Characterization of United Kingdom Isolates of 
*Corynebacterium pseudotuberculosis* Using 
Pulsed-Field Gel Electrophoresis

KATHLEEN M.CONNOR,* MALCOLM M. QUIRIE, GRAHAM BAIRD, 
AND WILLIAM DONACHIE

Moredun Research Institute, International Research Centre, Pentlands Science Park, 
Bush Loan, Penicuik, Midlothian EH26-OPZ, Scotland

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Caseous lymphadenitis is a chronic suppurative disease caused by *Corynebacterium pseudotuberculosis* and is responsible for serious economic losses to the sheep and goat industry. Caseous lymphadenitis was first reported for goats in the United Kingdom in 1990 and for sheep in 1991. Recent evidence suggests that the prevalence of the disease within the national flock is increasing. Fifty isolates of *C. pseudotuberculosis* from the United Kingdom comprising sheep and horse isolates, the original goat outbreak strain, and the type strain were characterized by biotyping, antimicrobial susceptibility, production of phospholipase D, and genotyping by pulsed-field gel electrophoresis using *Sfi*I and *Sma*I. All of the isolates were confirmed as *C. pseudotuberculosis*, and all produced phospholipase D but none reduced nitrates. Restriction with *Sfi*I generated 16 to 18 bands between 48.5 and 290 kb and differentiated six pulsotypes. We conclude that 80% of the strains tested were epidemiologically related to the outbreak strain and that the equine profile was distinct both phenotypically and genotypically.

*Corynebacterium pseudotuberculosis* is the causative organism of caseous lymphadenitis (CLA) and has been isolated from sheep, goats, cattle, horses, and more rarely man (1, 5, 8, 22, 29, 34). CLA is prevalent worldwide but incidence is higher in areas where intensive husbandry is practiced (5, 16; M. Edelsten, Letter, *Vet. Rec.* 141:631, 1997). Sheep and goat industries worldwide suffer significant economic loss due to the culling of infected animals, carcass condemnation, and decreased wool production (6, 23). As European border controls have become less stringent and livestock are moved more freely between nations, countries previously free from CLA have reported outbreaks. The first outbreak in The Netherlands was in 1984 and was linked to goats imported from France (27). This outbreak could not be contained, and CLA subsequently became established in the dairy goat industry (9). CLA was first reported in the United Kingdom in 1990 (12, 15, 18) in goats that had been in contact with Boer goats imported from Germany (19). Despite stringent restriction orders on the movement of livestock (K. C. Meldrum, Letter, *Vet. Rec.*, 126:369, 1990) and the tracing of in-contact animals (R. A. Laven, J. C. Fishwick, G. C. Pritchard, and P. G. G. Jackson, Letter, *Vet. Rec.* 141:479, 1997), the first occurrence of CLA in sheep was reported in 1991 (26). Since then, cases have been identified in sheep flocks in England (17; B. E. Preece, Letter, *Vet. Rec.* 141:527, 1997; S. Rizvi, L. E. Green, and M. J. Glover, Letter, *Vet. Rec.* 140:586, 1997), and Scotland (G. Baird, Letter, *Vet. Rec.* 140:611, 1997), and more recently in Northern Ireland (G. Baird, unpublished observations). United Kingdom national statistics indicate that twice as many new outbreaks of CLA occurred in 1998 as in 1997 (Veterinary Laboratories Agency, MAFF, p. 27, 1998). Reports of CLA outbreaks in United Kingdom flocks reveal a pattern of lesion distribution around the head and neck that had previously been reported as uncommon in sheep (Rizvi et al., *Vet. Rec.*). Extensive variability in the cultural and biochemical characteristics of *C. pseudotuberculosis* has been reported previously (21, 29, 30), and genotypic variation has been investigated by restriction endonuclease analysis and restriction fragment length polymorphism. Characterization studies using restriction endonuclease analysis have been hampered by the overabundance of banding patterns (31) and the poor discrimination between isolates from different animal species and geographical locations (16, 29, 30, 31). Pulsed-field gel electrophoresis (PFGE) is a powerful epidemiological tool that produces unique, discernible genomic fingerprints and has been applied to other veterinary pathogens and gram-positive organisms, such as *Listeria ivanovii* and *Listeria monocytogenes* (24). Correia et al. (7) published the first report on PFGE analysis of *Corynebacterium* (*Corynebacterium glutamicum*), and other authors have explored PFGE analysis of *Corynebacterium diphtheriae* using a variety of restriction enzymes (10, 25). The aims of this study were to characterize United Kingdom isolates of *C. pseudotuberculosis* on the basis of their biochemical, antimicrobial resistance, and PFGE patterns and to establish whether there was genetic diversity or a clonal arrangement of *C. pseudotuberculosis* within them. This paper describes the first characterization of isolates from the United Kingdom and, to the authors’ knowledge, the first report of *C. pseudotuberculosis* genotyping by PFGE.

**MATERIALS AND METHODS**

**Bacterial strains.** The isolates used in this study are described in Table 1. Forty-six field isolates of *C. pseudotuberculosis* were collected at random from the Borders Region, Scotland, by the Scottish Agricultural College, Veterinary Services Division, St. Boswells, over a period of 12 months. Isolates were identified by standard microbiological techniques (3) and sent to Moredun Research Institute for confirmation and typing. Three caprine isolates from the original United Kingdom outbreak were kindly supplied by Jean Shreeve, Central Veterinary Laboratory (CVL), Surrey, United Kingdom. They were isolated from goats on three separate premises in Buckinghamshire, Oxfordshire, and Cornwall, England, within a 2-week period in April 1990. The following National
TABLE 1. NCTC bacterial strains and field isolates of *C. pseudotuberculosis* used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate no.</th>
<th>Total no. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovine field isolates*</td>
<td>1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45</td>
<td>44</td>
</tr>
<tr>
<td>Caprine field isolates†</td>
<td>46, 47, 48</td>
<td>3</td>
</tr>
<tr>
<td>Equine field isolates‡</td>
<td>4, 49</td>
<td>2</td>
</tr>
<tr>
<td><em>C. pseudotuberculosis</em> NCTC 3450d (type strain)</td>
<td>45</td>
<td>1</td>
</tr>
<tr>
<td><em>C. jeikeium</em> NCTC 11913e (type strain)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>C. kutscheri</em> NCTC 11138f (type strain)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>C. ulcerans</em> NCTC 12077g</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Staphylococcus aureus NCTC 7428h</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Streptococcus agalactiae NCTC 818i</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*a* Isolates were obtained from Scottish Agricultural College, Veterinary Services Division, Greycrook, St. Boswells, Borders, Scotland.

*b* Isolate was obtained in Ireland.

*c* Isolates were obtained from CVL, New Haw, Addlestone, Surrey, United Kingdom.

*d* Strains were obtained from NCTC, PHLS, Central Public Health Laboratory, London, United Kingdom.

Collection of Type Cultures (NCTC) strains were used as comparators through out the biochemical characterization: *Corynebacterium jeikeium*, *Corynebacterium kutscheri*, *Corynebacterium ulcerans*, and *C. pseudotuberculosis*. *Streptococcus agalactiae* and *Staphylococcus aureus* strains were used for the CAMP inhibition test. Cultures were preserved by inoculating a Microbank vial (Pro-lab Diagnostics, Neston, Cheshire, England) according to the manufacturer’s instructions and were stored at −70°C. Isolates were passaged no more than six times from initial isolation to testing by PFGE.

### PLD production

Phospholipase D (PLD) was detected by the CAMP inhibition test as described by Barksdale et al. (2). Antimicrobial susceptibility. Isolates were tested for antimicrobial susceptibility using ATB-VE (bioMérieux), a kit intended for use with veterinary isolates. The antimicrobials used and the breakpoint concentrations were as follows: amoxicillin, 4 μg/ml; amoxicillin-clavulanic acid, 4 and 2 μg/ml, respectively; apramycin, 16 μg/ml; cefoperazone, 4 μg/ml; cephalothin, 8 μg/ml; chloramphenicol, 8 μg/ml; colistin, 1 μg/ml; flumequine, 4 μg/ml; gentamicin, 4 μg/ml; kanamycin, 8 μg/ml; lincomycin, 2 μg/ml; metronidazole, 4 μg/ml; nitrofurantoin, 25 μg/ml; oxacillin, 2 μg/ml; oxolinic acid, 2 μg/ml; penicillin, 0.25 μg/ml; pristinamycin, 2 μg/ml; rifampin, 4 μg/ml; spectinomycin, 64 μg/ml; streptomycin, 8 μg/ml; sulfamethoxydiazine, 100 μg/ml; tetracycline, 4 μg/ml; trimethoprim, 1 μg/ml; vancomycin, 1 μg/ml; nalidixic acid, 8 μg/ml. The tests were performed according to the manufacturer’s instructions with the exception of incubation time, which was extended from 24 to 48 h.

### RESULTS

#### Biotyping

*C. pseudotuberculosis* colonies were white, regular, and α-hemolytic, and there was a tendency for the entire colony to move when scraped. Approximately colony diameters after a 48-h incubation were 0.5 mm with the exception of isolates 4 and 49 (both 1.5 mm) 2 (0.2 mm), and 38 (0.3 mm). Growth on blood agar produced a mouse-like odor. All isolates were catalase positive. Of the ovine isolates, 43 of 45 had an identical API profile (0101324) and were confirmed as *C. pseudotuberculosis* (percent identity, 99.6). Ovine isolate 19 was identified as *C. jeikeium* due to its inability to hydrolyze urea and was removed from the study. The API yielded unexpected results in that caprine and ovine isolates were expected to be nitrate negative and equine isolates were expected to be nitrate positive (4). The equine isolates were nitrate negative, and repeat testing using the nitrate broth method yielded a negative result both times for all isolates. The equine isolates differed from the ovine and caprine isolates by producing alpha glucosidase and differed from each other in their production of alkaline phosphatase.

#### Antimicrobial susceptibility

Variation in susceptibility was observed with five antibiotics (Table 2): streptomycin, kana mycin, gentamicin, sulfamethazole, and furazolidone. In addition, all isolates were susceptible to penicillin G, amoxicillin, co-amoxiclav, oxacillin, cephalexin, cefoperazone, spectinomycin, apramycin, chloramphenicol, tetracycline, doxycycline, erythromycin, lincomycin, pristinamycin, tylodin, co-trimox azole, fluoroquinolone, enrofloxacin, fusidic acid, and rifampin.

### PLD production

All *C. pseudotuberculosis* isolates produced PLD.

#### PFGE

Of the 50 isolates tested, six pulsortypes were identified (Fig. 1; Table 2). Band size ranged from 48.5 to 240 kb, and the average number of bands within this range was 17. Three rare-cutting restriction enzymes were tested: *Sfi I*, *Apa I*, and *Spe I*. *Sfi I* proved to be the optimal choice under these running conditions. The reproducibility of the PFGE method was confirmed by repeated testing of a panel of five isolates.
Fig. 1, but in previous gels was confirmed to be pulsotype P3 single ovine isolates. Two of the caprine isolates typed as P2 ovine isolates. Pulsotypes P3, P5, and P6 were represented by both the equine isolates. Pulsotype P2 comprised 43 of 46 Dice coefficient. Pulsotype 1 was a unique profile produced by the Image Master Database 3D Elite. Figure 2 illustrates the sotypes were first identified by eye and were confirmed using patterns before and after 20 passages were identical. Pul-

vitro stability of the pulsotypes was confirmed in that the band-

eight different gels, yielded identical banding patterns. The in

Inserts prepared from the same batch of plugs, when run on eight different gels, yielded identical banding patterns. The in vitro stability of the pulsotypes was confirmed in that the banding patterns before and after 20 passages were identical. Pul-

sotypes were first identified by eye and were confirmed using the Image Master Database 3D Elite. Figure 2 illustrates the computer-generated dendrogram of best-fit analysis using the Dice coefficient. Pulsotype 1 was a unique profile produced by both the equine isolates. Pulsotype P2 comprised 43 of 46 ovine isolates. Pulsotypes P3, P5, and P6 were represented by single ovine isolates. Two of the caprine isolates typed as P2 and one as P4. Isolate 33 did not produce a clear fingerprint in Fig. 1, but in previous gels was confirmed to be pulsotype P3 (our unpublished observations). The PFGE profiles obtained clearly distinguished between the horse isolates and the goat and sheep isolates.

**DISCUSSION**

Previous characterization studies of *C. pseudotuberculosis* have been hampered by the wide variation in the biochemical characteristics of the organism (5, 6, 29). Some of these variations may be attributed to the different identification methods and techniques, but there may also be individual strain to strain variation.

This study showed that on the basis of biochemical and molecular characterization, the United Kingdom isolates of *C. pseudotuberculosis* are clonally arranged. Our results confirm the findings of others in that the ovine and caprine isolates belong to biovar *ovis* (4, 21) and support the findings in regard to the nitrate reduction variability of the equine isolates (4).

Of the restriction enzymes tested, SfiI proved superior in both the range of band sizes generated and the fragment res-

olution. The six pulsotypes were differentiated by the presence or absence of one or more bands at various positions. These differences can be caused by a point mutation or by an inser-

tion or deletion, resulting in three or two fragment differences, respectively (32). Kodjo et al. (14) demonstrated that such variations in banding patterns of *Pseudomonas aeruginosa* and *Yersinia pestis* could occur after in vitro passage or after pas-

sage through different hosts. However, after 20 consecutive passages, no such variation was evident in our *C. pseudotuber-

closis* isolates. Since there are no universally agreed criteria for designating pulsotypes, we have used Tenover’s guidelines as a working hypothesis (32). Tenover et al. designated isolates of the same pulsotype indistinguishable. We postulate that the modal pulsotype 2 is indistinguishable from the original out-

break strain and that the isolates differing by a single genetic event are clonally related. The equine profile, by contrast, differs by at least three genetic events and is deemed to be genetically different.

Two studies on CLA in sheep and goats are of particular relevance to this report. In Australia, Sutherland et al. (30) compared isolates from sheep and goats with severe visceral disease imported from North America with isolates from indigenous merino sheep with typical superficial lesions. The isolates were identical both biochemically and genetically. This was an unexpected result, as the animals were from different parts of the world and presented different clinical pictures. Studies in Europe and Japan demonstrated a high degree of similarity among ovine and caprine isolates of *C. pseudotuber-

closis* (16).

Peel et al. (22) reviewed 12 previously published human cases and reported on 10 new ones and suggested that due to clinical or laboratory misdiagnosis, infection might be more common than published accounts indicate. There would appear to be a strong zoonotic implication, as all but two of the cases were thought to be linked to occupational exposure.

The results of the antimicrobial susceptibility testing are largely in agreement with others (16, 21, 28), in that isolates were susceptible to the majority of the antimicrobial agents tested and most were resistant to streptomycin.

Having shown the applicability of PFGE in the character-

ization of *C. pseudotuberculosis*, future studies will include a wider range of isolates in order to extend our knowledge of the epidemiology of CLA in the United Kingdom.

In summary, 49 of 50 United Kingdom isolates were con-

firmed as *C. pseudotuberculosis* on the basis of their biochemical characterization. Six pulsotypes were identified by PFGE,
which clearly distinguished the equine isolates from the caprine and ovine isolates. The results suggest that there is a clonal arrangement of *Corynebacterium pseudotuberculosis* in the United Kingdom, and given that the three caprine isolates were from the original outbreak, it is probable that the source of infection was the imported Boer goats.

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REFERENCES


