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PCR–RFLP of outer membrane proteins gene of *Dichelobacter nodosus*: a new tool in the epidemiology of footrot

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SUMMARY

Currently only phenotypic epidemiological markers, serogrouping and virulence testing of *Dichelobacter nodosus*, are available for investigating footrot outbreaks in small ruminants. These methods have limitations in tracing the source of infection. In this study, a genotypic marker, PCR–RFLP of outer membrane protein gene, was used to characterize *D. nodosus*. The technique was evaluated in a controlled experiment involving two strains of bacteria. PCR–RFLP was found to be highly specific in differentiating isolates obtained from recipient animals infected with different strains. Subsequently, this technique was used to characterize isolates obtained from field cases of footrot in Nepal. A total of 11 patterns was recognized among 66 Nepalese *D. nodosus* isolates representing four different serogroups. PCR–RFLP also discriminated isolates with similar phenotypic characteristics. However, all isolates which, phenotypically, were virulent were represented by only two patterns irrespective of their serogroups. It is suggested that PCR–RFLP described here could be a useful epidemiological marker in the study of footrot.

INTRODUCTION

Footrot is a specific contagious disease of the feet of ruminants which causes lameness [1]. The disease is endemic in most sheep rearing countries with temperate climates. The infection is caused by a mixed bacterial population of which *Dichelobacter nodosus* [2] is the essential transmitting agent. *D. nodosus* is a strict parasite and cannot survive outside the host for more than a few days. Therefore, infected animals are the only source of infection, and the disease can be eradicated if all infected animals are identified and removed from the population [1].

Isolates of *D. nodosus* vary in their virulence which in part determines the severity of infection in a susceptible population. The virulent form of footrot (VFR) causes severe lameness in most affected animals and results in significant economic losses [3]. As a result, VFR is subjected to quarantine and eradication either on a property or on a regional basis in a number of countries in which it is endemic. In Nepal, VFR was introduced some 30 years ago, and has become endemic in parts of three districts [4]. A recent approach to the management of footrot in Nepal has been specific vaccination based on two virulent serogroups of organisms isolated from these flocks [5]. This has resulted in a marked reduction in the prevalence of VFR in these flocks. There have been no isolations of virulent strains of *D. nodosus* since the second year of specific vaccination. An intensive clinical and bacteriological surveillance to confirm the absence of VFR and strains targeted for vaccination is ongoing. For effective surveillance programmes,

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defining the identity of the strains causing the disease and hence the target for eradication becomes essential.

Conventionally, *D. nodosus* has been classified into different serogroups and serotypes on the basis of its fimbrial antigen [6]. Serogrouping and serotyping have been extensively used and have proved invaluable in vaccination studies to test whether isolates cultured from vaccinated animals are homologous with the vaccine strain. Virulence testing of *D. nodosus* based on its proteolytic activity has also been commonly used to correlate the clinical expression of footrot with an *in vitro* test [7, 8]. These phenotypic characteristics have been used to identify strains targeted for eradication [8, 9]. Serogrouping and virulence characteristics, however, are independent of each other. Furthermore, multiple antigenic [6] and virulence [10, 11] classes frequently occur within a flock and even in the same foot of an animal. No single phenotypic test has sufficient stringency for tracing the source of an outbreak in a population.

Several molecular typing methods which are highly sensitive and repeatable have been developed for epidemiological studies of human and animal diseases [12]. Though there is a great potential in using such molecular typing methods in footrot investigations, they are not yet sufficiently developed and tested. In this paper, we have described the use of polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) of the gene encoding outer membrane proteins (*omp1*) of *D. nodosus* in the epidemiology of footrot. This gene has previously been investigated for other strains of the bacterium [13, 14].

MATERIALS AND METHODS

Bacteria

D. nodosus isolates from two sources were used in the present study. The first set of isolates was derived during a challenge experiment described in detail elsewhere [15]. In short, sheep and goats were infected experimentally and separately with two virulent strains of *D. nodosus*, one from a sheep (VCS 1745, serogroup G) and the other (VCS 1030, serogroup F) from a goat. Infected animals from both challenge groups were then transferred to separate paddocks, and run together with healthy sheep and goats. *D. nodosus* was reisolated from recipient animals after transmission occurred. Seven isolates were obtained from recipient animals in each of the groups exposed to infection with VCS 1030 and VCS 1745, respectively. These isolates were used for PCR–RFLP analysis.

The second set of isolates was from cases of footrot in migratory flocks of Nepal [11, 15]. Isolates for PCR–RFLP analysis included 12 virulent isolates of serogroup E, 6 virulent isolates and 11 benign isolates of serogroup B, 33 benign isolates of serogroup C, and 4 benign isolates of serogroup M.

DNA preparation and amplification conditions

DNA from all isolates was extracted by the method of Anderson and colleagues. [16]. Two primers, A (5′ AAT CAA GGA ACT GAA GAA 3′) and C (5′ AATGCC GTA CAT TAA AGC A 3′), designed previously [13] to amplify all four copies of *omp1* gene that encodes major outer membrane proteins of *D. nodosus* isolate VCS 1001 [17] were used. Amplification reactions were performed in 20 μl volume in a capillary tube (Corbett Research, Mortlake, Australia). The reaction mixture contained a final concentration of 20 mM Tris-HCl, 50 mM KCl, 3 mM $MgCl_2$ 0·4 μM of each primers and 200 μM each of four nucleotides, and one unit of Taq polymerase (Gibco–BRL, Gaithersburg, MD, USA) and approx. 100 ng of DNA template. The initial denaturation was done at 94 °C for 2 min which was followed by the amplification cycle of 94 °C for 5 s, 55 °C for 5 s and 72 °C for 30 s for 30 cycles, and final extension at 72 °C for 2 min. Two μl of the products were electrophoresed in 2 % agarose gel for analysis of the product. DNA from *D. nodosus* VCS 1001 was used as a positive control.

Restriction endonuclease analysis of PCR products

In general, PCR products were used directly for endonuclease digestion. However, when there was insufficient DNA, products from two or more amplification reactions were pooled, ethanol precipitated and redissolved in 10 μl of TE or water. PCR products (1–5 μg) were mixed with 3 units of the restriction enzyme *Hpa*II (Gibco–BRL, Gaithersburg, MD, USA) in the appropriate buffer as recommended by the supplier, and incubated overnight at 37 °C. The digested products were electrophoresed in a 4–20 % gradient polyacrylamide minigel (Novex, San Diego,

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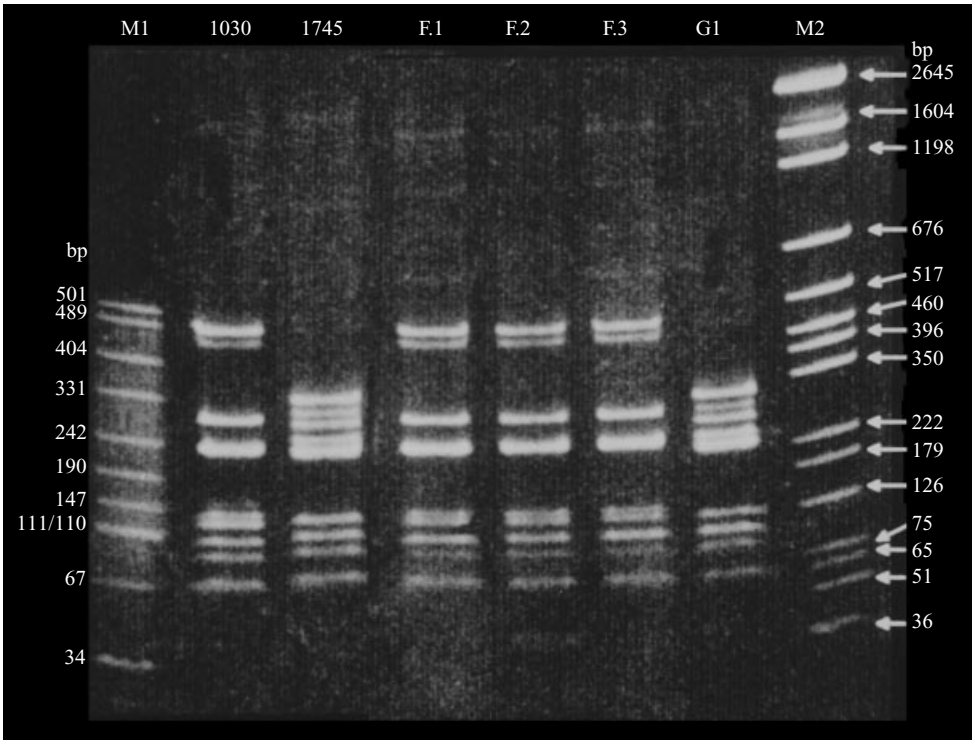

Fig. 1. PCR–RFLP fingerprints of challenge experiment isolates of *D. nodosus*. PCR–RFLP fingerprints from the isolates of serogroup F and G used to induce footrot in experimental animals were compared with those from isolates recovered from affected animals. PCR was used to amplify a region of chromosome internal to the *omp1* gene of each isolate. These products were digested with restriction enzyme *Hpa*II, subjected to electrophoresis in 4–20% gradient polyacrylamide and stained with ethidium bromide. In the figure, lanes marked M1 and M2 represent molecular weight markers pUC19/*Hpa*II and pGEM respectively. 1030 and 1745 are the fingerprints of the two challenge strains. F1, F2 and F3 are the fingerprints of three isolates recovered from infected animals after challenge with 1030. Lane G1 shows the fingerprint of an isolate from an animal challenged with 1745.

CA, USA) in TBE buffer and stained with ethidium bromide. The band sizes were estimated by visual comparison with the standard molecular weight markers. Each distinct fingerprint was assigned a PCR–RFLP pattern number.

RESULTS

PCR–RFLP patterns of isolates from the challenge experiment

With both strains, VCS 1030 and VCS 1745, PCR product(s) of about 525 bp were obtained. These products, when digested with the enzyme *Hpa*II, gave two distinct patterns, designated pattern F and G respectively. Pattern F had 9 bands of approx. 470, 450, 290, 230, 125, 120, 105, 95 and 65 bp. Pattern G also had 9 bands, but produced fragments of approx. 305, 290, 275, 235, 220, 120, 105, 95 and 65 bp (Fig. 1). Because multiple copies of the gene were amplified by the primers used [13], the sum of fragments in base pairs was always greater than the size of the PCR products obtained. The sum of fragment sizes were not indicative of whether 2, 3 or 4 copies of the genes were amplified. This was because a band identified in a fingerprint might have been composed of two fragments obtained from different copies of the genes. The fourth band of approx. 230 bp in pattern F, and the fifth band of 220 bp in pattern G (Fig. 1) are likely to be an example of a band composed of two fragments.

To determine whether the PCR–RFLP pattern of *D. nodosus* strains remained stable during natural transmission of the disease to other animals, 4 isolates recovered from sheep and 3 isolates from goats 1 month after they acquired footrot from donors infected with VCS 1030 were tested. Similarly, another 3 isolates recovered from sheep and 4 isolates from goats infected by VCS 1745 were also tested. All these isolates had PCR–RFLP patterns exactly the same as the strains from which the infection originated (Fig. 1). Thus, PCR–RFLP patterns of the *omp1* genes used

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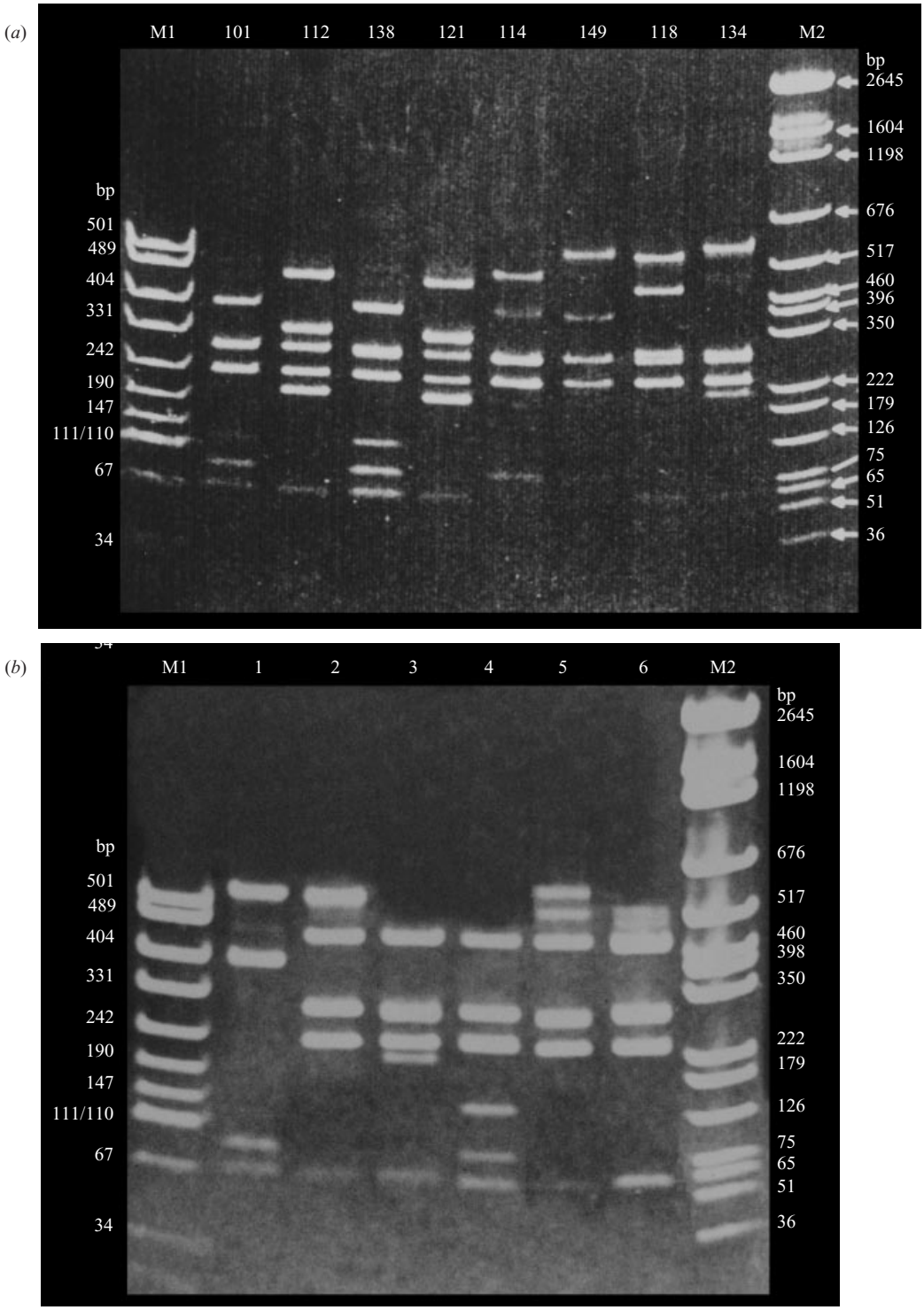

Fig. 2. PCR–RFLP fingerprints of field isolates of *D. nodosus* from Nepal. In both 2(a) and 2(b) M1 and M2 represent molecular weight markers pUC19/HpaII and pGEM respectively. (*a*) PCR–RFLP fingerprint patterns of eight isolates of *D. nodosus* are shown. There are 11 distinguishable patterns. Pattern 1 is illustrated in lanes marked 101 and 133, pattern 2 is shown in lanes marked 112 and 121. Isolates 101 and 112 are virulent and are of serogroup E while 133 and 121 are virulent serogroup B isolates. Lanes marked 114, 149, 113 and 134 illustrate patterns 3, 4, 5 and 6. Isolates representing these patterns were phenotypically serogroup B and were benign, although pattern 6 was also observed in some isolates of serogroup M (not shown). (*b*) Lanes marked 1, 2, 3, 5 and 6 illustrate additional PCR–RFLP patterns 7–11 from field isolates of *D. nodosus* which were from benign serogroup C isolates. Lane 4 has a similar pattern to 101 and 133, i.e. pattern 1 of (*a*) and is also a benign serogroup C isolate.

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in this study remained stable irrespective of their passage through sheep or goats.

PCR–RFLP patterns of field isolates of *D. nodosus*

A total of 66 isolates from field cases of footrot in Nepal, with varying antigenic and virulence characteristics, were examined by PCR–RFLP. PCR product(s) of approx. 525 bp were obtained from all isolates. When digested with *Hpa*II, 11 patterns were obtained (Fig. 2*a*, *b*). Fragments of 290, 230 and 65 bp were common to all isolates except that the former 2 were missing in 1 (pattern 7) and 65 bp band was missing in another pattern (pattern 3). In many cases 2 patterns differed from each other only by an extra band in 1 of them. Often the origin of 2 smaller bands in 1 isolate could be attributed to the digestion of a larger fragment present in another isolate. However, it was not possible to determine this with all isolates.

Serogroup B isolates

Serogroup B isolates were either benign or virulent phenotypically. They were also more diverse antigenically than isolates of serogroups E and C [11]. Six virulent B isolates which were tested, produced 2 PCR–RFLP patterns; 1 isolate produced pattern 1 fingerprints whereas the other 5 isolates were of pattern 2 (Fig. 2*a*). Four other patterns were produced by 11 benign B isolates which were designated patterns 3–6 (Fig. 2*a*). Pattern 3 was produced by 5 isolates, pattern 4 by 4 isolates, and patterns 5 and 6 by 1 isolate each. Pattern 6 was also produced by isolates of serogroup M (see below).

Serogroup C isolates

Thirty three serogroup C isolates which were tested produced 6 distinguishable patterns (patterns 1, 7, 8, 9, 10 and 11 of Fig. 2*b*). Pattern 1 was produced by 2 isolates, pattern 7 by 2 isolates, pattern 8 by 1 isolate, pattern 9 by 9 isolates, pattern 10 by 6 isolates, and pattern 11 by 13 isolates.

Serogroup E isolates

Serogroup E was the predominant antigenic group identified and was associated with most outbreaks of VFR in the Nepalese flocks [11]. All serogroup E isolates were phenotypically virulent. 12 E isolates tested produced two PCR–RFLP patterns which were similar to those obtained with virulent B isolates (Fig. 2*a*). 11 serogroup E isolates produced pattern 1 whereas 1 isolate was of pattern 2.

Serogroup M isolates

Four isolates of serogroup M tested, originating from two different flocks, had the same PCR–RFLP pattern (pattern 6). This pattern was shared by an isolate of serogroup B (Fig. 2*a*).

Relation between the origin and PCR–RFLP patterns of field isolates:

While isolates obtained from the same animal or the same location tended to have similar patterns, this was not always so. Patterns 2 and 11, for example, were produced by isolates obtained from different flocks and at different locations. Some patterns, like 2, 10 and 11, were shared by isolates from both sheep and goats. Additionally, some isolates obtained from the same animal and with similar phenotypic characteristics also produced distinguishable *omp* gene patterns.

DISCUSSION

One of the major aims of epidemiological investigation of disease is the identification of the aetiological agent to a level whereby it can be differentiated from other strains of the same species. This becomes especially important when targeted strains are to be monitored for their absence (or presence) in a population. If this is to be achieved by a molecular typing method like PCR–RFLP, the target gene must have several attributes, including a demonstrated heterogeneity that can produce discriminative fingerprints between isolates, yet be sufficiently conserved to provide uniform fingerprints within a clonal group [12]. The gene encoding outer membrane proteins of *D. nodosus*, isolate VCS 1001, has 4 variant copies linked together [17]. Of these 4 copies, only 1 is expressed at any time. Expression of any of the genes is mediated by gene inversion. It is postulated that the gene inversion in *D. nodosus* helps to overcome immune pressure from the host by switching the antigenic structure of outer membrane protein within a strain [17]. If the gene inversion, rather than mutation within the gene, is the main means of antigenic variation of Omp1, PCR–RFLP of *omp1* could be a useful test in differentiating isolates of *D. nodosus*.

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In the challenge experiment described here, fingerprints obtained from isolates recovered from recipient animals had similar PCR–RFLP patterns to those of isolates used to infect the donors. These patterns remained stable during their passage through susceptible animals by both experimental and natural transmission. Furthermore, these patterns were not affected by their transmission through the unrelated host species (sheep to goats and vice versa). Ovine and bovine isolates from the same property sharing a common fingerprint as reported by Allworth [14] would, therefore, be more likely to be a single isolate rather than their fingerprint similarity being a coincidence. Nepalese sheep and goat isolates also share common fingerprints. This, together with demonstration in another experiment [15] that *D. nodosus* isolates are not host specific, will have implications in the management of footrot. All ruminant species sharing a pasture with an infected animal are potential reservoirs of VFR strains of *D. nodosus*.

Among the 66 field isolates obtained from animals in Nepal, there were 4 antigenic and 2 virulence groups. Eleven PCR–RFLP patterns were recognized. Among these isolates there were 2 distinct patterns among serogroup E isolates, 6 among B isolates, 6 among C isolates and 1 among M isolates. Interestingly, there were only 2 patterns among virulent isolates irrespective of their serogroup. This is, however, not to say that only 2 PCR–RFLP patterns exist among virulent *D. nodosus* populations. Several distinct PCR–RFLP patterns had been recorded among virulent isolates obtained from Australia [14] which were different from the patterns reported here for virulent isolates from Nepal. As VFR in Nepal was introduced with a few imported rams three decades earlier [4], it may be that two virulent isolates were introduced with these rams, and their *omp1* gene has not mutated since then. Alternatively only one virulent isolate could have been introduced and its *omp1* could have gene mutated once within the last 30 years resulting in the two clones represented by patterns 1 and 2.

There could be several explanations for the limited patterns among virulent isolates in contrast to benign isolates, but the following two are more likely. First, pattern 1 and 2 could have been initially represented in separate serogroups (e.g. pattern 1 in serogroup E and pattern 2 in serogroup B isolates or vice versa). A single recombinational exchange of the fimbrial gene, *fimA* (which codes the agglutinating antigen used for serogrouping) or *omp1* gene between these isolates generated the four combinations detected (E isolates with patterns 1 and 2, and B isolates with patterns 1 and 2). While nothing is known about the recombination at or near the *omp1* gene locus, there is evidence [15, 18] that this occurs in or around the *fimA* locus in the *D. nodosus* chromosome. Secondly, patterns 1 and 2 were both represented only in isolates of one serogroup, most likely E, and two separate recombination events transferred the fimbrial gene of B isolates to E isolates having *omp1* RFLP pattern 1 and 2. Though the occurrence of two recombination events has low probability, this seems more likely to have occurred given the dissimilarity in agglutination test of the two virulent B isolates, NEP 133 and NEP 121 [11], the former being pattern 1 and the latter pattern 2. The latter is further supported by the fact that one benign B isolate with fingerprint pattern 3 (NEP 114) had very similar fimbrial gene sequences to that of virulent B isolate, NEP 133, with fingerprint pattern 1, whilst another virulent B isolate, NEP 121, with pattern 2 fingerprint has unrelated fimbrial gene sequences [15]. Evidence of frequent recombination at the fimbrial subunit gene region [15, 18] also strengthens this view. However, further analysis of DNA which could support these hypotheses has not been done.

Several patterns detected among benign isolates indicate that they could have been prevalent in these flocks for several years in Nepal. This also supports the hypothesis that benign isolates were present in Nepal before the introduction of VFR but were undetected due to the mild nature of the disease they produced in sheep and goats. This, however, remains speculative until the occurrence of benign isolates elsewhere in Nepal is proven. Meanwhile, although the origin of virulent isolates in Nepal remains unproven, an additional molecular typing tool is now available by which future isolates can be characterized.

PCR–RFLP described in this paper was easy to use and, at least in the challenge experiment described, was able to discriminate different isolates. However, while the test was able to differentiate the strains when compared with a known standard (e.g. challenge strains in pen experiments), it could not provide sufficient information alone when required to make decisions for disease management. Despite this, valuable information about an isolate could be obtained when the test is used in conjunction with other conventional typing methods. In addition, there are certain limitations associated with the PCR–RFLP

described here. Because the primers used amplify multiple copies of the gene, and some bands in the fingerprints could be composed of more than one fragment derived from different genes, simple addition of the fragment sizes cannot be used to cross check whether the bands derived are authentic or an artefact of incomplete digestion. This, however, was considered not to be a problem in this study, because the digestion of PCR products of isolate VCS 1001 under similar conditions produced a banding pattern similar to that expected from its sequence information (data not presented). Because characterization by PCR–RFLP could only be done with a single isolate in pure culture, this test will not alleviate the problem of tedious culturing associated with conventional typing methods. As with the conventional tests, this test also requires extensive sampling and examination of multiple isolates from each animal/flock to identify the range of isolates existing within the population. Furthermore, in its present form, its usefulness for tracing the source of an outbreak is considered to be limited [14]. Despite these shortcomings, this will certainly be an additional potential tool for characterizing strains of *D. nodosus*. Its applicability as an epidemiological marker however, requires further evaluation in a large number of isolates derived from both epidemiologically related and unrelated outbreaks.

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