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Case clusters of leproid granulomas in foxhounds in New Zealand and Australia

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Background – Canine leproid granuloma (CLG) characteristically presents as single to multiple circumscribed dermal to subcutaneous nodules in haired skin. An unidentified mycobacterium is considered be the aetiological agent of this entity.

Animals – Several cases of canine leproid granulomas occurred in dogs in New Zealand during 2010 and 2011. Cases appeared in clusters, affecting multiple closely related foxhounds domiciled in the same kennels. All affected hounds recovered after topical and/or systemic antimicrobial therapy. Two similar outbreaks that occurred in foxhounds near Melbourne, Australia are also reported.

Methods – Cases were investigated using cytological, histological, microbiological and several molecular techniques. An environmental epidemiological study was also performed.

Results – A diagnosis of CLG was established in 11 dogs. Molecular identification of the causative agent confirmed that it was a mycobacterial species with 100% sequence homology within the amplified regions of the 16S rRNA gene and internal transcribed spacer (ITS1) with that found in association with similar infections from the USA, Brazil and Australia.

Conclusion and clinical importance – This report details the first occurrence of multiple cases of CLG occurring in in-contact dogs and the first proven case of CLG in dogs in New Zealand.

Introduction

Canine leproid granuloma (CLG) characteristically presents as single to multiple circumscribed dermal to subcutaneous nodules in haired skin.[1–5] The head and dorsolateral pinnae are commonly, but not invariably, affected.[1,5] Affected dogs appear systemically healthy and usually show no clinical evidence of lymphadenomegaly or internal organ involvement. Lesions generally resolve spontaneously over weeks to months, without recourse to antimicrobial treatment.[3] The infection can, however, progress to produce disfiguring lesions that persist indefinitely.[4] Leproid granulomas are pyogranulomas in the subcutis and dermis that contain variable numbers of acid-fast bacilli (AFB), which do not grow on synthetic mycobacterial media using standard microbiological methods.[1,2] This prevents full chemotaxonomic analyses, susceptibility testing and experimental reproduction of the disease.

Recent publications using molecular techniques have shown genetic uniformity of the mycobacteria found in leproid granulomas from dogs in several states of USA, Brazil and Australia.[2,5,6] In contrast, several different mycobacterial species (which are characteristically difficult or impossible to culture *in vitro*) have been identified using molecular methods from mycobacterial granulomas in cats (the so-called feline leprosy syndromes),[7–9] and similar lesions can be seen in infections with the tuberculous species *Mycobacterium bovis* and *Mycobacterium microti* in cats.[10]

There have been several case reports of CLG, so the classical clinical presentations of ulcerated nodules on the head and pinnae are well known. As these areas are exposed to insect bite, including fly strike, previous investigators have speculated that biting insects acting as mechanical vectors are part of the disease pathogenesis.[1,11] However, precise mode(s) of transmission and environmental reservoir(s) of the organism remain unknown. There are similarities between CLG and skin infection ('Buruli ulcer') due to *Mycobacterium ulcerans*,

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which affects humans and animals in well-defined focal clusters in Australia.[12–14] *Mycobacterium ulcerans* DNA was detected by real-time PCR in a variety of plant, soil and water samples, including possum faeces from the endemic areas, compared with zero or very low levels detected in nonendemic areas.[12] Based on the methodology used in *M. ulcerans* testing, we investigated the environment in which the dogs lived and collected some ancillary samples (insect and faecal) for PCR testing.

Here, we present confirmation of CLG in New Zealand (NZ) dogs, occurring as case clusters affecting multiple foxhounds domiciled in the same kennels. A detailed description of the environmental factors surrounding the outbreaks is included. We also report two similar disease clusters that occurred 20 years earlier, in foxhounds near Melbourne, Australia.

Case report

Kennel environment

The hunt club to which the NZ hounds belonged is located in the middle of the north island of New Zealand, at latitude 37.5°S and longitude 175°E. The kennels are located on a low-lying ridge, with grassy paddocks, separated by prickly hedges and bordered by farms and lifestyle blocks. During the hunt season, which extends from March until late July, the hounds leave the property twice a week to hunt. The environment in which the pack is confined is shown in Figure 1. Forty English foxhounds are housed, randomly mixed, across seven 20 m × 40 m outdoor runs fenced with 8 cm wire mesh. The ground surface is a mixture of soil, grass and gravel. Weaned pups are kept separately, in one of the adjacent runs, until 8 months of age; thereafter, they are introduced to the pack to hunt the following season. Gravid and newly whelped bitches and suckling pups are kept in a different area. There is some interrelatedness between hounds within the pack. Strikingly, all affected hounds had the same sire or were from one entire litter that was one generation removed from him.

Figure 1. New Zealand hunt club showing the conditions in which the hounds were kept.

Clinical cases

In August 2010, at the end of the hunting season, an entire 3-year-old male hound (case 1) developed multinodular skin disease. There was no improvement after treatment with amoxicillin trihydrate (750 mg subcutaneously once daily for 5 days; Betamox LA®; Norbrook Laboratories Ltd, Newry, Northern Ireland), prescribed and administered by the huntsman. Six weeks later, (September 2010) an entire 2-year-old male hound (case 2) developed similar lesions. At this stage, the hunt club sought veterinary advice from one of the authors (R.W.).

The initial lesions were described as nodules, palpable within the dermis. Case 1 had lesions on the head, the lateral aspect of the thorax and all limbs. Lesions were notably absent from the pectoral region, the sparsely haired skin of the caudoventral abdomen and medial legs. The proximal, dorsolateral pinnae were affected by 4 cm ulcerated plaques. The ear folds were not affected. Several smaller nodules were present on the dorsal head and on the lateral muzzle. Nodules on the forelimbs were intact and extended from the shoulder to the metacarpus. They tended to be smaller distally. Hindlimb lesions, distributed from the thigh to the metatarsus, varied in size, with some up to 4 cm in diameter. These also showed reduction in size, distally (Figure 2a). Some had a central ulcer with thickened, hyperpigmented peripheral skin. The lesions did not appear to be painful. Although the second hound had a similar distribution of lesions, they were far fewer, and those on the ears were restricted to ulcerated nodules of 2 cm diameter. Both hounds were in good condition and showed normal demeanour and activity. They had neither enlarged peripheral lymph nodes nor pyrexia.

Fresh and formalin-fixed intact nodules from case 1 and aspirate smears of a nodule from case 2 were submitted to the diagnostic laboratory. The tissue specimen was routinely processed within 24 h of fixation in 10% neutral buffered formalin, embedded in paraffin wax and sectioned (4–6 μm). Deparaffinized sections were stained with haematoxylin and eosin (H&E) as well as by the Ziehl–Neelsen (ZN) method. Some aspirate smears were stained in the veterinary clinic using a rapid Romanovsky stain (Diff-Quik®; Siemens Healthcare Diagnostic Inc., Newark, USA) after methanol fixation, while others were stained in the laboratory (Leishman's stain; Merck, Germany).

Histology of two 1 cm nodules showed multifocal to coalescing infiltrates of macrophages and lymphoid cells, often centred on small groups of neutrophils. The macrophages were large and granular, with pale-staining nuclei. Epithelioid macrophages were apparent, and areas of the subcutaneous tissue were necrotic and had vascular proliferation with acute haemorrhage. In ZN-stained sections, AFB were found within macrophages and occasionally free within the interstitium.

The smears showed mostly neutrophils and macrophages, with low numbers of background plasma cells and some lymphocytes. There were variable numbers of bacterial rods, both in the smear background and the cytoplasm of macrophages. Some of these were refractile and thus appeared eosinophilic, when moved in and out of focus, whilst others appeared as negatively stained clefts. These were seen in smears stained with either

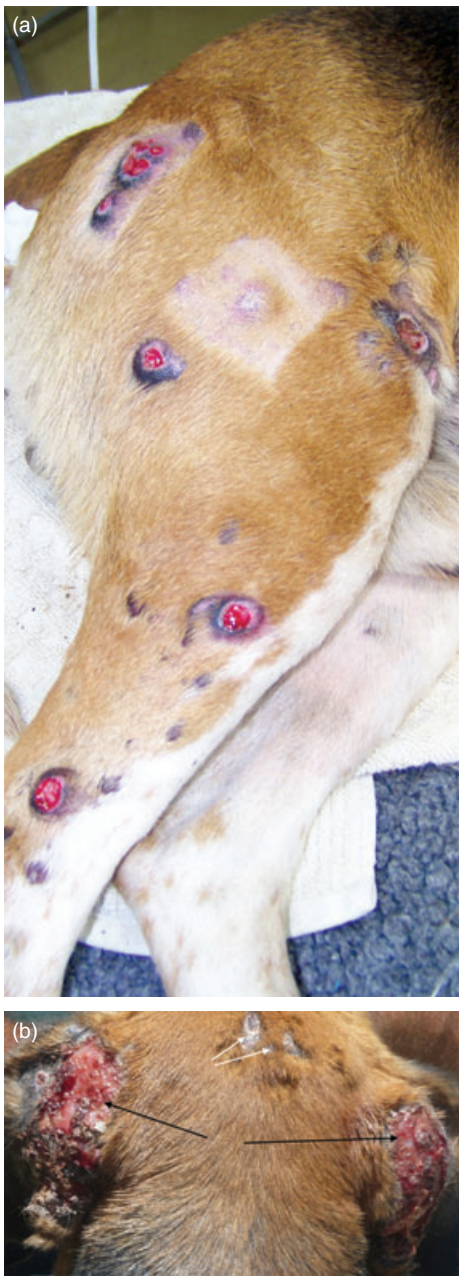

Figure 2. Clinical photographs: foxhound, New Zealand. (a) Case 1, 6 weeks after onset of lesions. Note that the hindlimb nodules are ulcerated and hyperpigmented. (b) Case 1, 3 months after onset of lesions. Note the bilateral ulcerative plaques (black arrows) and smaller healing nodules (white arrows).

Diff-Quik® or Leishman's stains. Modified ZN staining of smears and, in some cases, Fites staining, confirmed AFB. The diagnosis was cutaneous mycobacteriosis in both hounds. Culture and PCR, eventually involving three different laboratories, was undertaken to determine the specific aetiology.

Both affected hounds were isolated from the pack and treated orally with ciprofloxacin hydrochloride [30 mg/kg per os (p.o.), once daily for 21 days; Ciprofloxacin®; Rex Medical Ltd, Auckland, New Zealand]. The lesions in case 2 reduced in size and began to heal. Three weeks after commencing treatment, before complete resolution, the dog was reintroduced to the pack. Case 1, however, showed no improvement and was kept isolated in an open-wire run, 4 m away from the rest of the hounds. Signalment, diagnostic tests and lesions of these and subsequent cases are summarized in Table 1.

Compared with the preceding spring, September to November 2010 had less than half the usual rainfall. The hounds were exercised on the hunt club property in grassy paddocks adjacent to the kennels. Skin trauma was less likely to occur in this environment, and there were no natural water sources. Resident horses and a pig were also present in these paddocks.

During the third week in November 2010, a second cluster of cases emerged, with four more hounds developing lesions. Lesions in three of these were confined to the dorsal head and dorsolateral pinnae, whilst the fourth showed more generalized lesions, similar to cases 1 and 2. Blood from one of these hounds was submitted for haematological examination, which proved unremarkable. Fresh and formalin-fixed skin samples were collected. Faecal specimens were collected from the rectum of cases 1, 5 and 6 and from an unaffected hound in the pack. Earwigs found inside the shelters and flies netted from around the meat preparation area (within metres of the open-air kennels) were frozen at –20°C and subjected to PCR testing. Faeces from a clinically normal dog, living at an entirely different location, were used as a negative control.

Case 1, which was still clinically affected (Figure 2b), and the four newly affected hounds were all treated with clarithromycin (10 mg/kg p.o. twice daily; Klacid®; Abbott Laboratories, Maidenhead, UK) and rifampicin (10 mg/kg p.o. once daily; Rifadin®; Sanofi-Aventis, Anagni, Italy). Lesions in three hounds resolved after 6 weeks, while resolution was incomplete in cases 1 and 4. The same antibiotic regime was continued in these two hounds, and daily topical silver sulfadiazine 1% w/w (Flamazine Cream®; Smith & Nephew, Hull, UK) and dimethyl sulfoxide (DOMOSO Roll-on®, 100 g; Jurox Pty Ltd, Rutherford, NSW, Australia) were added to the regimen, and after a month of this treatment, lesions finally healed. From the time of first appearance, lesions in case 1 took 8 months to heal completely and were, at one stage, so disfiguring that euthanasia was considered.

A month after the second outbreak, in mid-December, the huntsman reported lesions in another 2-year-old entire male, littermate to cases 2, 3 and 6. With this latest case, one entire litter of four hounds had now been affected. Case 7 developed several cutaneous nodules (1–2 cm) on the head and ears. This dog was treated by the huntsman with topical silver sulfadiazine and dimethyl sulfoxide. After a few weeks, the lesions resolved, without ulcerating.

In November of the following year (2011), within 3 days, four new cases of CLG occurred in previously unaffected hounds. Case 8 was an entire 3-year-old male, which displayed nodules affecting the lateral aspects of the head, ears and legs. The number and distribution of lesions were similar to those of case 1. However, lesions on the dorso-lateral pinnae presented as firm plaques with an uneven surface that was cool and oozed serum. This appeared to be due to coalescing nodules covered

Table 1. Summarized clinical data, diagnostic tests and treatment of foxhound canine leproid granuloma cases, New Zealand

Case no.	Age (years), sex*	Month and year of onset	Lesions	Diagnostic test	Treatment/s	Outcome
1	3, M	August 2010	Severely affected, generalized nodules, 4 cm ulcerated plaque on pinnae	Fixed tissue ZN +ve	Amoxicillin 750 mg, s.c., 5 days	NR
				PCR +ve	Ciprofloxacin†, 21 days	NR
				Faeces +ve	Clarithromycin‡ + rifampicin§, 6 weeks	Incomplete healing
				Faeces +ve February 2012	Clarithromycin/ rifampicin continued, added silver sulfadiazine and DMSO topically, 4 weeks	From onset of lesions, 8 months to heal
2	2, M	September 2010	Moderately affected, generalized nodules, ulcerated pinnae	Smears AFB +ve	Ciprofloxacin, 21 days	Healing underway at 3 weeks
3	2, M	November 2010	Moderately affected, multiple nodules on head and pinnae	Smears AFB +ve Fresh tissue PCR +ve	Clarithromycin + rifampicin, 6 weeks	Healing apparent at 6 weeks
4	2, M	November 2010	Moderately affected, generalized nodules, ulcerated pinnae	Fixed tissue ZN +ve	Clarithromycin + rifampicin, 6 weeks	Incomplete healing
					Clarithromycin/ rifampicin continued, added silver sulfadiazine and DMSO topically, 4 weeks	From onset of lesions, 10 weeks to heal
5	2, M	November 2010	Mildly affected, nodules on head and pinnae	Smears AFB +ve Faeces +ve	Clarithromycin + rifampicin, 6 weeks	Healed at 6 weeks
6	2, F	November 2010	Mildly affected, nodules on head and pinnae	Fixed tissue ZN +ve Faeces –ve	Clarithromycin + rifampicin, 6 weeks	Healed at 6 weeks
7	2, M	December 2010	Mildly affected, nodules on head and pinnae	Not tested	Silver sulfadiazine + DMSO topically	Healed after 2–3 weeks
8¶	3, M	November 2011	Severely affected, generalized nodules, multiple coalescing ulcerated nodules on pinnae	Smears AFB +ve Smears AFB +ve February 2012	Clarithromycin + rifampicin + silver sulfadiazine + DMSO topically	Worsened after 2 weeks on treatment; some healing apparent after 4 weeks on treatment. Slow resolution, with small ulcers remaining February 2012. Completely healed by end of March 2012
9¶	2, F	November 2011	Single nodule, dorsum of each pinna	Smears AFB +ve	Clarithromycin + rifampicin + silver sulfadiazine + DMSO topically	Ulcerated after 2 weeks on treatment; healing apparent after 4 weeks on treatment
10¶	2, M	November 2011	Single ulcerated nodule, pinna and single nodule lateral thigh	Smears AFB +ve	Clarithromycin + rifampicin + silver sulfadiazine + DMSO topically	NR after 2 weeks on treatment; healing apparent after 4 weeks on treatment
11¶	2, F	November 2011	Bilateral pinnal nodules	Smears AFB +ve	Clarithromycin + rifampicin + silver sulfadiazine + DMSO topically	NR after 2 weeks on treatment; healing apparent after 4 weeks on treatment

Amoxicillin, ciprofloxacin and rifampicin were administered once daily; clarithromycin was administered twice daily; all were administered per os.
Abbreviations: +ve, positive; AFB, acid-fast bacilli; DMSO, dimethyl sulfoxide; NR, no response; s.c., subcutaneous; and ZN, Ziehl–Neelsen.
*All hounds were entire.
†Dosage 30 mg/kg.
‡Dosage 10 mg/kg.
§Dosage 10 mg/kg.
¶Two of three faecal samples from the isolation run containing these four dogs were PCR positive.

by necrotic skin. Nodules on the thigh were intact, but the central skin of some also appeared necrotic. The other three hounds had fewer lesions. These four were otherwise clinically normal and in good condition. The affected hounds were immediately isolated in one of the outdoor runs and treated with clarithromycin and rifampicin at the aforementioned oral doses, as well as topical silver sulfadiazine and dimethyl sulfoxide.

The popliteal and submandibular nodes of these hounds were not enlarged, but those from one hound were aspirated for examination. There was no evidence of inflammation in the smears and no organisms were seen.

Three fresh faecal samples were collected from this isolation run. All faeces sampled were positive for *Uncinaria* spp., with 850, 300 and 250 eggs per gram (e.p.g.) recorded. *Trichuris vulpis* was also present, with 150 and 7900 e.p.g. in two samples and none in the third. The faecal samples were also submitted for PCR testing.

Despite treatment, lesions in cases 8 and 9 progressed. After 14 days of treatment, case 8 had coalescing craterous, painful ulcers on his pinnae (Figure 3) and thighs. The pinnal nodules in case 9 had also ulcerated. Nodules in cases 10 and 11 were unchanged. After a further 14 days (a month from presentation and initial treatment), all cases showed marked improvement. The pinnal ulcers in case 8 healed gradually, and smears were AFB positive in February 2012. Complete healing was reported by the end of March 2012.

In all the cases, although presenting lesions would enlarge, ulcerate or regress, it was the attending veterinarian's impression that no new lesions developed after the initial presentation.

Australian foxhound report

Another two CLG case clusters, clinically similar to the NZ scenario described above, were seen by one of the authors in foxhounds of two hunt clubs on the outskirts of Melbourne, Australia in 1992. The climate in this area is similar to the affected region in NZ. The geographic location was 37.5°S and 145°E, with 30 year average temperature means of 13.9°C in July and 26.5°C in February, and average long-term humidity of 63–80% (http://www.bom.gov.au/jsp/ncc/cdio/cvg/av; accessed November 2011). These cases have not been reported previously, but details were recorded at the time and are presented here as likely, but unproven CLG. The clinical and diagnostic details are presented, along with attempts of two of the authors to determine the aetiological agent.

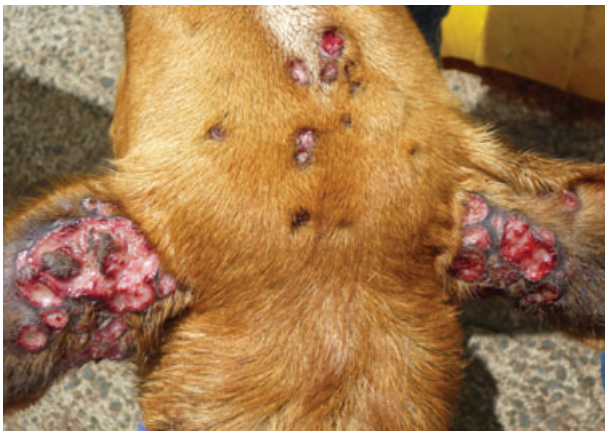

Figure 3. Clinical photograph: foxhound, New Zealand. Case 8, pinnal lesions after 2 weeks on antibiotic therapy.

Large, ulcerated granulomatous lesions were observed on the dorsal pinnae of multiple foxhounds, with smaller lesions occurring on the trunk and lower legs. In both kennels, managers reported seeing similar lesions seasonally for several years, although no hound was affected more than once. Generally, lesions appeared in the spring as small nodules. Those on the pinnae, in particular, became large, ulcerated masses, which then slowly regressed and resolved by the autumn. Lesions elsewhere on exposed areas of the trunk and limbs were much less severe. Although there were about 50 hounds in each kennel, only two or three of them were affected each year. The familial relatedness of the dogs is unknown. Treatment details are incomplete, and a variety of antibiotics were used. Trimethoprim/sulfadiazine and, after no improvement, enrofloxacin were given to two hounds by the author (V.S.). With enrofloxacin there was minor improvement that lasted only while the hound was receiving treatment.

The kennels were geographically separated by more than 50 km, but there had been casual contact between some hounds at a jointly held event at a time when lesions were occurring in one kennel, but not yet in the other. Tissue aspirates, taken from multiple hounds during two successive seasons, showed AFB in smears. One formalin-fixed tissue specimen was also positive for AFB in ZN-stained sections of cutaneous granulomas. One of two fresh pinna samples, submitted to a mycobacteria reference laboratory, tested positive after about 4 weeks of incubation at 32°C in Bactec 460® media (Becton Dickinson, Franklin Lakes, NJ, USA). The identity of this Bactec isolate remained elusive, despite subculture and a variety of standard mycobacterial identification biochemical tests routinely in use at that time (1992). In 2011, this isolate was subcultured from the frozen stock and identified as *Mycobacterium colombiense*,[15] a novel member of the *Mycobacterium avium* complex, by sequence analysis of the 16S rRNA gene. It was not possible to locate the formalin-fixed paraffin-embedded tissue blocks from these cases to confirm that the lesions from these hounds contained *Mycobacterium* spp. CLG DNA.

Materials and methods (NZ cases)

DNA extraction of formalin-fixed paraffin-embedded tissue specimens

At least six paraffin sections of lesions from case 1, 10–20 μm in thickness, were dewaxed in a 1.5 mL tube by extracting with 1 mL of Histolene (Fronine Pty Ltd, Riverstone, NSW, Australia), followed by the addition of 1 mL of absolute ethanol and centrifugation (14,000 ***g*** for 5 min) in a microfuge (Heraeus, Hanau, Germany). DNA was extracted from the tissue pellet using the QIAamp DNA minikit (Qiagen Inc., Valencia, CA, USA) using their DNA clean-up protocol, preceded by 24 h incubation at 56°C in digestion buffer (50 mmol/L Tris–HCl pH 7.5, 10 mmol/L EDTA, 0.5% w/v SDS, 50 mmol/L NaCl and 300 μg/mL proteinase K).

Fresh tissue

An approximately 2 g biopsy specimen from case 3 was homogenized in 10 mL phosphate-buffered saline in a

Tenbroek grinder and frozen at –20°C. The homogenate was thawed, and 500 μL was transferred to a 2 mL Lysing Matrix B tube containing 0.1 mm silica spheres (Bio101 Systems/Q-Biogene Inc., Carlsbad, CA, USA) and 100 μL buffer composed of 250 mmol/L Tris, 50 mmol/L EDTA and 2.5% SDS. The homogenate was lysed in a Ribolyser (FastPrep Cell Disrupter; ThermoSavant, Holbrook, NY, USA) set on 6.5 for 40 s and then cooled in ice. Proteinase K (12 μL of 10 mg/mL) was added, and the homogenate was incubated at 56°C for 3 h. The homogenate was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and once with chloroform/isoamyl alcohol (24:1, v/v). The DNA was ethanol precipitated, washed with 70% ethanol and resuspended in 50 μL 10 mmol/L Tris, 1 mmol/L EDTA.

Faeces

DNA from 200 mg aliquots of the seven faecal samples and the control were extracted using the FastDNA® SPIN Kit for soil with the FastPrep®-24 Instrument (MP Biomedical, Solon, OH, USA), according to the manufacturer's instructions. DNA extracts were stored at –20°C.

Insects

One female *Calliphora vicina* (European bluebottle fly), five female *Calliphora hilli* (Hill's brown blowfly), three *Calliphora stygia* (golden blowfly, a common strike fly) and four earwigs (*Forficula auricularia*, the European earwig) were collected from the kennel premises. They were identified according to morphological characteristics by an experienced entomologist. The nine flies were randomly divided into two pools. The earwigs were pooled to make a third one. Each of the three resulting pools was extracted in two different ways. One method used overnight incubation at 37°C; the other did not.

PCR amplification and sequence analysis

Amplification of the mycobacterial internal transcribed spacer (ITS1) region using DNA extracted from the formalin-fixed paraffin-embedded sample and subsequent sequence analysis of the PCR product were performed as described previously.[7] Primers, 5′- CGTGCTTAACACATG CAAGTCGAAC-3′ (46–70, forward) and 5′-CACGAACAA CGCGACAAACCACC-3′ (597–575, reverse; *M. tuberculosis* H37Rv, GenBank accession number NC000962) were designed to be selective for mycobacteria and to amplify the variable region of the 16S rRNA gene. Amplification conditions and subsequent sequence analysis of the PCR product were performed as described previously.[16]

TaqMan real-time PCR (qPCR)

To design specific assays suitable for detecting DNA of *Mycobacterium* spp. canine leproid granuloma (*Mycobacterium* spp. CLG) in clinical and environmental samples, primers and TaqMan® MGB probes (Applied Biosystems, Foster City, CA, USA) were selected from unique regions relative to other known mycobacterial sequences of the ITS1 and *hsp*65 gene sequences of *Mycobacterium* spp. CLG strain 'Metcalfe' (GenBank accession no. EF611177 and EF611178, respectively) using the Primer Express® Software v2.0 program (Applied Biosystems). Primer and probe sequences for each assay are shown in Table 2. Real-time PCR mixtures contained 1 μL of template DNA, 0.9 μM concentrations of each primer, 0.25 μM concentration of the probe, TaqMan® Gene Expression Mastermix and TaqMan® Exogenous Internal Positive Control (IPC) reagents in a total volume of 25 μL. Amplification and detection were performed with the ABI Prism® 7500 Fast Real-Time PCR System (Applied Biosystems) using the following programme: one cycle of 50°C for 2 min, one cycle of 95°C for 15 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. DNA extracts were tested at least in duplicate, and negative controls were included in each assay. DNA extracts were screened initially using the CLG-ITS assay, and samples giving a positive result were confirmed using the CLG-hsp65 assay.

Results

Table 1 summarizes the clinical data, diagnostic tests and treatment regimes. Culture of a nodule from case 1 grew moderate numbers of coagulase-negative *Staphylococcus* spp., β-haemolytic *Streptococcus* spp. and a heavy growth of *Staphylococcus aureus*. Anaerobic culture was negative. Samples of fresh tissue from this dog were submitted to a mycobacteria reference laboratory in a human hospital. No mycobacteria were detected by PCR or culture.

A mycobacteria reference laboratory with expertise in CLG infections tested the paraffin block from a formalin-fixed lesion of case 1. *Mycobacterium* spp. CLG was confirmed in this index case via sequence analysis of a 224 bp amplicon within the ITS1 region that was identical to the sequence in GenBank (accession no. EF611177). This specimen also tested positive in both real-time PCR assays.

DNA matching *Mycobacterium* spp. CLG was detected using both real-time PCR assays in the faeces of case 1. The cycle threshold (C_T) value obtained from the faeces of case 1 was 29.7. DNA extracted from faeces of case 5

Table 2. Primers and probes designed for real-time PCR assays targeting the internal transcribed spacer (ITS1) region and *hsp65* gene of *Mycobacterium* spp.

Primer or probe	Sequence (5′–3′)	Nucleotide positions (GenBank accession no.)	Amplicon size
CLG-ITSTF	CACTATTGGGCCCTGAGACAAC	98–119 (EF611177)	60 bp
CLG-ITSTR	AAGACGGAGGGACACCACTTC	137–157 (EF611177)	
CLG-ITSTP	6FAM-CTCGGCCGGCTTG-MGBNFQ	121–133 (EF611177)	
CLG-hspTF	CCGAGACCCTGCTGAAGTCA	216–235 (EF611178)	79 bp
CLG-hspTR	TCACCGGCGGAAATCG	279–294 (EF611178)	
CLG-hspTP	6FAM-CAAGGATCAGATCGCT-MGBNFQ	253–268 (EF611178)	

Abbreviations: CLG, canine leproid granuloma; ITS, internal transcribed spacer; TF, forward primer; TP, probe; TR, reverse primer.

gave a very weak signal (C_T 39) in the ITS real-time assay, while that of case 6 was negative. The one unaffected, in-contact dog tested was also negative. Three separate, random faecal samples from the isolation run of the 2011 episode were also tested by the same method. Two were weakly positive and one was negative. The clinically normal control dog faeces were negative at all times. Faeces of case 1 tested positive again in February 2012, a year after lesions were healed (C_T 37.5). At this time, the dog was back in with the pack and in contact with case 8, which still had AFB in small pinnal ulcers. Case 1 was subsequently isolated in a concrete run, and after 14 days two faecal samples (one collected from the ground and one per rectum) were negative.

Although DNA extracts of one of the pooled fly preparations (without overnight incubation) screened weakly positive using the CLG-ITS assay (C_T 36), the CLG-hsp65 assay (which is slightly less sensitive that the CLG-ITS assay) was not able to confirm this result. The earwig pool was negative.

Fresh tissue from case 3 was sent to a veterinary mycobacteria reference laboratory and cultured for 30 days in Bactec® media. Although ZN-stained smears of the specimen were positive for AFB, no mycobacterium was isolated from this skin nodule. The partial 16S rRNA sequence amplified from DNA extracted from this sample was identical to that reported for cases of CLG infections (GenBank accession no. 144747).[6]

Two different laboratories showed that fresh or formalin-fixed skin from two of the NZ foxhounds (cases 1 and 3) contained nucleic acid identical to that described in previous CLG reports. This provides support for the assertion that the AFB seen in aspirates and biopsies from all these affected hounds was the same infectious agent. The clinical presentation, characteristic histological features of the lesions and molecular studies all confirm CLG in these foxhounds.

Discussion

This report details the first occurrence of multiple cases of CLG occurring in in-contact dogs and the first proven case of CLG in dogs in New Zealand. The only other published information on the status of this disease in NZ derives from cases received between 1982 and 1992 by the University of Sydney, Department of Veterinary Pathology and Bacteriology and a survey of veterinary practitioners in Australia, which included one response indicating the presence of this condition in NZ.[1] However, there was no documented follow-up of that particular case and there are no publications in peer-reviewed literature or nonpeer-reviewed NZ publications of this condition occurring in NZ. *Mycobacterium bovis* is endemic in focal areas of NZ, sustained in a feral population including Australian brushtail possums, pigs and deer. The presence of AFB alone in canine granulomas does not confirm CLG in a NZ setting.

We describe the appearance, progression, treatment and healing of multiple cases of CLG in hounds in the hope that this will provide information and motivation to persevere with treatment of persistent or severe cases. After the first two cases, the huntsman became particularly observant, alerting his veterinarian promptly to subsequent cases. This facilitated documentation and observation of the progression of the lesions and responses to treatment. As all affected hounds received treatment, it is not possible to comment on spontaneous resolution. It was the clinician's impression that resolution was more rapid in hounds with fewer nodules. To what degree the numbers of nodules were related to the individual's immune response, the challenge dose of organisms (or other factors) is unknown. In hounds with widespread lesions and large ulcerated granulomas, resolution occurred more rapidly when a combination of systemic and topical treatment was used. Compared with the response in less affected hounds, these cases appeared to have a refractory period of some weeks in which no response was initially seen. This may be due to these hounds receiving a larger dose of mycobacteria, or the fact that significant tissue damage had already occurred by the time treatment commenced.

It is striking that the only reports of multiple in-contact dogs affected in consecutive years occurred in foxhounds. Although confirmation of the suspected Melbourne cases is unfortunately not possible, clinical and epidemiological features of the cases closely matched the ones in New Zealand. The organism isolated (*M. colombiense*) from one Melbourne sample almost certainly represents a contaminant present on the skin surface rather than the causal agent present within the lesion. Although it is well documented that at least four different agents are capable of causing feline cutaneous mycobacteriosis (feline leprosy),[7–10,17–21] a uniform molecular identity has emerged in CLG cases where biopsy specimens are carefully collected. Ideally, the surface skin is removed prior to paraffin embedding to circumvent spurious contaminants being identified. In addition to the Melbourne cases, another cluster of CLG cases occurring in a kennel of foxhounds in Georgia, USA (Craig E. Greene, personal communication 2002) was made known to one author (R.M.). Although these case details are not retrievable and a definitive diagnosis could only be made in the NZ hounds, the apparent foxhound cases suggest two things: (i) there may be a common environmental trigger pertaining to the lifestyle of hunting foxhounds; and (ii) this breed of dog may have a genetic predisposition to infection by the CLG organism or inadequate immune response to it. How much either or both of these factors are part of the epidemiology and pathogenesis of the disease is unknown.

Although the lesions became clinically apparent shortly after the end of the hunting season, inoculation of the skin may have occurred during the preceding hunting season or the previous summer. Some mycobacterial infections are known to have long incubation periods following exposure.[22–24] It seems reasonable to assume that the CLG agent likewise has an incubation period of several weeks or months. The unknown incubation period makes it difficult to correlate the hounds' activities and occurrence of disease. Restriction of lesions to the lateral body, head and limbs and absence of them on the softer, ventral skin suggests a cutaneous route of infection in exposed skin. The environment usually inhabited by the CLG organism is unknown. It may be present normally in the dogs' runs

and gain entry to the skin only in specific circumstances. No affected hounds showed clinical signs of systemic infection, and the lack of inflammation or organisms in the draining lymph nodes of one hound tested suggests that the infection in that hound remained localized to the skin. Although lymph nodes draining lesions were examined carefully in only one hound, if this a consistent finding in affected dogs it supports the hypothesis that entry of the organism is percutaneous and infection then remains localized to superficial structures.

The fact that only hunting hounds were affected, and no cases occurred in the adolescent unhunted hounds nor in the weaned pups kept in an adjacent run, suggests that the environment in which the dogs are confined may be less important than the environments in which they hunted over the preceding winters. The general hunting area has widespread use of prickly hedges (barberry hedges (*Berberis* spp.) and hawthorne (*Crataegus monogyna*)] delineating field boundaries and water troughs or natural bodies of water where the dogs cool off. Movement through the hedges and undergrowth could cause abrasions to the areas where lesions developed, but should also affect the pectoral regions, which were, however, not clinically affected. The huntsman noted that the dogs do not usually show gross evidence of skin trauma after a hunt, although minor abrasions may well occur. The lesion distribution was also noted to be mostly sites of contact with the ground, when the dogs were laterally recumbent. The finding of hookworm eggs in the last outbreak introduces the possibility that larval penetration of the skin may be associated with inoculation of organisms from contaminated soil into the skin. If this were the case, however, one would expect the feet and ventrum to be affected preferentially, given the predilection sites for larval penetration.[25,26]

In other reports, the distribution of lesions on the dorsal ears and head, as well as the propensity for shorthaired dogs to be affected, has led some authors to propose biting flies as potential mechanical vectors.[1,11] Biting flies are proven transmitters and/or mechanical carriers of a variety of infectious agents, including bacteria and protozoa.[27–29] Horses grazed in surrounding paddocks before and during the clinical episodes of CLG in the NZ hounds. Freshly slaughtered horse carcasses, destined to be hound food, were hung within metres of the hounds' runs. *Stomoxys* (stable flies) were likely to have been in the area. *Stomoxys calcitrans* is a blood-feeding fly widespread in NZ, which feeds on the blood of cattle and horses. The blood-feeding *Tabanus* genus (horse flies) are not present in NZ.[30,31] Most Diptera species in NZ have a seasonal activity pattern, with more of them seen in spring and summer. In a study commissioned to determine the distribution of *S. calcitrans* in regions of NZ, *S. calcitrans* numbers in the area where the kennels are located peaked in January–May.[32] None was captured in traps in August, but numbers slowly increased from September. It is possible that the hounds were infected by flies in the previous fly season of January–May but did not show clinical signs until August–September.

There are some features of these cases that do not support biting insect involvement, as follows.

1 The huntsman was specifically questioned about the hounds' behaviour, and biting flies were not considered a problem. There had been no cases of fly-bite dermatitis observed.
2 There was a marked, distinct and consistent restriction of lesions to the head, lateral aspects of the body and legs and, in particular, a lack of lesions on the sparsely haired axillae, inguinum and medial thighs. Although different species of biting fly are likely to have preferred sites of attack, no scientific data could be found alluding to canine attack sites of *Stomoxys* or, indeed, any other biting flies. The anecdotal information of ear tips, margins and folds being targeted is well known, but scientific data on this are lacking. A study of *S. calcitrans* feeding behaviour in dairy cattle reports that the insects 'usually alight upon the lower portions of the front legs. When abundant, *Stomoxys* is commonly seen on other parts of the animal's body'.[33] *Stomoxys* feeding behaviour is influenced by host defensive response in dogs as well as other species.[34–36]
3 The 10 weaned puppies present in an adjacent run throughout the period remained unaffected. If biting insects are vectors of infection, one might expect that the puppies would have also been affected. It is possible that their behaviour prevented them being bitten as frequently, because they may have been less tolerant of the pain of bites and actively tried to prevent insects landing and feeding. As of March 2012, none of the pups from 2010, by now mixed with the adult hounds, showed clinical disease.

Most of the hounds, and all of those affected, had been born on the property. The sudden occurrence of disease suggests that the organism or a vector was newly introduced to the hounds' environment or, conversely, that the hounds were newly introduced to the mycobacteria's environment. We cannot discount the possibility that the first affected hound brought the organism into the kennels and served as a source of infection either through his contaminated faeces or ulcerated lesions. If this was the case, it is difficult to explain the sporadic and episodic nature of the clinical presentation over the following year unless the natural cycle of the organism and/or its vector in nature is also intermittent. Although some insects were tested from the premises, none were biting insects. Nonbiting flies are also important mechanical vectors for some diseases,[37] but given the barrier action of intact skin it is unlikely that nonbiting flies represent a primary source of infection unless they transmit infection to damaged skin.

The CLG qPCR assays are still under development and not yet fully validated for use on insects, so results should be considered preliminary. However, similar technology has been successfully developed to detect *M. ulcerans* from environmental samples, including insects and animal faeces.[12,38] A DNA extract prepared from a fly pool gave a weak positive real-time PCR signal for the CLG ITS region, but was negative when tested using the CLG-hsp65 assay. No conclusions can be drawn from these results until further samples are tested. Earwigs were found in the kennel shelters but were unlikely to have

close interaction with the animals. Tests were therefore expected to be negative. In other PCR assays, we found the CLG agent in faeces of two identified hounds (cases 1 and 5) and in two of three faecal piles collected from the isolation run in 2011. Positive faecal results may reflect either oro-anal contamination after licking lesions or ingestion and transit of the organism. The organism may be ingested following licking of lesions or from a source in the environment. The finding, a year later, of PCR-positive faeces in case 1, and then negative faeces after his isolation, suggest either that there was ingestion of the organism from the environment and/or infected wounds of case 8 or that intermittent shedding of the organism came from the dog's gastrointestinal tract. It is unknown whether the organism can colonize or invade the gastrointestinal tract. Clinically, there was no indication of concurrent gastrointestinal disease, and necropsy of one case of CLG failed to detect alimentary or internal organ involvement.[11] The first faecal sample collected from case 1 was strongly PCR positive. Based on the real-time PCR cycle thresholds, this hound was likely to be shedding the organism at 100-fold higher concentrations than the other positive hounds. His later faecal test result was more weakly positive. The significance of this finding is yet to be determined.

As the incubation period of infection is unknown, it is impossible to correlate weather and insect patterns with disease occurrence. The NZ outbreak began at winter's end and occurred in distinct episodes. Weather data recorded over the entire period are available (see Table S1 in Supporting Information). Such data may be of importance in the future should incubation periods or a vector be determined. The Melbourne foxhound clusters, like the sporadic Brazilian cases,[5] occurred mostly in the hottest months of the year. In another report, largely based on a postal survey of Australian clinicians, the authors noted a different seasonal pattern, with slightly more cases occurring in the colder months of the year.[1] Known locations of CLG cases are mapped on a shaded background of summer temperatures (northern hemisphere) in Figure 4. The overall tendency for cases to be reported from temperate to subtropical climatic zones suggests that the environment required for infection is climate specific. Although the NZ and Australian outbreaks both occurred at very close latitudes (37.5°S), other reports from Australia and Brazil spread the latitude lines more widely. An informal survey of members of the International Society of Veterinary Dermatopathology (ISVD) by list-serve email (sent 28 November 2011) did not produce any anecdotal reports of diagnoses of CLG from countries outside the latitude regions indicated in Figure 4. Specifically, despite targeted queries, no cases were reported to the author (B.S.) from Canada, northern Europe, Russia, UK or Scandinavia. The most northerly cases were in California, USA; the most southerly were in Tasmania, Australia.

Figure 4. Global bioclimatic temperature range showing distribution of canine leproid granuloma cases reported in the literature since 1976. Note the tendency of cases to occur in temperate and subtropical locations. The continuous red line marks the equator. The responses to International Society of Veterinary Dermatopathology listserve query do not fall outside of the most extreme latitude lines of the cases marked X.

As in previous reports, these dogs were shorthaired.[1,2,5] There is a clear bias in this case study towards a particular type of shorthaired dog, namely the foxhound. There were about 40 adult hounds in the kennel. Four of the 11 affected hounds constituted one entire litter and were one generation removed from the dog that sired all the other cases. However, the same sire also produced unaffected siblings within affected litters. Whilst other sires were used at the kennel, none of their progeny was clinically affected. The familial significance is tantalizing but debatable. The foxhound breed is suggested to have a predisposition to visceral leishmaniosis, and transplacental transmission has been demonstrated.[39] Leishmaniosis is a problem in many foxhound kennels in the USA, particularly those on the east coast.[40,41] Protective immunity to *Leishmania* infection is thought to be cell mediated.[41] It is likely that this is also the mechanism of action against mycobacteria in CLG, given the phagocytic response induced and the pathogenesis of other mycobacterial diseases.

We confirm that CLG occurred in episodic clusters in foxhounds of a NZ hunt club. The clinical and epidemiological details are remarkably similar to those seen in two Melbourne hunt clubs 20 years previously. Some hounds had severely disfiguring lesions and protracted recovery times, possibly related to the severity of the presenting lesions. A variety of treatment protocols are reported, and PCR techniques previously used to identify environmental *Mycobacteria* species also show presence of the CLG DNA in pooled insect and faeces from the kennels. A major obstacle to understanding CLG pathogenesis remains the unknown incubation period of the disease.

Addendum

In Spring of 2012, the NZ hunt club described above had two more cases of CLG in hounds that hunted with the pack over the winter. These were hounds not previously affected. Lesions again involved the ears and lateral limbs. Thus for three consecutive years, in this one NZ

hunt club, the disease became clinically apparent each spring and never recurred in the same hound.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Weather conditions during 12 month period of the NZ outbreak. Tabulated parameters from the New Zealand National Institute of Water and Atmospheric Research from the closest weather recording station to the hunt club.

Résumé

Contexte – Le granulome léproïdes canin (CLG) se présente typiquement comme des nodules uniques à multiples, circonscrits, dermiques à sous-cutané de la peau velue. Une mycobactérie non-identifiée est considérée comme l'agent étiologique de cette entité.

Sujets – Plusieurs cas de granulomes léproïdes canins sont apparus chez les chiens en Nouvelle Zélande en 2010 et 2011. Les cas sont apparus en groupe, affectant plusieurs chiens fox-hound vivant dans la même cage. Tous les chiens atteints ont guéri après un traitement antimicrobien topique et/ou systémique. Deux épisodes semblables ont également été rapportés chez des fox-hounds près de Melbourne, en Australie.

Méthodes – Les cas ont été étudiés par cytologie, histologie, microbiologie et plusieurs techniques moléculaires. Une étude environnementale épidémiologique a également été réalisée.

Résultats – Un diagnostic de CLG a été établi chez 11 chiens. L'identification moléculaire de l'agent causal a confirmé qu'il s'agissait d'une espèce de mycobactérie avec 100% d'homologie de séquence au sein des régions amplifiées du gène 16S rRNA et de ITS1 (internal transcribed spacer) avec celle trouvée en association avec des infections similaires aux USA, au Brésil et en Australie.

Conclusion et importance clinique – Cet article détaille pour la première fois l'apparition de multiples cas de CLG apparus chez des chiens en contact et le premier cas prouvé de CLG chez des chiens en Nouvelle Zélande.

Resumen

Introducción – el granuloma leproide canino (CLG) se presenta de forma característica como nódulos solitarios o múltiples, circunscritos, dérmicos a subcutáneos en la piel con pelo. Se considera que se producen por un agente micobacteriano no identificado.

Animales – varios casos de granuloma leproide se produjeron en perros en Nueva Zelanda durante el periodo 2010–2011. Los casos aparecieron en grupos afectando numerosos perros Foxhound con cercano parentesco y alojados en las mismas perreras. Todos los perros afectados se recuperaron tras aplicación tópica y/o sistémica de antimicrobianos. Dos brotes similares que ocurrieron en perros Foxhound cerca de Melbourne en Australia también se incluyen en el artículo.

Métodos – los casos se investigaron utilizando técnicas citológicas, histológicas, microbiológicas y varias técnicas moleculares. Se desarrolló un estudio epidemiológico ambiental.

Resultados – el diagnóstico de CLG fue establecido en 11 perros. La identificación molecular del agente causal confirmó que era una especie de micobacteria con 100% de homología en las regiones amplificadas del gen 16S rRNA y el espaciador interno de transcripción (ITS1) con los encontrados en infecciones similares en casos de los EEUU, Brasil y Australia.

Conclusión e importancia clínica – este artículo detalla los primeros casos de CLG descritos en múltiples perros en contacto y el primer caso probado de CLG en perros en Nueva Zelanda.

Zusammenfassung

Hintergrund – Das canine leproide Granulomsyndrom (CLG) zeigt sich typischerweise in Form von einzelnen oder multiplen umschriebenen dermalen bis subkutanen Knoten in der behaarten Haut. Ein bislang nicht identifiziertes Mycobacterium wird für das ätiologische Agens dieser Gebilde betrachtet.

Tiere – Mehrere Fälle von caninem leproiden Granulomsyndrom traten bei Hunden in Neuseeland zwischen 2010 und 2011 auf. Die Fälle traten geballt auf, wobei mehrere nahe miteinander verwandte Foxhounds, die in denselben Zwingern untergebracht waren, betroffen waren. Alle betroffenen Hunde erholten sich nach topischer und/oder systemischer antimikrobieller Therapie. Zwei ähnliche Ausbrüche, die bei Foxhounds in der Nähe von Melbourne auftraten, werden aus Australien berichtet.

Methode – Die Fälle wurden zytologisch, histologisch, mikrobiologisch und mit mehreren Molekulartechniken untersucht. Eine epidemiologische Studie wurde ebenfalls durchgeführt.

Ergebnisse – Eine Diagnose von CLG wurde bei 11 Hunden erstellt. Auf molekularem Weg wurde das verursachende Agens als mycobakterielle Spezies identifiziert. Dieses zeigte eine 100%ige Sequenzhomologie innerhalb der amplifizierten Regionen des rRNA Gens und des so genannten ''internal transcribed Spacer'' (ITS1) mit jener Sequenz, die im Zusammenhang mit ähnlichen Infektionen aus den USA, Brasilien und Australien beschrieben wurde.

Schlussfolgerungen und klinische Bedeutung – Dieser Report beschreibt das erste Auftreten von multiplen Fällen von CLG im Detail, welches bei Hunden, die miteinander in Kontakt waren, auftrat und die ersten bestätigten Fälle von CLG bei Hunden in Neuseeland.

要約

背景–犬のレプラ様肉芽腫（CLG）は有毛部皮膚に生じる単独から複数の限局性の真皮から皮下の結節として認められる。未同定のマイコバクテリウムがこの疾患の病因と考えられる。

供与動物-2010年から2011年の間にニュージーランドで見られた複数の犬のレプラ様肉芽腫が生じた症例。症例は集団で認められ、同じ犬舎に居住する複数の密接に関係するフォックスハウンドが罹患していた。罹患したすべての犬は外用/あるいは抗菌剤の全身投与後に回復した。オーストラリアのメルボルン近郊で，フォックスハウンドに2つの類似した発生が報告された。

方法-症例を細胞学的、組織学的、微生物学的、いくつかの分子学的な手技によって調査した。環境疫学的な調査も実施した。

結果—１１頭の犬がCLGと診断された。アメリカ、ブラジル、オーストラリアでの類似した感染から発見された原因病原体の分子学的同定で増幅した，16S rRNA遺伝子と領域内と遺伝子間スペーサー領域(ITS1)と100％の配列相同性を示す抗酸菌が同定された。

結論と臨床的な重要性-この報告は接触した犬にみられた多発性のCLGの症例の最初の発生を詳細に記述し，またニュージーランドの犬でCLGが証明された最初の症例である。

摘要

背景 – 犬麻风样肉芽肿（CLG）的典型表现为有毛皮肤单个至多个界限分明的皮肤至皮下结节。一般认为本病的病因是一种未经确认的分支杆菌。

动物 –在2010和2011年间新西兰犬上出现的几例犬麻风样肉芽肿病例。

方法 – 病例采用细胞学、组织学、微生物学和一些分子生物学技术进行研究。也进行环境流行病学研究。

结果 –11只犬被诊断为CLG。病原体分子鉴定确认是一种分支杆菌，16S rRNA基因和内转录间隔区（ITS1）扩增区域内序列同源性100%，发现与来自从美国、巴西和澳大利亚相似的感染相关。

结论和临床价值 – 这篇报道第一次详细说明了在传染病接触犬中多个CLG病例的出现，第一次证明了在新西兰有犬的CLG病例。