J. Vet. Malaysia (1998) 10(1): 5-9

VIRULENT CHARACTERISTICS OF *DICHELOBACTER NODOSUS* ISOLATED FROM OVINE FOOT-ROT IN MALAYSIA

Z. Zunita[1], A.R. Sheikh-Omar[1], A.R. Mutalib[1], P.G. Joseph[2] and R. Son[3]

[1]Faculty of Veterinary Medicine and Animal Science,
[2]Faculty of Biomedical and Health Sciences and
[3]Faculty of Biotechnology and Food Science
Universiti Putra Malaysia, Serdang, 43400, Selangor, Malaysia

SUMMARY

Twelve *Dichelobacter nodosus* isolates obtained from ovine foot-rot lesions in Malaysia were characterised by the elastase and the gelatin gel tests to determine the virulence of each isolate. Although all isolates were obtained from mild foot-rot lesions with score 2, they varied in their virulence. Six isolates were found to be virulent, four were intermediate and two were benign. Both tests showed similar results in assessing the virulence of *Dichelobacter nodosus* isolates. None of the isolates were found to contain plasmid.

Keywords: *Dichelobacter nodosus,* foot-rot, sheep

INTRODUCTION

Dichelobacter nodosus (formerly known as *Bacteroides nodosus)* (Dewhirst, 1990) is the causative organism of severe foot-rot, a contagious debilitating disease which affects ruminants particularly sheep, goats and cattle. The disease is characterised by separation of the horn of the hoof from the underlying soft tissue resulting in lameness, loss of body condition and reduced wool production (LaFontaine *et al.,* 1993; Rood *et al.,* 1996).

Dichelobacter nodosus is a Gram-negative, non-spore-forming, rod-shaped obligate anaerobe with bulb ends. An important feature of *D. nodosus* is its diversity in virulence, which ranges from virulent, intermediate and benign (Egerton and Parsonson, 1969; Stewart *et al.,* 1986). Conventional laboratory tests such as the elastase and gelatin gel tests have been used to determine the laboratory virulence of various isolates of *D.nodosus.*

The elastase test relies on detection of elastase activity on the elastin plates, in which the highly virulent strains digest elastin particles more readily and rapidly than the less virulent, while the benign strains do not. On the other hand, the gelatin gel test depends on the differentiation of the stability of protease enzymes produced by various strains at 68°C for 8 and 16 min.

This study determines the virulence of twelve *D. nodosus* isolates obtained from sheep in Malaysia using the elastase and gelatin gel tests.

MATERIALS AND METHODS

Bacterial isolates

Twelve local isolates of *D. nodosus* were used in this study (Table 1). The foot-rot lesions, from where samples were taken, were scored according to Stewart and Claxton (1993). Isolations were made on 4% hoof agar (HA), pH 7.8-8.0 incorporated with 1.5% of finely ground hoof powder (Skerman, 1975).

In preparing the hoof agar, the horns of the hoof were manually removed from the feet, washed and soaked in water to soften. The hooves were then cut into narrow strips and dried either under the sunlight or in an oven at 37°C. The dried hoof strips were then grounded in a grinder (hammer mill) with a 2mm sieve screen before it was added into the medium. Other ingredients included Lab Lemco powder 4g/L (Oxoid), yeast extract 1g/L (BBL), protease peptone 10g/L (Oxoid), sodium chloride 5g/L and Difco-Bacto agar at either 40g/L or 20g/L.

Swab samples taken from the necrotic foot lesions were immediately streaked onto dried 4% HA and transported to the laboratory. The inoculated agar were then incubated in an anaerobic jar with 90% hydrogen and 10% $C0_2$ with sufficient catalyst provided by the commercial gas generating kit (GasPak *Plus;* BBL) at 37°C for four to seven days. After incubation, suspected colonies were transferred onto 4% HA and subcultured until free from contaminating bacteria. Reference virulent (A 1001) and benign (G 1674) strains were used as controls.

Gelatin gel test

(a) Preparation of substrate gel

The substrate gel consisted of 1% agarose (Boeringher), 0.75% (w/v) gelatin (Difco), 20mM Tris base and 0.5mM $CaCl_2$. These ingredients were dissolved by boiling, adjusted to pH 8.8 and poured into a glass plate at 1.5mm thick. Each sample to be tested required an area of 25mm x 75mm, into which 3mm diameter holes were punched in lines, 25mm apart. Samples were prevented from spreading by allowing the surface of the gel to dry at room temperature for 2 h.

(b) Sample preparation

Cultures of *D. nodosus* isolates were grown in trypticase arginine serine (TAS) broth (Skerman, 1975) for 3 days at 37°C and checked for purity by using a microscope. Pure cultures, which normally achieved a count of 10^8/mL, were diluted 0.5 into 1.5 mL buffer (Hepes 0.1M, $CaCl_2$ O.O1M pH 8.5). Dilutions of protease stable (virulent strain A 1OO1) and unstable (benign strain G I674) *D. nodosus* were also prepared.

(c) Gelatin test procedure

A 20 µL aliquot of the unheated diluted sample was placed into one well of the gelatin gel before the remaining sample was placed in a water bath (DT Hetoterm) at 68±0.1°C. A further 20 µL aliquot was pipetted out after 8 and 16 min of incubation, and placed into appropriate wells. A similar procedure was applied to all isolates. The gel was then placed in a moist chamber and incubated at 37°C for 18 h.

At the end of the incubation period, the unhydrolysed gelatin was precipitated by immersing the gel into saturated ammonium sulphate solution at 60°C. The clear zones of proteolysis were measured whilst viewing the gel against a black background with oblique lighting. The percentage stability of protease at 8 and 16 min was determined by comparing the diameter of the clear zone of digestion before and after heating using the following formula:

$$\text{Percentage stability} = \frac{\text{Heated zone size}}{\text{Unheated zone size}} \times 100$$

Tests showing partial clearing after 16 min heating were recorded as equivocal. Tests with an unheated zone diameter of less than 12mm (insufficient initial protease) were recorded as invalid. The procedure was repeated for isolates yielding either equivocal or invalid result.

Elastase test

The elastase test was performed using the trypticase arginine serine (TAS) medium containing 1.5% Bacto agar (Difco) and 0.3% elastin powder from bovine neck ligament (Sigma Chemical, USA). Calcium chloride (0.15%) was added to stabilise the protease and enhance clearing of elastin particles. The elastin agar was poured into petri dishes as a layer of 3 mm thick, stored in an anaerobic environment (GasPak Plus, Becton Dickinson, USA) prior to use and dried to remove surface moisture immediately prior to inoculation. Each isolate was inoculated in a straight line on the surface of the agar, using an up-and-back stroke with a wire loop, with up to 3 isolates per plate in separate streaks. Inoculated elastin agar plates were incubated with minimum delay under anaerobic environment at 37°C. The virulent and the benign reference strains were included with each batch of the test. Plates were examined at 7, 14, 21 and 28 days after inoculation. Positive results were recorded at the days on which plates showed clearing of elastin particles.

Plasmid isolation

The test organisms were screened for plasmid DNA by the procedure of Birnboim and Doly (1979). Products were subjected to electrophoresis for 2 h at 35 mA in a 0.7% agarose gel in TBE (89 mmol/L Tris base, 2.5 mmol/L disodium EDTA, 89 mmol/L boric acid) as described by Meyers *et al.* (1976). *E. coli* V517 harbouring plasmids of known molecular weight, was used as a molecular weight marker (Macrina *et al.*, 1978).

RESULTS

All twelve isolates were obtained from score 2 foot-rot lesions, which was a necrotising inflammation of the interdigital skin involving either a portion or the entire soft horn of the axial wall of the digit. In spite of the dense, mixed bacterial growth, colonies of *D. nodosus* or parts of colonies could still be identified by their flat, spreading appearance and characteristic fimbriated outer areas with ground glass texture. *D. nodosus* colonies basically consisted of three zones; central (beaded/mucoid), mid-zone (clear/transparent) and the outer zone which spread with either fimbriated/smooth edges. *D.nodosus* cells from pure culture were rod-shaped with bulb ends.

When proteases from non-benign and benign strains were compared, the former produced larger initial clear zone of proteolysis and still produced a clear zone even after being heated at 68°C for 16 mins. Six (50%) isolates were thermostable (>60% stability), 4 (33%) were intermediate (11-60% stability) while 2 (17%) were unstable (<10% stability) (Table 1). Both virulent and benign controls showed thermostable and unstable proteolysis respectively.

Seven (58%) isolates were confirmed as elastase positive while the rest (42%) were elastase negative (Table 1). The virulent control strain was elastase positive before day 7 and the benign control was negative even after 28 days. Each isolate yielded similar results on three replicate tests, indicating high qualitative repeatability of the test.

None of the isolates studied contained any plasmids, as determined by a standard alkaline lysis procedure and by pulsed field gel electrophoresis of undigested DNA,

Table 1: Comparative examination of twelve *D. nodosus* strains using elastase and gelatin-gel tests.

Isolate[a]	Source[b]	Elastase[c]	Gelatin Gel[d]
Mal 1	IHK	+ (14 days)	76%
Mal 2	IHK	-	0%
Mal 3	IHK	-	45%
Mal 4	PPBGM	-	52%
Mal 5	PPBGM	+(14 days)	66%
Mal 6	PPBGM	+(21 days)	58%
Mal 7	PPBGM	+(21 days)	62%
Mal 8	PTBC	+(7 days)	75%
Mal 9	PTBC	-	0%
Mal 10	PTBC	+(14 days)	62%
Mal 11	PTBC	-	50%
Mal 12	PTBC	+(7 days)	76%
A1OO1	virulent control	+(7days)	76%
G1674	benign control	-	0%

[a]All lesion were of score 2
[b]IHK: Institut Haiwan Kluang; PPBGM: Pusat Pembiakan Bebiri Gajah Mati; PTBC: Pusat Ternakan Bebiri Chalok
[c]+(x days): incubation days before detection of positive result
[d]Isolates with proteases of thermostability of >60% are regarded as thermostable proteases (or virulent), 11- 60% as intermediate protease and <10% as unstable proteases (or benign) (Liu *et. al.*, 1994)

DISCUSSION

In this study, isolations of *D. nodosus* were successfully achieved on 4% HA. High concentration of agar enhances the development of *D. nodosus* colonies as well as suppressing the growth of contaminants (Stewart and Claxton, 1993), while the incorporation of hoof powder promotes and stimulates the growth of *D. nodosus,* giving its characteristic colony morphology (Skerman, 1975). In addition, this medium provides a suitable environment for the micro-aerophilic nature of this obligate anaerobe since exposure to oxygen does not interfere with its viability. This allows easy handling and manipulation of the bacteria on the bench (Gordon, 1981).

The relationship between the laboratory and clinical characteristics of ovine foot-rot has not been established in causing foot-rot since *D. nodosus* demonstrates a wide range of *in vitro* virulence (Liu *et al.*, 1994). Moreover, the phenotypic expression of *D. nodosus* is often influenced by factors such as seasonal and local conditions, that affects the growth and ability of the bacteria to produce disease (Egerton *et al.*, 1983; Stewart, 1989; White, 1991).

The elastase and gelatin gel tests are the most common diagnostic tests used in assessing the virulence of *D. nodosus* (Stewart, 1979; Liu and Yong, 1993; Palmer, 1993). The elastase test, although notoriously time consuming and lacking in precision, is very useful in differentiating isolates into virulent, intermediate and benign strains. This, in general, agrees with clinical characteristics (Liu and Yong, 1993). The gelatin gel test was developed for a more rapid characterisation of isolates (Kortt *et* al., 1983; Depiazzi *et al.,* 1991). There are variations in the incubation temperature, ranging from 66.8 to 68°C, used by different researches for the gelatin-gel test (Liu and Yong, 1993; Palmer, 1993; Liu *et al.,* 1994; Links and Morris, 1996). However, the bacteria are interpreted as either thermostable or thermolabile proteases.

Reproducibility of each test depends on the consistency of test conditions, as slight change may cause some variations in the results. Liu *et al.* (1994) observed that even different brands or batches of elastin used might influence the results. Maintaining an accurate temperature (68°C $\pm$ 0.1) is also crucial for the gelatin gel test. The results of the present study suggest that there is a close correlation between the elastase and the gelatin gel tests. Isolates that were elastase positive were likely to produce more proteases which were thermostable compared to isolates with low or negative elastase activity.

It is of interest to note that despite the same clinical characteristics whereby all the isolates were isolated from score 2 lesions, they vary in their laboratory virulent characteristics. Mal 1, Mal 8 and Mal 12, which are virulent *in vitro* and have the ability to cause severe foot-rot due to the positive elastase and thermostable protease, however, only caused a mild form of the disease with lesion score 2. This manifestation was probably due to the constant topical treatment regimen adhered to and the results of the vaccination programme which suppressed the bacteria from fully expressing their virulence. It was known

that *D. nodosus* strains with the capacity to produce severe foot-rot might only cause a clinical phenomenon resembling intermediate or even benign foot-rot under unfavourable conditions (Liu *et al.*, 1994).

Bacterial plasmids have been shown to confer a variety of phenotypic modifications and genetic flexibility upon their hosts by carrying genes that may code for antibiotic resistance, toxin production, ion sequestration, adhesiveness and serum resistance (Novick, 1982). The determination of plasmid profiles and virulent tests allow us to correlate virulence with the presence or absence of plasmid DNA in the host organisms. In this study, all twelve isolates of *D. nodosus* were found to contain no plasmid. This shows that the virulence properties of the *D. nodosus* isolates as determined by their positive results for elastase and gelatin tests were not plasmid mediated. Genomic region coding for virulence of *D. nodosus* has been located on the chromosome which includes the virulence related locus (*vrl*) and virulent associated regions (*vap*) (Katz *et al.*, 1992; Billington *et.al.*, 1996). However, the presence of plasmid in *D. nodosus* was first reported only recently by Billington *et al.* (1996). Out of 102 *D. nodosus* strains tested, only one isolate was found to contain a 10 kb native plasmid designated as pJIR 896. It was shown to be carrying *vap* sequences and a putative insertion sequence indicating that this plasmid may represent a replicating progenitor of the chromosomal *vap* sequence found in many *D. nodosus* isolates.

Both elastase and gelatin gel tests possess parallelism in assessing virulence. In conclusion, conventional laboratory tests such as the elastase and gelatin gel tests can be used to determine the virulence of *D. nodosus* strains. This is helpful in clarifying foot-rot situations so that safety measures could be undertaken especially if there are virulent strains present.

ACKNOWLEDGEMENTS

We express our gratitude to the Department of Veterinary Services, Malaysia for the supply of samples. We also thank Prof. Egerton of University of Sydney for donating prototype strains and antisera. This study was supported by IRPA grant No. 51011, Malaysia.

REFERENCES

Billington, S.J., Sinistaj, M., Chathem, B.F., Ayres, A., Moses, E.K., Katz, M.E. and Rood, J.I. (1996). Identification of a native *Dichelobacter nodosus* plasmid and the evolution of the *vap* regions. *Gene* **172**: 111-116

Birnboim, H.C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**: 1513-1523.

Dewhirst, F.E., Faster, B.J., La Fontaine, S. and Rood, J.I. (1990) Transfer of *Kingella indogenes* (Snell and Lapage 1976) to the genus *Suttonella* gen. Nov. as *Sultonella indogeles* comb. nov.; Transfer of *Bacteroides nodosus* (Beveridge 1941) to the genus *Dichelobacter* gen. nov. as *Dichelobacter nodosus* comb. nov.; and assignment of the genera *Cardiobacterium, Dichelobacter, and Suttonella to Cardiobacieriaceae* fam. Nov. in the gamma division of Proteobacteria based on 16S ribosomal ribonucleic acid sequence comparisons. *Int. J. Syst. Bacteriol.* **40**: 426-4533.

Depiazzi, L.J., Richards, R.B., Henderson, J., Rood, J.I., Palmer, M. and Penhale, W.J. (1991). Characterisation of virulent and benign strains of *Bacteroides nodosus*. *Vet. Microbiol.* **26**: 151-160.

Egerton, J.R., Ribeiro, L.A., Meran, P.J. and Thorley C.M. (1983). Onset and remission of ovine footrot. *Aust. Vet. J.* **60**: 334-336

Egerton, J.R. and Parsonson, I.M. (1969). Benign footrot - A specific interdigital dermatitis of sheep associated with infection by less proteolytic strains of *Fusiformis nodosus*. *Aust. Vet. J.* **55**: 124-129.

Gordon, L. (1981). Experiences with laboratory diagnosis of footrot in a control area. *In:* Ovine Footrot – Report of Workshop at University of Sydney. Beveridge, W.I.B. and Egerton, J.R. (Eds). pp70-79

Katz, M.E., Strugnell, R.A. and Rood, J.I. (1992), Molecular characterisation of a genomic region associated with virulence in *Dichelobacter nodosus*. *Infect. Immun.* **60**: 4586-4592.

Kortt, A.A., Burns, J.E. and Stewart, D.J. (1983). Detection of the extracellular protease of *Bacteroides nodosus* in polyacrylamide gels: a rapid method of distinguishing virulent and benign ovine isolates. *Res. Vet. Sci.* **35**: 171-174.

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

Links, I.J. and Morris, S. (1996). Assessment of gelatin gel and elastase tests for detection of protease activity of *Dichelobacter nodosus* isolates from ovine footrot. *Vet. Microbiol.* **51**: 305-318.

Liu, D. and Yong, W.K. (1993). *Dichelobacter nodosus*: differentiation of virulent and benign strains by gene probe based dot blot hybridisation. *Vet. Microbiol.* **38**: 71-79.

Liu, D., Roycroft, C., Samuel, J. and Webber, J. (1994). A retrospective study of clinical and laboratory characteristics of ovine footrot. *Vet. Microbiol.* **42**: 373-381.

Macrina, F.L., Kopecko, D.J., Jones, K.R., Ayers, D.J. and McCowen, S.M. (1978). A multiple plasmids-containing *Escherichia coli* strains: Convenient source of size reference plasmid molecules. *Plasmid* **1**: 417-420.

Meyers, J. A., Sanchez, D., Elwell, L.P. and Falkow, S. (1976). Simple agarose gel electrophoretic method for the identification and characterisation of plasmid deoxyribonucleic acid. *J. Bacteriol.* **127**: 1529-1537

Novick, R.P. (1982). Plasmid. *Sci. Ann.* **246**: 77-90.

Palmer, M.A. (1993). A gelatin gel test to detect activity and stability of proteases produced by *Dichelobacter (Bacteroides) nodosus. Vet. Microbiol.* **36**: 113-122.

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. and Vaughan, J.I. (1996). Comparison of gene probe and conventional methods for the differentiation of ovine footrot isolates of *Dichelobacter nodosus. Vet. Microbiol.* **52**: 127-141.

Skerman, T.M. (1975). Determination of some *in vitro* growth requirements of *Bacteroides nodosus. J. Gen. Microbiol.* **87**:107-119.

Stewart, D.J. and Claxton, P.D. (1993). Ovine Footrot -Clinical Diagnosis and Bacteriology. *In:* Australian Diagnostic Techniques For Animal Disease. CSIRO, pp 1-26.

Stewart, D.J. (1989). Footrot of sheep. *In:* Footrot and Foot Abscess of Ruminants. J.R. Egerton, W.K. Yong and G.G. Riffkin (Eds), CRC Press, Boca Raton, pp 5-45

Stewart, D.J., Peterson, J.E., Vaughan, J.A., Clark, B.L., Emery, D.L., Cladwell, J.B. and Kortt, A.A. (1986). The pathogenicity and cultural characteristics of virulent, intermediate and benign strains of *Bacteroides nodosus* causing ovine footrot. *Aust. Vet. J.* **63**: 317326.

Stewart, D.J. (1979). The role of elastase in the differentiation of *Bacteroides nodosus* infections in sheep and cattle. *Res. Vet. Sci.* **27**: 99-105.

White, A. (1991). Footrot in sheep. *Vet. Ann.* **31**: 85-89.

RINGKASAN

CIRI VIRULENS DICHELOBACTER NODOSUS *YANG DIPENCILKAN DARIPADA REPUT KAKI OVIN DI MALAYSIA*

Dua belas isolat Dichelobacter nodosus *diperolehi daripada lesi reput kaki ovin di Malaysia telah dicirikan melalui ujian elastase dan gelatin untuk menentukan kevirulenan setiap isolat. Walaupun kesemua isolat diperolehi daripada lesi reput kaki ringan berskor 2, virulensnya berbeza. Enam isolat di dapati bervirulens, empat sederhana dan dua benigna. Kedua-dua ujian ini menunjukkan hasil sama dalam penilaian kadar virulens isolat* Dichelobacter nodosus. *Tiada satupun isolat di dapati mengandungi plasmid.*