml proteinase K at 50°C for 24 h. The plugs were then washed

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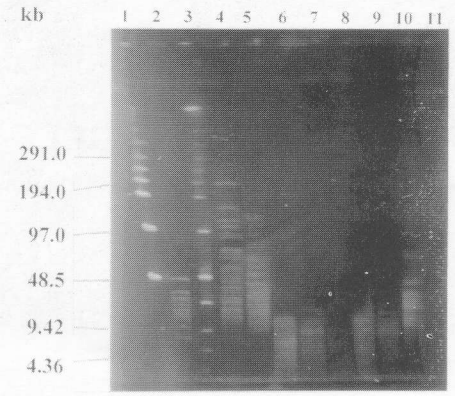

Figure 1. PFGEseperation of restriction fragments of the *P. multocida* genome. Lane 1 is d DNA ladder, lane 2 is 8-48 kb marker and lane 3 is low range PFGE marker. lanes 4 through 11 are, respectively, total digests of the genome of *P. multocida* serotype B:2 with *Not*I, *Sfi*I, *Sac*II, *Bg*lI, *Bst*Z I, *Xma*I and *Apa*I

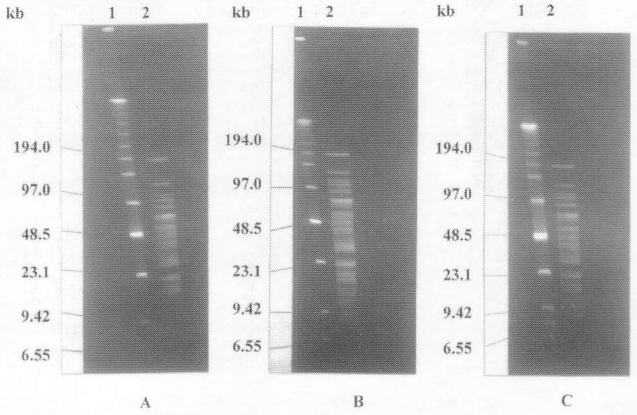

Figure 2. The effects of agarose gel concentration. A composite gel with agarose of (A) a.0% (B) 1.2% and (C) 1.5% shows PFGE separation of *P. multocida* genome diagested with *Not*I. Lane contain (1) low range PFGE marker and (2) *Not*I digested *P. multocida* genome.

thoroughly with TE buffer (10 mM Tris-HCl, pH8.0, 1 mM EDTA, pH 8). A slice of the DNA plug was then digested with 10 U *Not*I in the restriction buffer according to the manufacturer's instruction.

PFGE analysis was performed using the Gene Navigator by Pharmacia Biotech, with pulsed times as determined by the desired fragment size range. Low Range PFG marker (from New England Biolabs, Inc.) was used as molecular weight size standards. After electrophoresis, the gels were stained with ethidium bromide followed by destaining in distilled water and photographed with UV illumination using black & white Polaroid film (Polapan 665_{PN}).

Eight restriction endonucleases having G+C recognition sequences i.e. *Not*I, *Sfi*I, *Sac*II, *Nar*I, *Bgl*I, *Bst*ZI, *Xma*I and *Apa*I were screened to see which would cut the *Pasteurella multocida* chromosomal DNA into a convenient number of large fragments. Two out of these eight restriction enzymes, i.e. *Not*I and *Sfi*I, both with eight base recognition sequences produced a suitable range of the DNA fragments. The other enzymes produced many fragments that were smaller than 40 kb (Figure 1) and therefore unsuitable for PFGE analysis. For further analysis, only *Not*I enzyme was evaluated.

The effects of different agarose concentrations on DNA separation, that is, 1%, 1.2%, and 1.5% agarose gel was carried out and it was observed that resolution was improved with increasing agarose concentrations. Figure 2 shows the effects of different agarose concentrations run at the 200 V and pulsed times of 1-25 s. The 1.5% agarose gel concentration was most ideal as with the 1% and 1.2%, the resolution decreased especially for fragments less than 48 kb.

Different voltage i.e. 100 V for 72 h, 200 V for 26 h and 300 V for 8 h of total run time were tested. Different run times were selected to achieve a comparable separation between the various voltage tested. Figure 3 shows the effects of the different voltage used. It was observed that 200 V gave the best resolution for DNA fragments ranging between 194 kb to about 6 kb.

The effect of pulsed time was carried out on a 1.5% agarose gel at 200 V for 30 h of total run time. The different range of ramped pulsed time i.e. 1-25 s, 1-1.8 s and 0.5-1 s was performed to enable observation of the size range that can be separated (Figure 4). It was observed that DNA molecules ranging from 47–190 kb are best separated when pulsed time was ramped from 1-25 s while DNA molecules ranging from 23-49 kb are best separated at 1-1.8 s and 7-12 kb fragments should be ramped at 0.5-1 s for optimal resolution.

In this study, parameters affecting the resolution of PFGE were tested for optimal separation of restricted DNA fragments. We have tested the suitability of eight restriction enzymes and identified two, that is, *Not*I and *Sfi*I as the enzymes that are useful to analyse and estimate the size of the *P. multocida* genome. These enzymes can generate discernible number of fragments while the other six enzymes tested generated too many small fragments. Townsend *et al.*(1997) also selected *Not*I and *Sma*I in their analysis but found that *Not*I produced simpler restriction patterns. Therefore we are reporting the initial analysis of this study which involves *Not*I. We are currently optimising the resolution with *Sfi*I. Two other enzymes, *Hha*I and *Hpa*II which have not been investigated in this study, were found to efficiently

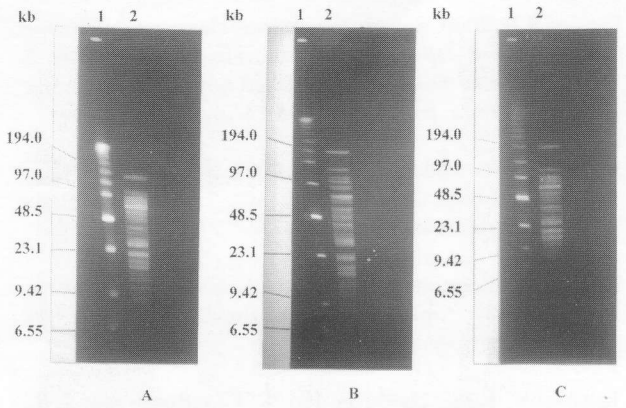

Figure 3. The effects of voltage gradient. PFGE seperation of *P. multocida* genome was analysed using (A) 100 V for 72 hourse, (B) 200 V for 26 hours and (C) 300 V for 8 hours of total run time. Lane contain (1) low range PFGE marker and (2) *Not*I digested *P. multocida* genome.

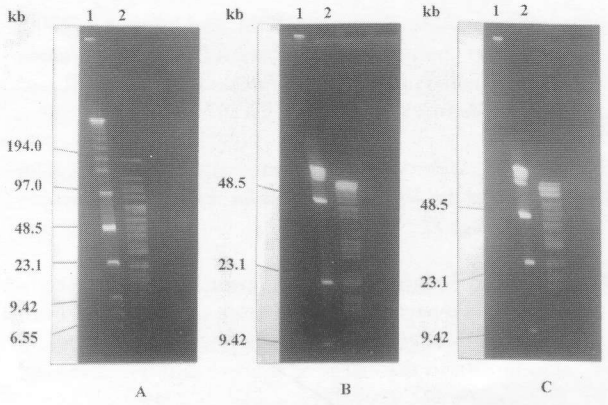

Figure 4. The effects of pulse time. PFGE separation of *P. multocida* genome diagested with *Not*I. Pulse time: (A) 1-25 s, (B) 1-1.8 s and (C) 0.5-1 s. lanes contain (1) low range PFGE marker and (2) *Not*I digested *P. multocida* genome.

discriminate isolates of serogroup B *P. multocida* associated with haemorrhagic septicaemia (HS). It would be interesting to test these two enzymes in the Malaysian isolates associated with HS.

From the experiments conducted to study the effects of gel concentration, our results suggested that there is an improvement in resolution with the increase in agarose concentration. This is in accordance with the results of Mathew *et al.*, (1988) where there was a small but significant improvement in resolution when the agarose concentration was increased from 0.9% to 1.2% for the separation of large DNA fragments.

The velocity of all sizes of DNA is proportional to the voltage gradient. Birren *et al.* (1988) observed that a lower voltage gradient produce better separation over a narrower size range. Therefore in a restriction enzyme analysis that generates fragments which are in a moderate size range of 5-194 kb, we observed that a voltage of 200 V was optimal in achieving good band resolution.

The pulsed time has been shown to have an important effect on the mobility of large DNA fragments in PFGE gels. The separation of molecules is based on the rate at which they alter their shape inside the gel matrix. With the different pulsed times the molecules will change their shape, that is, each time the field direction changes, the molecules change to achieve a configuration that will allow it to efficiently move through the gel. The molecules also spend some time to relax before they make the change in their configuration. DNA relaxation times are approximately proportional to the DNA size. Therefore as relaxation times match the pulsed duration, the mobility of these molecules varies very sharply with molecular weight and very high electrophoretic resolution is achieved. However if pulsed time is increased beyond that which is necessary for good separation of fragments then little additional change in velocity will be observed. As shown in our results different pulsed times were optimal for specific sizes of the DNA fragments in the *P. multocida* genome.

Townsend *et al.*, (1997) made similar conclusions that are for restriction fragments of 10-550 kb, optimal pulsed time was from 0.5 to 35.5 s and for fragments of 100-150 kb, optimal pulsed time was from 10 to 150 s.

Therefore, from the study, the optimised conditions for PFGE analysis were: 1.5% agarose gel concentration, 200 V, ramped pulsed time of 1-25 s and a run time of 26 h. The data obtained is important for molecular epidemiological studies to discriminate between isolates of similar serotype of *Pasteurella multocida.*

ACKNOWLEDGEMENT

This work was supported by Vot. F No. 0250/98 and 0221/99, University of Malaya.

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