ELSEVIER

Veterinary Microbiology 62 (1998) 243–250

veterinary microbiology

Short communication

Molecular analysis of *Dichelobacter nodosus* isolated from footrot in sheep in Malaysia

Z. Zakaria[a], Son Radu[b,*], A.R. Sheikh-Omar[a], A.R. Mutalib[a], P.G. Joseph[c], G. Rusul[b]

[a] *Faculty of Veterinary Medicine and Animal Science, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*

[b] *Department of Biotechnology, Faculty of Food Science and Biotechnology, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*

[c] *Faculty of Medicine and Health Sciences, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*

Received 10 December 1997; accepted 11 June 1998

Abstract

Pulsed field gel electrophoresis analysis of genomic DNA was used to investigate genetic diversity among *Dichelobacter nodosus* from footrot in sheep in Malaysia. Twelve *Dichelobacter nodosus* strains isolated from lesion materials from infected sheep were confirmed as *Dichelobacter nodosus* by polymerase chain reaction technique using the species-specific *Dichelobacter nodosus* 16S RNA sequence Ac and C as primers. Pulsed field gel electrophoresis banding profiles using restriction enzymes *Apa*I (5′GGGCCC3′), *Sfi*I (5′GGCCNNNNNGGCC3′)and *Sma*I (′5CCCGGG3′) enabled the 12 *Dichelobacter nodosus* strains to be differentiated into eight different PFGE patterns and thus genome-types, with F (coefficient of similarity) values ranging from 0.17 to 1.0 (*Apa*I), 0.14 to 1.0 (*Sfi*I) and 0.22 to 1.0 (*Sma*I). Strains with origin in different farms were shown to have different PFGE patterns (two strains, M7 and M8 were the only exception). On the basis of their PFGE, all field strains used in the study differed from the reference strains. Our data revealed that there are several clonal types of *Dichelobacter nodosus* isolates and indicated that there is probably more than one source of this pathogen on the farms studied. The study showed that strains of *D. nodosus* exhibited considerable genetic diversity using this method and that genomic analysis by pulsed field gel electrophoresis was useful in discriminating the *D. nodosus* strains.

Keywords: *Dichelobacter nodosus*; Sheep-bacteria; PCR; PFGE

* Corresponding author. Tel.: +60-3-9486101; fax: +60-3-9423552; e-mail: son@fsb.upm.edu.my

0378-1135/98/$19.00
PII S0378-1135(98)00219-3

1. Introduction

Dichelobacter nodosus (formerly known as *Bacteroides nodosus* (Dewhirst et al., 1990)) is essential causative agent of footrot, a highly contagious disease characterised by inflammation of the interdigital skin and hoof matrix leading to separation of the hoof from the underlying epidermal tissues and resulting in severe lameness, loss of body condition and reduced wool production (Egerton et al., 1969; Stewart et al., 1984). The clinical disease profile assumes a continuum ranging from virulent footrot, through the intermediate range, to benign footrot (Egerton et al., 1969; Stewart et al., 1982), depending on the climatic conditions and the virulence of the invading *D. nodosus* strain.

Traditionally, the identification of *D. nodosus* has relied on the isolation of *D. nodosus* from footrot lesion material and biochemical tests on the resultant isolates (Skerman, 1989; Pitman et al., 1994). Recent studies showed that PCR method base on the 16S RNA sequences can be used as a practical method for direct detection of *D. nodosus* isolates, but such analysis is not intended for strain differentiation (La Fontaine et al., 1993; Rood et al., 1996). In applying sensitive methods to epidemiological studies, however, it is important to understand the clonal nature of the organism under investigation, as well as the genetic diversity that exists within the population being studied. Data from genetic diversity studies could be used to monitor trends in the occurrence of pathogenic strains, and to identify possible sources of infection (Bowditch et al., 1993). Thus, a technique that is able to distinguish among *D. nodosus* strains could be extremely valuable. Pulsed field gel electrophoresis analysis has been used successfully to characterise bacterial isolates involved in epidemiological investigations (Single and Martin, 1992; Bert et al., 1997; Thomas et al., 1997). Thus, the objective of our research was to use PCR in the identification of *D. nodosus* isolates and reports here the diversity of strains isolated by pulsed field gel electrophoresis.

2. Materials and methods

2.1. Bacterial strains

Dichelobacter nodosus strains were isolated from sheep suffering from footrot from three government sheep farms: Institut Haiwan Kluang, Johor; Pusat Pembiakan Bebiri Gajah Mati, Kedah and Pusat Ternakan Bebiri Chalok, Terengganu. Lesion materials from affected sheep was spread on 4% hoof agar (HA) plates (Egerton and Roberts, 1971), placed in anaerobic jars which were gassed (BBL Gas Pak Plus, Becton Dickinson, Microbiology Systems, Cockeysville, MD) and transferred to the laboratory. After 3–5 days of incubation at 37°C, suspected Gram negative, strict anaerobes with typical colony (Thorley, 1976) and cell (Beveridge, 1941) morphology were sub-cultured onto a series of 4% HA plates until they were free of contaminating bacteria. They were sub-cultured onto 2% HA plates (Thomas, 1958) for further biochemical characterisation as *D. nodosus*. Standard *D. nodosus* strains 25549 (A1001) and G1674 and *E. coli* K 12 were used as controls.

2.2. DNA isolation and PCR

Genomic DNA from confirmed *D. nodosus* strains grown on HA plates was extracted according to the method of Anderson et al. (1984). Identification and confirmation of field isolates of *D. nodosus* by PCR were performed with forward primer Ac (5′CGGGGTTATGTAGCTTGC5′) corresponding to positions 67–84 and reverse primer C (5′TCGGTACCGAGTATTTCTACCCAACACCT3′) (each manufactured by Gibco, BRL) corresponding to positions 821–849 of the *D. nodosus* 16S RNA sequence (Dewhirst et al., 1990). The control strains were *D. nodosus* ATCC 25549 (A 1001) and G 1674, and *E. coli* K 12. Target DNA was amplified through 35 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s) and polymerisation (72°C, 35 s), using a microprocessor-controlled thermal cycler (GeneAmp PCR system 2400, Perkin Elmer, Norwalk, CT). Amplification products were separated by electrophoresis on 2% agarose gels stained with ethidium bromide (final concentration, 0.5 μg/ml).

2.3. PFGE

The pulsed field gel electrophoresis (PFGE) technique of contour-clamped homogenous electric field (CHEF) electrophoresis (Chu et al., 1986) was used for genomic typing of *D. nodosus* isolates. Genomic DNAs were digested in agarose plugs with *Apa*I (5′GGGCCC3′), *Sfi*I (5′GGCCNNNNNGGCC3′) and *Sma*I (5′CCCGGG3′) (Promega, Madison, WI) as recommended by the manufacturer. The resulting fragments were resolved by CHEF-PFGE by using a CHEF-DRIII apparatus (Bio-Rad Laboratories, Richmond, CA) in gels of 1% agarose in 0.5X TBE (0.1 M Tris, 0.1 M boric acid, 0.2 mM EDTA) at 6 V/cm for 20 h at 14°C with ramped pulse time varying according to the enzymes used (ranging from 0.1 to 30 s). Gels were stained with ethidium bromide and visualised with a UV transilluminator (Spectroline, 320 nm). Lambda concatamers (Promega, Madison, WI) were used as DNA size standards. The number and size of the DNA fragments were used as criteria for categorising distinct patterns. Several electrophoretic runs with ramped pulsed times varying between 0.1 and 30 s were performed to obtain optimal times in which individual bands were clearly separated.

2.4. Data analysis

DNA fragments patterns were visually assessed and distinct patterns were assigned an arbitrary PFGE pattern and genome-type. Strains were considered genetically similar or identical if there was a complete concordance of DNA fragment profiles and considered different if there was difference of one or more DNA bands. Patterns generated by PFGE for all strains were compared, and the similarity of fragment length patterns between two strains was scored by the Diced coefficient, also known as coefficient of similarity (El-Adhami et al., 1991). This coefficient F, expresses the proportion of shared DNA fragments in two isolates and was calculated by using the formula $F = 2n_{xy}/(n_x + n_y)$, where n_x is the total number of DNA fragments from isolate X, n_y is the total number

from isolate Y, and n_{xy} is the number of fragments identical in two isolates. An F value of 1.0 indicates that two isolates have identical PFGE patterns or genome-type.

3. Results and discussion

Isolates from footrot were analysed in this study. One isolate was recovered from each of the footrot samples and was hypothesised to be the causal strain responsible for the infection. All strains of *D. nodosus* from footrot in sheep used in this study showed identical biochemical characteristics to the reference strains. Confirmation as *D. nodosus* was made using the PCR primers of La Fontaine et al., 1993 shown to amplify a common 784 bp sequence from the *D. nodosus* DNA or lesion materials of footrot in sheep. A combination of the forward and reverse primers Ac and C generated a single PCR product with DNA from all the tested *D. nodosus* strains (12 of 12) as well as from the control strains ATCC 25549 (A1001) and G1674. The PFGE patterns obtained with the three enzymes differed from one another but remained stable and reproducible. As can be seen in Table 1, strains isolated from the same flock had up to three different genome patterns. Coincidently, in this limited study each flock had patterns different from one another. PFGE analysis of *Apa*I-digested DNA yielded 11–12 well-separated DNA fragments ranging in size from 27 to 380 kpb (Fig. 1); PFGE analysis of *Sfi*I-digested DNA yielded 4–10 DNA fragments ranging in size from 44 to 470 kbp (Fig. 2); and PFGE analysis of *Sma*I-digested DNA yielded 8–15 DNA fragments ranging in size from 60 to 560 kbp (Fig. 3). Analysis of these PFGE patterns showed that between three and five different PFGE patterns were present among the 12 *D. nodosus* strains, with coefficient F (coefficient of similarity) values ranging from 0.17 to 1.0 (*Apa*I), 0.14 to 1.0 (*Sfi*I), and 0.22 to 1.0 (*Sma*I). Thus when the results of the PFGE analysis of genomic DNA from the

Table 1
Comparison of the *D. nodosus* strains by their pulsed field gel electrophoresis patterns

Strain no.	Place[a]	PFGE pattern			Genome pattern
		*Apa*I	*Sfi*I	*Sma*I	
M1	IHK	1	4	8	1
M2	IHK	2	5	9	2
M3	IHK	3	5	11	3
M4	PPBGM	3	4	11	4
M5	PPBGM	3	6	11	5
M6	PPBGM	3	6	11	5
M7	PPBGM	3	7	12	6
M8	PTBC	3	7	12	6
M9	PTBC	2	5	12	7
M10	PTBC	2	5	12	7
M11	PTBC	2	7	12	6
M12	PTBC	2	7	12	6

[a]IHK, Institut Haiwan Kluang; PPBGM, Pusat Pembiakan Bebiri Gajah Mati, Kedah; PTBC, Pusat Ternakan Bebiri Chalok, Terengganu.

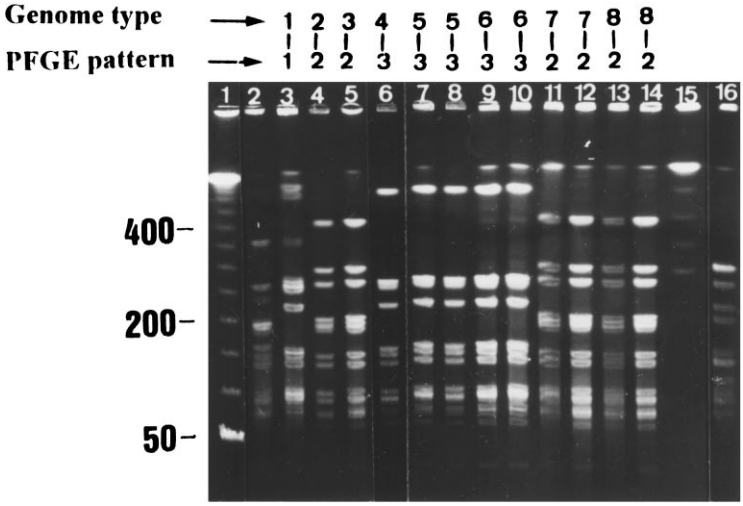

Fig. 1. PFGE patterns of *Apa*I digests of *D. nodosus* genomic DNA. Lanes: 1, lambda concatamers molecular size markers (kbp) are indicated by numbers on the left; 2, *D. nodosus* ATCC 25549 reference strain; 3, M1; 4, M2; 5, M3; 6, M4; 7, M5;8, M6; 9, M7; 10, M8; 11; 12, M10; 13, M11; 14, M12; 15, *S. cerevisiae*; 16, *D. nodosus* G1674 reference strain. Numbers across the top indicates the PFGE patterns and genome-types.

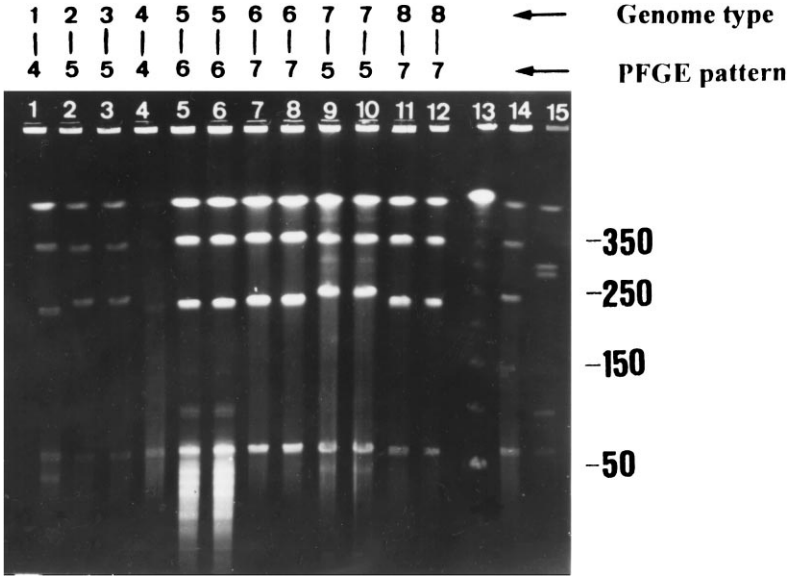

Fig. 2. PFGE patterns of *Sfi*I digests of *D. nodosus* genomic DNA. Lanes: 1, M1; 2, M2; 3, M3; 4, M4; 5, M5; 6, M6; 7, M7; 8, M8; 9, M9; 10, M10; 11, M11; 12, M12; 13, lambda concatamers molecular size marker (kbp) are indicated by numbers on the right; 14, *D. nodosus* ATCC 25549 reference strain, 15; *D. nodosus* G1674 reference strain. Numbers across the top indicates the PFGE patterns and genome-types.

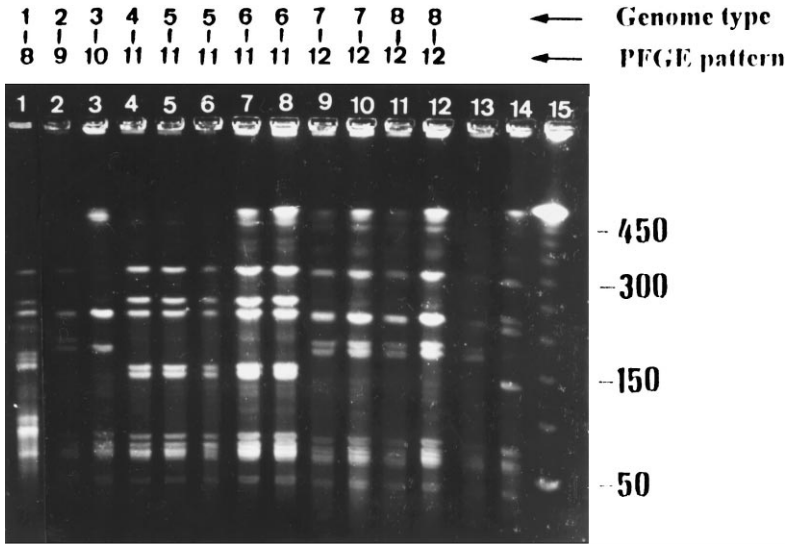

Fig. 3. PFGE patterns of *Sma*I digests of *D. nodosus* genomic DNA. Lanes:1, M1; 2, M2; 3, M3; 4, M4; 5, M5; 6, M6; 7, M7; 8, M8; 9, M9; 10, M10; 11, M11; 12, M12; 13, *D. nodosus* ATCC 25549 reference strain; *D. nodosus* G1674 reference strain; 15, lambda concatamers molecular size marker (kbp) are indicated by numbers on the right. Numbers across the top indicates the PFGE patterns and genome-types.

12 *D. nodosus* strains using the three different enzymes (*Apa*I, *Sfi*I and *Sma*I) were taken together (with a total of 12 PFGE genome patterns), eight genome-types were observed (Table 1) among the three different flocks studied. On the basis of their PFGE patterns, all the *D. nodosus* strains in the study area differed from the reference strains ATCC 25549 and G1674. The results may indicate that the Malaysian strains belongs to different clonal lineages. Therefore, considerable genetic diversity was found among the *D. nodosus* strains studied (eight genome-types from 12 strains isolated). We also established that *D. nodosus* strains from the same flock has different PFGE profiles. However, because of the sampling bias of the study (a single strain studied from each footrot lesion) the above described PFGE results for the 12 *D. nodosus* strains does not imply that a particular genome-type is the most disseminated or epidemic strains among footrot infection in the study area. In addition, to further confirm our results, PFGE with more *D. nodosus* strains as well as digestion with more restriction enzymes may be necessary.

The successful use of PFGE fragments in the differentiation between strains of *D. nodosus* suggests other potential uses for this technique. For example, specific PFGE fragments could be used to generate strain-specific or PFGE group-specific probes. Anecdotally, to the best of our knowledge, this is the first report on the isolation of *D. nodosus* from footrot in sheep in Malaysia. PFGE could be useful in the analysis of the level of diversity among strain that originated in different geographical areas and on different hosts. Because of its potential for strain differentiation, it could be used for

monitoring the pathogenic capability of *D. nodosus* strains that may pose threat to the sheep farmers.

Acknowledgements

The authors are greatly indebted to Dr. Egerton, J.R. of the Department of Animal Health, University of Sydney, Australia, for his kindness in supplying the standard *D. nodosus* strains ATCC 25549 (A1001) and G1674 and the Department of Veterinary Services, Malaysia for providing samples. This work was financially supported by the Malaysian Government through the IRPA mechanism, grant number 51011.

References

Anderson, B.J., Sills, M.M., Egerton, J.R., Mattick, J.S., 1984. Cloning and expression in *Escherichia coli* of the gene encoding the structural subunit of *Bacteroides nodosus* fimbriae. J. Bacteriol. 160, 748–754.

Bert, F., Brange, C., Lambert-Zechovsky, N., 1997. Pulsed-field gel electrophoresis is more discriminating than multilocus enzyme electrophoresis and random amplified polymorphic DNA analysis for typing pyogenic Streptococci. Curr. Microbiol. 43, 226–229.

Beveridge, W.I.B., 1941. Footrot in sheep: A transmissible disease due to infection with *Fusiformis nodosus*. In: Bull. 140. Council for Scientific and Industrial Research. Melbourne, Australia, pp. 1–53.

Bowditch, B.M., Albright, D.G., Williams, J.G.K., Braun, M.J., 1993. Use of randomly amplified polymorphic DNA markers in comparative genome studies. In: Zimmer, E.A., White, T.J., Cann, R.L., Wilson, A.C. (Eds.), Methods in Enzymology. Academic Press, NY, pp. 294–309.

Chu, G., Vollrath, D., Davis, R.W., 1986. Separation of large DNA molecules by contour-clamped homogenous electric field. Sci. 234, 1582–1585.

Dewhirst, F.E., Paster, B.J., La Fontaine, S., Rood, J.I., 1990. Transfer of *Kingella indologenes* (Snell and Lapage, 1976) to the genus *Suttonella* gen. Nov. *Suttonella indologenes* comb. Nov.; transfer of *Bacteroides nodosus* (Beveridge, 1941) to the genus *Dichelobacter nodosus* comb. Nov.; and assignment of the genera *Cardiobacterium*, *Dichelobacter*, and *Suttonella* to *Cardiobacteriaceae* fam. Nov. in the gamma division of *Proteobacteria* based on 16S ribosomal ribonucleic acid sequence comparisons. Intern. J. Syst. Bacteriol. 40, 426–433.

Egerton, J.R., Roberts, D.S., 1971. Vaccination against ovine footrot. J. Comp. Path. 81, 179–185.

Egerton, J.R., Roberts, D.S., Parsonson, I.M., 1969. The aetiology and pathogenesis of ovine footrot. A histological study of the bacterial invasion. J. Comp. Path. 79, 207–216.

El-Adhami, W., Roberts, L., Vickery, A., Inglis, B., Gibbs, A., Stewrat, P., 1991. Epidemiological analysis of a methicillin-resistant *Staphylococcus aureus* outbreak using restriction fragment length polymorphisms of genomic DNA. J. Gen. Microbiol. 35, 101–117.

La Fontaine, S.L., Egerton, J.R., Rood, J.I., 1993. Detection of *Dichelobacter nodosus* using species-specific oligonucleotide as PCR primers. Vet. Microbiol. 35, 101–117.

Pitman, D.R., Palmer, M.A., Depiazzi, L.J., 1994. The laboratory culture of *Dichelobacter nodosus* in a footrot eradication program. Aust. Vet. J. 71, 109–112.

Rood, J.I., Howarth, P.A., Haring, V., Billington, S.J., Yong, W.K., Liu, D., Palmer, M.A., Pitman, D.R., Links, I., Stewart, D.J., Vaughan, J.A., 1996. Comparison of gene probe and conventional methods for the differentiation of ovine footrot isolates of *Dichelobacter nodosus*. Vet. Rec. 52, 127–141.

Single, L.A., Martin, D.A., 1992. Clonal differences within M-types of the group A Streptococcus revealed by pulsed field gel electrophoresis. FEMS Microbiol. Lett. 91, 85–90.

Skerman, T.M., 1989. In: Egerton, J.R., Yong, W.K., Riffkin, G.G. (Eds.), Footrot and Foot Abscess of Ruminants. CRC Press, Boca Raton, pp. 84–104.

Stewart, D.J., Clark, B.L., Jarrett, R.G., 1982. Observations on strains of *Bacteroides nodosus* of intermediate virulence to sheep. In: Cooper, M.G. (Ed.), Australian Advances in Veterinary Science. Australian Veterinary Association, pp. 219–222.

Stewart, D.J., Clark, B.L., Jarrett, R.G., 1984. Differences between strains of *Bacteroides nodosus* in their effects on the severity of footrot, bodyweight and wool growth in Merino sheep. Aust. Vet. J. 61, 349–352.

Thomas, J.H., 1958. A simple medium for isolation and cultivation of *Fusiformis nodosus*. Aust. Vet. J. 34, 411.

Thomas, L.M., Long, K.A., Good, R.T., Panaccio, M., Widders, P.R., 1997. Genotypic diversity among *Campylobacter jejuni* isolates in a commercial broiler flock. Appl. Environ. Microbiol. 63, 1874–1877.

Thorley, C.M., 1976. A simplified method for the isolation of *Bacteroides nodosus* from ovine footrot and studies on its colony morphology and serology. J. Appl. Bacteriol. 40, 301–309.