IS900 Restriction Fragment Length Polymorphism (RFLP) Analysis of Mycobacterium avium subsp. paratuberculosis Isolates from Goats and Cattle in Norway

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Introduction
Paratuberculosis is a chronic intestinal inflammation in ruminants caused by Mycobacterium avium subsp. paratuberculosis (M. a. paratuberculosis). The infection is widely distributed in domestic cattle, sheep and goats, and the prevalence varies in different parts of the world (Kennedy & Benedictus 2001). In Norway, paratuberculosis has been quite common in goats, whereas cattle have been almost free of the infection. From 1966 to 2000, M. a. paratuberculosis was isolated in 898 goats from 186 different herds. During the same period, M. a. paratuberculosis was isolated only in 20 cattle on 12 different farms (Djønne et al. 2001). The different prevalence of the infection in goats and cattle has led to speculations about the existence of M. a. paratuberculosis strains that are non-pathogenic for cattle. Saxegaard (1990) carried out an experimental infection where M. a. paratuberculosis isolated from Norwegian goats was administered to cattle. Based on the results of this trial, it was concluded that paratuberculosis in goats in Norway is caused by an apparently specific goat-pathogenic strain of the bacterium. Variation in virulence between different iso-
lates of a bacterial species can be caused by genetic variation that is detected by phenotypic or genotypic characterisation. *M. a. paratuberculosis* strains from Norwegian goats do not differ phenotypically from strains isolated from cattle in Norway or other parts of the world (Gunnarson & Fodstad 1979). There is little information available on the genotypic variation of *M. a. paratuberculosis* isolated from animals in Norway. Collins et al. (1990) performed genotypic examinations of three *M. a. paratuberculosis* isolates originating from Norwegian goats. Two of these showed marked differences from the 48 other strains from sheep, goat and cattle examined, in that they lacked a repetitive *M. a. paratuberculosis* sequence and also showed a very different restriction fragment pattern compared with the other strains (Collins et al. 1990). Thus, the authors suggested that these Norwegian strains might be uniquely adapted to goats. In other studies, however, only minor genotypic differences between Norwegian goat isolates and strains isolated from cattle in other parts of Europe have been found (Thoresen & Olsaker 1994, Pavlik et al. 1999). Molecular typing has shown that, in comparison with other pathogens, there is relatively little genetic variability in *M. a. paratuberculosis* (Stevensen et al. 2002). Therefore, the potential of many different methods, such as IS900 restriction fragment length polymorphism (RFLP) (Whipple et al. 1990, Collins et al. 1990, Pavlik et al. 1995), pulsed-field gel electrophoresis (PFGE) (Feizabadi et al. 1997, Stevenson et al. 2002), random amplified polymorphic DNA patterns (Scheibl & Gerlach 1997, Pillai et al. 2001), and multiplex PCR typing (Bull et al. 2000) have been investigated. RFLP has been found to be one of the best methods to differentiate between *M. a. paratuberculosis* isolates, and many different RFLP patterns have been found (Whipple et al. 1990, Collins et al. 1990, Pavlik et al. 1995, Moreira et al. 1999, Pavlik et al. 2000, Cousins et al. 2000, Whittington et al. 2000). In 1999, Pavlik et al. (1999) standardised the RFLP typing and nomenclature of the RFLP types, enabling a comparison of isolates from different parts of the world.

The aim of the present study was to investigate the genotypic variation among *M. a. paratuberculosis* isolates from goats and cattle in Norway, by use of IS900 RFLP analysis.

**Materials and methods**

*M. a. paratuberculosis* strains

Fifty-one *M. a. paratuberculosis* strains from goats and four from cattle were examined; they originated from 51 goat and four cattle herds, and were collected during the period 1983-2000. The goatherds were distributed in Western Norway. Three cattle herds located in Eastern Norway had imported animals from Denmark and Finland in 1993, and the fourth cattle herd, located in Western Norway, had previously had goats with paratuberculosis. From most of these herds, *M. a. paratuberculosis* was isolated more than once, but in this study, only the last detected isolate from each herd was included. From the combined cattle and goat herd, one isolate from cattle and one isolate from goat were included. The strains were isolated from either clinically ill animals or animals in the subclinical stages of paratuberculosis.

*M. a. paratuberculosis* isolation and identification

The *M. a. paratuberculosis* strains examined were either fresh or low passage number isolates. They had primarily been isolated after cultivation on selective and non-selective Dubos medium with mycobactin (2 µg/ml) and pyruvate (4 mg/ml) as described by Saxegaard (1985). At the time of isolation, the isolates were identified by colony morphology, degree of acid-fast staining with the Ziehl-Neelsen
method and mycobactin dependency. All isolates were nonpigmented and were stored as glycerol stocks at –70 °C. Before further examination, the isolates were confirmed as *M. a. paratuberculosis* by detection of the insertion segment IS900 by PCR (Sigurðardóttir et al. 1999).

**IS900 restriction fragment length polymorphism analysis (RFLP)**

RFLP analysis was performed as described by Pavlik et al. (1999). Briefly, DNA was extracted from the isolates with lysozyme, sodium dodecyl sulfate and proteinase K, purified by chloroform isoamylalcohol extraction and precipitated with isopropylalcohol. The DNA was digested by restriction endonucleases *Pst*I and *Bst*EII and hybridised with a standard PCR generated IS900 probe. The DNA fingerprints were analysed and the types were designated as described by Pavlik et al. (1999). The fingerprints were scanned by a CCD camera (UltraLum KS4000, USA), and analysed by Gel Compare software (Applied Maths, Kortrijk, Belgium).

**Results**

Three different profiles were detected when using restriction endonuclease *Pst*I (B, N and O) whereas five profiles were found with *Bst*EII (C1, C5, C20, C24 and C26) (Figure 1). The combination of typing with *Pst*I and *Bst*EII was able to differentiate seven RFLP types (Table 1). All the cattle isolates and 43 goat isolates were type B-C1. Other identified RFLP types were B-C5 (n=2), B-C24 (n=1), B-C26 (n=1), N-C20 (n=2), O-C5 (n=1) and O-C24 (n=1).

**Discussion**

In the present study, all *M. a. paratuberculosis* isolates from cattle and 84% of the isolates from goats in Norway were of the B-C1 RFLP type. Other identified RFLP types were B-C5 (n=2), B-C24 (n=1), B-C26 (n=1), N-C20 (n=2), O-C5 (n=1) and O-C24 (n=1).

**Table 1. IS900 restriction fragment length polymorphism patterns detected in Norwegian isolates of *Mycobacterium avium* subsp. *paratuberculosis***

| RFLP pattern | *Pst*I | *Bst*EII | No. of strains | Isolated from |
|--------------|--------|----------|----------------|---------------|
| B C1         | 4      |          |                | Cattle        |
| B C1         |        | 43       |                | Goat          |
| B C5         |        | 2        |                | Goat          |
| B C24        | 1      |          |                | Goat          |
| B C26        | 1      |          |                | Goat          |
| N C20        |        | 2        |                | Goat          |
| O C5         | 1      |          |                | Goat          |
| O C24        | 1      |          |                | Goat          |

Figure 1. IS900 restriction fragment length polymorphism patterns detected with the enzymes *Bst*EII and *Pst*I in *Mycobacterium avium* subsp. *paratuberculosis* isolates from Norwegian cattle and goats.
pattern. Four cattle isolates are very few, but B-C1 is also the most common RFLP pattern detected in cattle in Europe and the United States (Whipple et al. 1990, Pavlik et al. 2000). The B-C1 type was distributed throughout the area where paratuberculosis in goats is common. Five RFLP types found in the present study have not yet been described; these types were O-C24, O-C5, B-C26, N-C20 and B-C24. However, only minor differences in the RFLP patterns were found for these types, and the difference was usually the absence or gain of one band. Except for B-C5 and N-C20, only one isolate of each RFLP type was found. The two N-C20 isolates were from the same district, while the two B-C5 isolates were from two different counties.

RFLP analysis with other restriction enzymes might have enabled a better differentiation of the Norwegian isolates. Cousins et al. (2000) examined Australian M. a. paratuberculosis isolates with four different restriction endonucleases, and some additional information was gathered by using the restriction endonucleases BamHI and PvuII in combination with BspEII and PstI. Thoresen & Olsaker (1994) used RFLP with restriction endonuclease PvuII to analyse 16 isolates from Norwegian goats and five isolates from Danish cattle. All but one goat isolate had the same RFLP pattern. These results are in accordance with the results reported in the present investigation, where the majority of the isolates were identical to the most common isolates from European cattle, although some variation between the different isolates was found. Collins et al. (1990) examined two Norwegian isolates that differed in many aspects from the majority of other M. a. paratuberculosis isolates. These strains were so different that it was concluded they might belong to another species.

Other typing methods might have enabled a better differentiation of the isolates. Pulsed-field gel electrophoresis (Feizabadi et al. 1997, Stevenson et al. 2002) has been used to differentiate between isolates of M. a. paratuberculosis. Stevenson et al. (2002) found that multiplex PFGE gave additional information to RFLP, and they concluded that combining both techniques might improve the discrimination of M. a. paratuberculosis isolates.

Our investigation did not detect any genotypic variation between the isolates from cattle and the majority of the Norwegian goat isolates. This lack of genetic variation may indicate that the most common strain of M. a. paratuberculosis in Norway is able to infect both cattle and goats. This finding is in accordance with observations from other countries, where M. a. paratuberculosis isolates from one animal species are known to infect others. In the Netherlands, sheep grazing on the same pastures as cattle infected by M. a. paratuberculosis were also found to be infected (Muskens et al. 2001). In Iceland and the Czech Republic, infection from sheep to cattle has been reported (Pavlik et al. 1995, Fridriksdottir et al. 2000, Whittington et al. 2001), and in the Czech Republic transmission of M. a. paratuberculosis from pastured cattle to free living wild ruminants has been documented (Pavlik et al. 2000). Our observations do not exclude that these M. a. paratuberculosis isolates have different pathogenicity for cattle and goats, as RFLP might not detect the genetic background for this difference. However, there are factors other than strain specificities that should be considered when evaluating the pathogenicity of M. a. paratuberculosis for cattle and goats. These factors include management conditions and breed resistance. The management conditions are quite different for cattle and goats in Norway; small cattle units, early separation of calves from their mothers, and a low average age of the cows (Holstad et al. 2005). All of
these management factors have been shown to reduce the spread of infection in a herd (Johnson-Ifeearulundu & Kaneene 1998, Obasanjo et al. 1997, Rossiter & Burhans 1996). The goat kids, however, are often born in pens where several goats are housed, and they might suckle several dams. Therefore, the risk of being exposed to faecal material from a bacterial shedder are higher in goats than in cattle.

Paratuberculosis was considered to be a clinical problem in the Norwegian cattle population during the first part of the 20th century. At that time, different local cattle breeds made up the cattle population in Norway. After 1970, the majority of the population was drawn from the Norwegian red cattle breed, which is a hybrid of many different breeds, and speculations that the Norwegian red cattle breed is more resistant to clinical infection with \textit{M. a. paratuberculosis} than the local breeds have been put forward (Holstad et al. 2005).

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