1 **Improved diagnosis of virulent ovine footrot using the *intA* gene**

2 B.F. Cheetham[a], L.R. Tanjung[a], M. Sutherland[a], J. Druitt[a], G. Green[b], J. McFarlane[b],

3 G. D. Bailey[c], J.T. Seaman[c] and M.E. Katz[a]

4 [a]Molecular and Cellular Biology, University of New England, Armidale, NSW, 2351

5 [b]Rural Lands Protection Board, Armidale, NSW, 2350

6 [c]NSW Department of Primary Industries, Orange, NSW, 2800

7 Corresponding Author: Assoc. Prof. B.F. Cheetham, tel: 61 2 6773 3394, fax: 61 2

8 6773 3267

9 Address for correspondence: Assoc. Prof. B.F. Cheetham, Molecular and Cellular

10 Biology, the University of New England, Armidale, NSW, 2351, Australia. Email:

11 bcheetha@une.edu.au.

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1   **Abstract**

2   Footrot is a mixed bacterial infection of the hooves of sheep. The Gram-negative

3   anaerobic bacterium *Dichelobacter nodosus* is the principal causative agent, with

4   different strains causing disease of different severity, ranging from benign to virulent.

5   In Australia, in the state of New South Wales (NSW), only virulent footrot is subject

6   to regulatory action, including quarantine. However, it is often difficult to distinguish

7   benign footrot from virulent footrot in the initial stages of infection, or under adverse

8   climatic conditions. The gelatin gel test, which measures the thermostability of

9   secreted bacterial proteases, is the laboratory test most widely used in Australia to aid

10   in the differential diagnosis of footrot. The proteases of virulent strains are, in

11   general, more thermostable than the proteases of benign strains. However, there are

12   some false positives in the gelatin gel test, which may lead to unnecessary quarantine

13   procedures. We used Southern blot analysis on 595 isolates of *D. nodosus* from 124

14   farms on which sheep had benign or virulent footrot to test for the presence of the *intA*

15   gene. We found that for *D. nodosus* strains which are stable in the gelatin gel test,

16   there is a high correlation between the presence of the *intA* gene and the ability of the

17   strain to cause virulent footrot. We also developed a PCR-based assay for the rapid

18   detection of *intA*, which can be used to test DNA extracted from colonies grown on

19   plates, or DNA extracted from cotton swabs of culture plates.

20

21   **Keywords**: Footrot; *Dichelobacter*; PCR, virulence, protease.

22

1   Introduction

2   Footrot of sheep is a contagious disease caused by a mixture of bacteria with

3   *Dichelobacter nodosus* the essential transmitting agent (Beveridge, 1941). Different

4   strains of *D. nodosus* cause disease of different severity, ranging from mild (benign)

5   to severe (virulent). The severity of the disease is influenced by the breed of sheep,

6   with Merinos being more susceptible than British breeds (Emery et al., 1984).

7   Environmental conditions are also important, with warm, moist conditions and lush

8   pasture favouring expression of the disease (Stewart et al., 1984). In the early stages

9   of infection, or under adverse climatic conditions, it is often difficult to distinguish

10   between benign and virulent footrot. In Australia, in the state of New South Wales

11   (NSW), a footrot control program has been in place since 1988 with the aim of

12   eradicating virulent footrot. Under the program, diagnosis of virulent footrot is

13   essentially a field diagnosis with virulent footrot subject to regulatory action,

14   including quarantine.

15

16   A variety of laboratory tests have been used to assist in the diagnosis of footrot. In

17   general, the proteases secreted by virulent strains are more thermostable than those

18   secreted by benign strains. This is the basis of the gelatin gel test (Palmer, 1993),

19   which is the most commonly used laboratory test in Australia for the diagnosis of

20   footrot. Using this test, strains are classified as stable, and considered to be capable of

21   causing virulent footrot, or unstable, and considered likely to cause benign footrot.

22   Some strains are classified as equivocal, as they give intermediate results in the

23   gelatin gel test. The gelatin gel test has been used extensively as an aid to diagnosis

24   of footrot in NSW. However, it has become apparent that there are some strains of *D.*

25   *nodosus* which secrete thermostable proteases but are incapable of causing virulent

1   footrot as defined in the NSW Footrot Eradication Manual (Anon, 1995). We have

2   termed these strains "gel stable, field benign". Although such strains have been noted

3   previously (Depiazzi et al., 1991), this is the first report showing a large number of

4   farms with gelatin gel stable isolates from sheep with benign footrot.

5

6   To improve the laboratory diagnosis of virulent footrot, we have been analysing

7   genetic differences between virulent and benign strains of *D. nodosus*. This work has

8   led to the identification of a series of genetic elements, the *intA*, *intB*, *intC* and *intD*

9   elements, which integrate into the *D. nodosus* chromosome (Cheetham et al., 1995;

10   Bloomfield et al., 1997; Cheetham et al., 1999). These genetic elements consist of an

11   integrase (*int*) gene, and a series of adjacent genes, and they integrate into two

12   different *tRNA-ser* genes. The distribution of some of these genetic elements between

13   virulent and benign strains is non-random, and we have proposed a model whereby

14   the integration of these genetic elements modulates virulence (Whittle et al., 1999).

15

16   In this study, we analysed by Southern blotting 595 isolates of *D. nodosus* from 124

17   farms on which sheep had benign or virulent footrot for the presence of the *intA* gene

18   in order to establish a correlation between the presence of *intA* and virulence. We

19   also developed and validated a PCR-based test for the detection of *intA*.

20

Materials and Methods

Footrot diagnosis

Footrot diagnosis was based on field assessment by District Veterinarians and/or Rangers from the Rural Lands Protection Boards in NSW, according to the NSW Footrot Eradication Manual (Anon, 1995). Briefly, the severity of the damage to the hoof is scored on a scale from 1 to 5 (Whittington and Nicholls, 1995), and virulent footrot is usually diagnosed if lesions are severe (score 4 or 5) or where a significant proportion of the flock show underrunning. The climatic conditions are also taken into account. The procedures are described in detail in Agfact A0.9.56: Footrot - sheep and goats, available at the following website: http://www.agric.nsw.gov.au/reader/sheep-footrot/a0956.pdf. For this study, in many cases, follow-up visits were carried out over months or years to confirm the diagnosis.

Gelatin gel tests

Gelatin gel tests (Palmer, 1993) were carried out at the Regional Veterinary Laboratory, NSW, Department of Primary Industries, Orange, NSW.

Southern blot analysis

D. nodosus isolates were grown for four days at 37°C in an atmosphere of 10% (v/v) CO_2 in N_2 on Eugonagar (Becton-Dickinson) plates supplemented with 5% defibrinated horse blood. Cells from two confluent plates were suspended in 10 ml of

Eugonbroth (Becton-Dickinson) and harvested by centrifugation. DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega), according to the manufacturer's instructions. Genomic DNA was digested with *Eco*RI, separated by electrophoresis on a 1% agarose gel, and Southern blot analysis was carried out using a digoxygenin non-radioactive labelling and chemiluminescent detection kit, as described previously (Cheetham et al., 1995). The probes used consisted of part of the *intA* gene (nucleotides 924-1423 from the sequence identified by GenBank accession number L31763) or part of the *pnpA* gene (nucleotides 1-677 from the sequence identified by GenBank accession number X98545), cloned into the plasmid pUC18.

PCR analysis

PCR was used to test for the presence of the *intA* and *pnpA* genes. Each PCR run included three amplification controls – DNA extracted from strain A198 (ATCC 27521 held by CSIRO Sydney and isolated originally from a flock at Goulburn, NSW; (Claxton et al., 1983) which contains both the *intA* and *pnpA* genes, DNA extracted from strain C305 (Yong and Gordon, 1986), which contains *pnpA*, but not *intA*, and a negative control using TE buffer instead of DNA. For amplification of part of the *intA* gene, primers used were ACATCATGCGACTCACTGAC and TCTCTGGTCGGTCGTACAAT, while primers for amplification of part of the *pnpA* gene were ACCGAACAGACGGGAACAAC and CGCGTACATCATTAACCCG. The 25 µl amplification reactions contained 120 ng of each primer, 1 x reaction buffer (supplied by the manufacturer, Fisher Biotec), 1.8 mM MgCl$_2$, 1 U *Taq* DNA polymerase and approximately 100 pg of template DNA. Quantification of template

6

DNA was carried out using agarose gel electrophoresis against a lambda standard containing a known amount of DNA. The reaction mixtures were amplified for 31 cycles, each cycle consisting of 90 s at 94°C, 60 s at 60°C and 120 s at 72°C in a Corbett FTS-320 thermal cycler (Corbett Research). A 10 µl sample of the amplification products was analysed by agarose gel electrophoresis.

DNA preparation for PCR from D. nodosus *cultures on plates*

Using a sterile toothpick, *D. nodosus* cells were transferred from an agar plate into 100 µl of sterile distilled water in a 1.5 ml microfuge tube. The tube was placed in a boiling water bath for 10 min., cooled on ice for one min. and then spun at 12,000x*g* for 10 min. 1 µl of the supernatant was used for PCR.

DNA preparation for PCR from cotton swabs

D. nodosus isolates were transported from the Regional Veterinary Laboratory in Orange, NSW, to the University of New England in Stewart's transport medium (Stewart and Claxton, 1993). Cotton swabs were used to collect *D. nodosus* cells from culture plates, then placed in Stewart's transport medium in glass bottles for shipping. On arrival, swabs were used to inoculate culture plates, and DNA was extracted directly from the swabs by swirling the swabs in 100 µl of sterile distilled water, removing the swabs, and processing the aqueous sample as described above. Swabs could be stored at 4°C for several weeks before processing.

Results and Discussion

Presence of intA *is associated with virulence*

Isolates of *D. nodosus* were obtained from affected sheep on 124 farms in NSW, mostly between the years 2000 and 2005. The results are summarised in Tables 1 and 2, and presented in detail in Tables 3 and 4. Based on gelatin gel tests and field clinical diagnosis, the footrot type on each farm was classified as **stable benign**, if there were stable or equivocal isolates and the field clinical diagnosis was benign (Table 1, 37% of farms); **virulent**, if the field clinical diagnosis was virulent, with stable or equivocal isolates (Table 1, 41% of farms), or **unstable benign**, if the field clinical diagnosis was benign and all isolates were unstable (Table 1, 22% of farms).

From the 124 farms, single isolates of *D. nodosus* were obtained on 37 farms (Table 1), and multiple isolates on the remaining 87 farms, with an average of 6.3 isolates per farm with multiple isolates. More than one gelatin gel type was found on 30 farms (Table 1, e.g farms A1, A4, B19, B26 and B27, in Tables 3 and 4), suggesting that more than one strain of *D. nodosus* was present. The presence of more than one strain of *D. nodosus* on a farm is not uncommon. For example, up to six different *D. nodosus* serogroups have been found in isolates from a single flock, with up to four different serogroups in isolates from a single foot (Claxton et al., 1983).

To test the hypothesis that the presence of the *intA* gene is associated with virulence, DNA was prepared from 595 isolates of *D. nodosus*, and Southern blot analysis was used to detect the *intA* gene. As a positive control, the DNA samples were probed for

1   the *pnpA* gene, which is present in all strains analysed so far (Whittle et al., 1999). A

2   sample of this data is shown in Fig. 1A, where there are bands in lanes 1, 3 and 11,

3   showing that these three strains contain one or more copies of the *intA* gene. There

4   are bands in all lanes in Fig. 1B, showing that all strains contain the *pnpA* gene.

5

6   *intA* was not detected in 166 out of 178 (93.2%) of the stable isolates from farms with

7   stable benign footrot, while *intA* was detected in 116 out of 154 (75.3%) of stable

8   isolates from farms with virulent footrot. Since multiple strains of *D. nodosus* may be

9   isolated from flocks with footrot (Claxton et al., 1983), it is likely that sheep from

10   farms with virulent footrot may carry benign strains in addition to virulent strains.

11   This may explain the presence of strains that do not contain *intA* on farms with

12   virulent footrot. Similarly, unstable isolates, which are usually associated with benign

13   footrot, were found on 7 out of 51 properties with virulent footrot (Table 1, Table 4).

14

15   *intA* was not detected in 54 out of 55 (98.2%) of equivocal isolates from farms with

16   stable benign footrot, but was detected in 30 out of 47 (63.8%) of equivocal isolates

17   from farms with virulent footrot. Overall, when stable and equivocal isolates were

18   considered, *intA* was not detected in any isolates from 42 out of 46 (91.3%) of farms

19   with stable benign footrot, but was detected in at least one isolate from 47 out of 51

20   (92.1%) of farms with virulent footrot, and thus there is a strong correlation between

21   the presence of *intA* and virulence, and the absence of *intA* and benign footrot.

22

23   For four farms with virulent footrot (B22, B36, B43 and B46, Table 4), stable isolates

24   negative for *intA* were found in the absence of stable isolates positive for *intA*. This

25   may indicate that there are stable isolates which are *intA* negative, but are capable of

causing virulent footrot. Alternatively, the field diagnosis may be incorrect, or the sheep on these farms may carry stable benign strains as well as stable virulent strains. A mixture of *intA* negative and *intA* positive strains is seen on several other farms with virulent footrot (Table 1, e.g. farms B6, B39, B41, B47, B48 and B49 in Table 4). If this were the case, wider sampling with testing of more strains may have led to the identification of *intA* positive isolates.

Similarly, stable or equivocal isolates which were *intA* positive were found on four farms with stable benign footrot (farms A8, A20, A41 and A43, Table 3). Again, these may be genuine false positives i.e. *intA* positive isolates which are not capable of causing virulent footrot. Alternatively, the field diagnosis may be incorrect. This is more likely for a diagnosis of benign footrot than virulent footrot because expression of the disease may be low under unfavourable environmental conditions.

If the *intA* DNA test had been used for footrot diagnosis, 39 of the 42 farms in group A with stable isolates would have been classified as having benign footrot, even though the gelatin gel test indicated virulent footrot. The *intA* DNA test would have avoided costly quarantine procedures in these cases. In addition, compliance with footrot control programs is likely to be higher if farmers have confidence in the laboratory tests.

For the 27 farms with unstable benign footrot, 75 out of 90 (83%) of the isolates were negative for *intA* (Table 2). 21 out of 27 (78%) of farms had only isolates which were negative for *intA*, while 6 out of 27 (22%) had positive isolates. While this result follows the same trend as for stable isolates, the correlation between the presence of

1 *intA* and virulent footrot is not as high for unstable isolates. The combined data

2 support the use of the *intA* DNA test as an adjunct to the gelatin gel test, to distinguish

3 between stable benign and stable virulent isolates. The *intA* test is also very valuable

4 for equivocal isolates.

5

6 *Development of a PCR-based test*

7

8 The Southern blot test is time consuming and labour intensive, and is therefore not

9 well suited for routine diagnosis. To overcome these limitations, we designed primers

10 to amplify a 530 bp fragment of the *intA* gene, and a 300 bp fragment of the *pnpA*

11 gene (see Materials and Methods) in a PCR-based assay. The *intA* PCR assay reliably

12 amplified 0.02 pg of *D. nodosus* genomic DNA, corresponding to approximately 15

13 copies of genomic DNA per reaction. The *pnpA* PCR assay was approximately ten

14 fold less sensitive, reliably amplifying 0.2 pg of *D. nodosus* genomic DNA. The

15 greater sensitivity of the *intA* assay compared to the *pnpA* assay is useful to avoid

16 false negatives which could result from insufficient template DNA in samples.

17

18 These PCR assays were applied to 221 DNA samples which had been used for

19 Southern blot analysis. A sample of the data from PCR assays is given in Fig. 2A,

20 where there are bands in lanes 1, 3 and 11, which are *intA* positive. There are bands

21 in all lanes 1-13 in Fig. 2B, which show amplification of the *pnpA* gene. In all 221

22 cases, the results from the PCR assays were in agreement with the results from

23 Southern blot analysis.

24

1   *D. nodosus* cultures were routinely shipped on cotton swabs in Stewart's transport

2   medium (Stewart and Claxton, 1993). In some cases, cultures failed to grow when

3   inoculated onto media plates, so it was not possible to prepare sufficient quantities of

4   DNA for Southern blot analysis. To overcome these problems, we developed a PCR

5   method which can be used directly from the cotton swabs. DNA for PCR analysis

6   was extracted from the swabs by agitating them in sterile water, boiling the bacterial

7   suspension and clarifying it by centrifugation. PCR analysis of samples from 232

8   swabs gave identical results to Southern blot analysis of DNA prepared after culturing

9   the organisms.

10

11   Finally, a method was developed for rapid analysis of cultures growing on plates (see

12   Materials and Methods). Again, 150 samples assayed by PCR from DNA prepared

13   from cell suspensions gave identical results to Southern blot analysis.

14

15   Methods have been developed for PCR analysis of swabs taken directly from infected

16   hooves (La Fontaine et al., 1993; Zhou et al., 2001; Moore et al., 2005). However, the

17   *intA* test we have developed is more reliable for isolates which are stable or equivocal

18   in the gelatin gel test than for unstable isolates, so we recommend the *intA* test as an

19   adjunct to the gelatin gel test, rather than as a primary test. Since the gelatin gel test

20   requires the prior isolation of pure *D. nodosus* cultures, these can then be used for

21   PCR analysis, and thus detection by PCR of *intA* directly from swabs of lesions is not

22   necessary.

23

24   DNA probes have been developed previously for the differentiation of virulent and

25   benign footrot. These include the *vap* and *vrl* probes (Katz et al., 1991; Rood et al.,

1   1996) and benign-specific and virulent-specific probes developed by Liu and Webber,

2   (1995). The *vap* and *vrl* probes are not currently used for routine diagnosis of footrot

3   in Australia, possibly because they were not more specific than the gelatin gel test.

4   The virulent-specific probe of Liu and Webber is part of the *vap* region identified by

5   (Katz et al., 1991).   The *vap* regions are found in almost all virulent strains, but are

6   also present in approximately 30% of benign strains. The *intA* gene is found next to

7   the *vap* region in many strains (Cheetham et al., 1995), but some strains have the *vap*

8   regions in the absence of *intA* (Tanjung, L.R., Katz, M.E. and Cheetham, B.F.,

9   unpublished). This may explain the greater specificity of the *intA* probe compared

10 with *vap* probes for the diagnosis of virulent footrot. The benign- specific probe (Liu

11 and Webber, 1995) did not react with the virulent strains tested, but gave a positive

12 result for 20/25 isolates classified as high intermediate.   This may limit its use, as it

13 may be undesirable to exclude these high intermediate strains from footrot eradication

14 programs.

15

16 *Conclusions*

17

18 The results presented here support the use of the *intA* DNA test in NSW as an adjunct

19 to the gelatin gel test to distinguish stable benign footrot from stable virulent footrot.

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5

1   Table 1. Distribution of isolates between farms with stable benign, virulent and

2   unstable benign footrot.

Type of footrot	No. of farms	No. of farms with single isolates	No. of farms with multiple isolates		
			Int A -	*intA* +& -	*intA* +
Stable benign(Group A)					
Stable only	27	8	16	2	1
Stable & equivocal	9	-	8	1	0
Stable & unstable	4	-	4	0	0
Equivocal only	1	0	1	0	0
Equivocal & unstable	3	-	2	1	0
Stable, unstable & equivocal	2	-	1	1	0
Total	46	8	32	5	1
Virulent (Group B)					
Stable only	35	15	3	5	12
Stable & equivocal	5	-	0	3	2
Equivocal only	4	1	0	1	2
Equivocal & unstable	1	-	0	1	0
Stable, unstable & equivocal	6	-	0	5	1
Total	51	16	3	15	17
Unstable benign (Group C)					
Unstable	27	13	12	2	0
Total	27	13	12	2	0

3

4

1

2   Table 2. Summary of *intA* tests on 595 isolates of *D. nodosus*.

3

4

Footrot type on farm (n = No. of farms)	Type of isolate	No. of isolates	No. *intA* negative	No. *intA* positive
Stable benign	Stable	178	166	12
(Group A)	Equivocal	55	54	1
n = 46	Unstable	47	45	2
Virulent	Stable	154	38	116
(Group B)	Equivocal	47	17	30
n = 51	Unstable	24	8	16
Unstable benign	Stable	0	0	0
(Group C)	Equivocal	0	0	0
n = 27	Unstable	90	75	15

5
6

1 Table 3. Detection of *intA* in DNA of *D. nodosus* isolates from farms with stable

2 benign footrot

Farm Code	No. of isolates	Stable	*intA* -ve	*intA* +ve*	Unstable	*intA* -ve	*intA* +ve	Equiv.	*intA* -ve	*intA* +ve
A1	14	5	5	0	9	9	0	0	0	0
A2	1	1	1	0	0	0	0	0	0	0
A3	11	11	11	0	0	0	0	0	0	0
A4	11	2	2	0	4	4	0	5	5	0
A5	1	1	1	0	0	0	0	0	0	0
A6	7	3	3	0	0	0	0	4	4	0
A7	2	2	2	0	0	0	0	0	0	0
A8	10	6	3	3	0	0	0	4	3	1
A9	7	0	0	0	3	3	0	4	4	0
A10	1	1	1	0	0	0	0	0	0	0
A11	1	1	1	0	0	0	0	0	0	0
A12	1	1	1	0	0	0	0	0	0	0
A13	17	0	0	0	7	7	0	10	10	0
A14	4	4	4	0	0	0	0	0	0	0
A15	13	10	10	0	0	0	0	3	3	0
A16	14	10	10	0	0	0	0	4	4	0
A17	9	2	2	0	3	2	1	4	4	0
A18	3	3	3	0	0	0	0	0	0	0
A19	1	1	1	0	0	0	0	0	0	0
A20	3	3	2	1	0	0	0	0	0	0
A21	17	7	7	0	10	10	0	0	0	0
A22	2	2	2	0	0	0	0	0	0	0
A23	6	3	3	0	3	3	0	0	0	0
A24	4	2	2	0	2	2	0	0	0	0
A25	4	4	4	0	0	0	0	0	0	0
A26	5	3	3	0	0	0	0	2	2	0
A27	1	1	1	0	0	0	0	0	0	0
A28	4	4	4	0	0	0	0	0	0	0
A29	7	0	0	0	6	5	1	1	1	0
A30	3	2	2	0	0	0	0	1	1	0
A31	6	6	6	0	0	0	0	0	0	0
A32	3	2	2	0	0	0	0	1	1	0
A33	14	10	10	0	0	0	0	4	4	0
A34	7	7	7	0	0	0	0	0	0	0
A35	7	0	0	0	0	0	0	7	7	0
A36	3	3	3	0	0	0	0	0	0	0
A37	9	9	9	0	0	0	0	0	0	0
A38	4	4	4	0	0	0	0	0	0	0
A39	5	5	5	0	0	0	0	0	0	0
A40	4	4	4	0	0	0	0	0	0	0
A41	6	6	4	2	0	0	0	0	0	0
A42	7	6	6	0	0	0	0	1	1	0
A43	6	6	0	6	0	0	0	0	0	0
A44	1	1	1	0	0	0	0	0	0	0
A45	7	7	7	0	0	0	0	0	0	0
A46	7	7	7	0	0	0	0	0	0	0
Total - Group A	280	178	166	12	47	45	2	55	54	1

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Table 4. Detection of *intA* in *D. nodosus* isolates from farms with virulent footrot.

Farm Code	No. of isolates	Stable	*intA* -ve	*intA* +ve	Unstable	*intA* -ve	*intA* +ve	Equiv.	*intA* -ve	*intA* +ve
B1	7	7	0	7	0	0	0	0	0	0
B2	4	4	0	4	0	0	0	0	0	0
B3	5	5	0	5	0	0	0	0	0	0
B4	4	4	0	4	0	0	0	0	0	0
B5	6	0	0	0	0	0	0	6	2	4
B6	12	12	2	10	0	0	0	0	0	0
B7	8	8	0	8	0	0	0	0	0	0
B8	2	0	0	0	1	1	0	1	0	1
B9	1	1	0	1	0	0	0	0	0	0
B10	1	1	0	1	0	0	0	0	0	0
B11	1	1	0	1	0	0	0	0	0	0
B12	1	1	0	1	0	0	0	0	0	0
B13	1	1	0	1	0	0	0	0	0	0
B14	1	1	0	1	0	0	0	0	0	0
B15	1	1	0	1	0	0	0	0	0	0
B16	1	1	0	1	0	0	0	0	0	0
B17	1	1	0	1	0	0	0	0	0	0
B18	1	1	0	1	0	0	0	0	0	0
B19	11	1	0	1	2	2	0	8	3	5
B20	6	5	0	5	0	0	0	1	0	1
B21	1	1	0	1	0	0	0	0	0	0
B22	1	1	1	0	0	0	0	0	0	0
B23	1	1	0	1	0	0	0	0	0	0
B24	1	1	0	1	0	0	0	0	0	0
B25	1	1	0	1	0	0	0	0	0	0
B26	10	5	0	5	2	0	2	3	0	3
B27	17	1	0	1	14	3	11	2	1	1
B28	2	2	0	2	0	0	0	0	0	0
B29	6	1	1	0	1	1	0	4	1	3
B30	1	0	0	0	0	0	0	1	0	1
B31	3	3	0	3	0	0	0	0	0	0
B32	6	3	0	3	0	0	0	3	2	1
B33	3	0	0	0	0	0	0	3	0	3
B34	4	1	0	1	0	0	0	3	0	3
B35	2	0	0	0	0	0	0	2	0	2
B36	3	3	3	0	0	0	0	0	0	0
B37	7	5	0	5	0	0	0	2	1	1
B38	10	1	0	1	3	0	3	6	6	0
B39	3	2	1	1	0	0	0	1	0	1
B40	4	2	0	2	1	1	0	1	1	0
B41	6	6	5	1	0	0	0	0	0	0
B42	6	6	0	6	0	0	0	0	0	0
B43	2	2	2	0	0	0	0	0	0	0
B44	2	2	0	2	0	0	0	0	0	0
B45	4	4	0	4	0	0	0	0	0	0
B46	3	3	3	0	0	0	0	0	0	0
B47	14	14	13	1	0	0	0	0	0	0
B48	7	7	2	5	0	0	0	0	0	0
B49	6	6	5	1	0	0	0	0	0	0
B50	5	5	0	5	0	0	0	0	0	0
B51	9	9	0	9	0	0	0	0	0	0
Total - Group B	225	154	38	116	24	8	16	47	17	30

1 **Legends to Figures.**

2

3 Figure 1. Southern blot analysis of genomic DNA from 13 strains of *D. nodosus*.

4 Lanes 1-13 contain genomic DNA from 13 different isolates of *D. nodosus* probed

5 with *intA* (panel A) or *pnpA* (panel B). Strains in lanes 1 (strain A198), 3 and 11 are

6 virulent, while strains in lanes 2, 4-10, 12 and 13 are stable benign. Sizes of

7 molecular weight standards in kb are indicated on the left hand side.

8 Figure 2. PCR analysis of genomic DNA from 13 strains of *D. nodosus*. Primers

9 used were specific for *intA* (panel A) or *pnpA* (panel B). M. wt = molecular weight

10 markers, lanes 1-13 – genomic DNA from the 13 strains of *D. nodosus* analysed in

11 Fig. 1. Strains in lanes 1 (strain A198), 3 and 11 are virulent, while strains in lanes 2,

12 4-10, 12 and 13 are stable benign. The sizes in kb of the molecular weight markers

13 are indicated on the left hand side.

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