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Pyogranuloma caused by *Mycobacterium asiaticum* infection in a steer

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Abstract. In 2003, a steer carcass was condemned at a Central Queensland abattoir because of metastatic tumors. In addition, a granulomatous lesion was found in the mediastinal lymph node. Histological examination showed this to be a pyogranuloma, typically associated with *Rhodococcus* or the *Nocardia/Streptomyces* group. However, in this case, the only etiological agent was an acid-fast bacillus, which would normally be associated with a more fibrous lesion. A number of nucleic acid–based techniques were used, and the isolate was identified as *Mycobacterium asiaticum.* This organism is a rarely encountered opportunistic pathogen of humans, associated with subtropical climates. This is the first report of this organism causing infection in cattle. The similarities between this case and cases of human disease are discussed.

Key words: Cattle; molecular diagnosis; *Mycobacterium asiaticum*; opportunistic infection.

Mycobacterium asiaticum was first described in 1971,[14] isolated from lymph nodes and viscera of healthy monkeys. Subsequently, this bacterium was found to be virulent and contagious for mice, with intrauterine transmission occurring.[13] Historically, clinical isolates have been rarely encountered but when recovered have usually been the cause of disease in humans.[12] In 1983, pulmonary mycobacteriosis caused by this organism was reported in Queensland, Australia.[1] Similar reports were later made in the United States[10] and Thailand.[16] During the 1990s, there were reports of ophthalmic keratitis,[4] flexor tenosynovitis,[5] and olecranon bursitis[3] associated with *M. asiaticum.* The pathogen has been increasingly recognized as a cause of pulmonary and nonpulmonary infection in Queensland human patients with the application of molecular biology tools in mycobacterial identification. Since 2003, *M. asiaticum* has been isolated from 5 Queensland patients. Four of these have been pulmonary isolates, and there was 1 isolate from elbow tissue. In most, if not all, described cases, the patients had some predisposing condition that suggested a predilection to mycobacterial infection.[12] All isolates reported by Wayne and Sramek[12] could be traced to subtropical climate environmental sources.[1]

This article describes the first reported isolation of *M. asiaticum* associated with pyogranulomas in cattle. The identification of the isolate was confirmed using 3 different molecular techniques, in addition to conventional growth characteristic analyses conducted concurrently in the Queensland Mycobacterium Reference laboratory.

In May 2003, a steer carcass was condemned during routine inspection at a Central Queensland abattoir because of metastatic tumors. In addition, a granulomatous lesion was observed in the mediastinal lymph node. The latter lesion was excised and submitted for investigation under the National Granuloma Submis-

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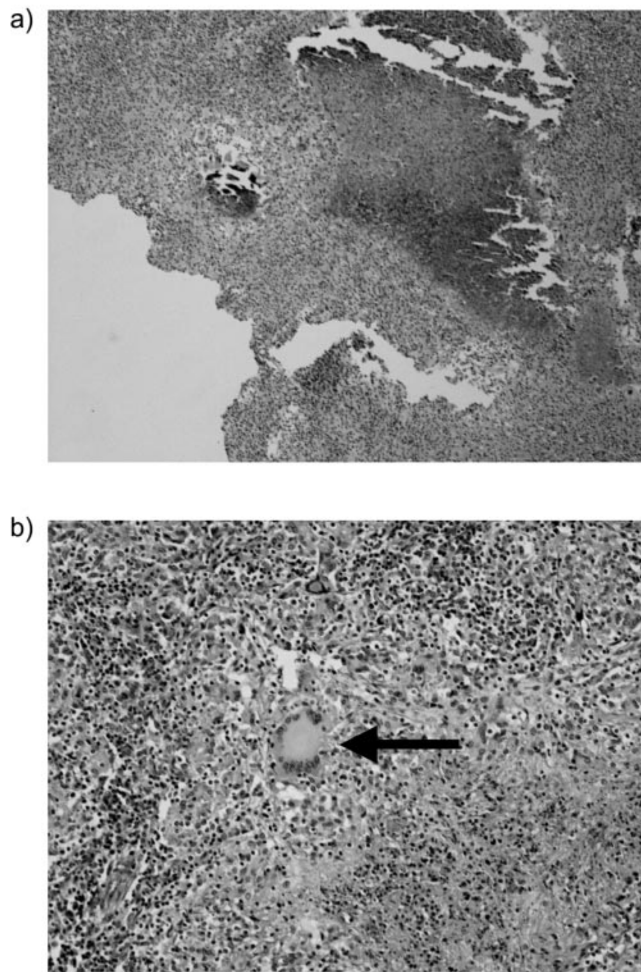

Figure 1. **a**, Area of pyogranulomatous inflammation showing large area of central necrosis on the HE slide (10×) and **b**, area of chronic inflammation characterized by Langerhan's giant cells (arrow), macrophages, and lymphocytes (HE, 40×).

sion Program. Histological examination of a hematoxylin and eosin (HE)–stained slide from a section of the lesion showed a typical pyogranuloma (Fig. 1a). The irregular central necrotic area of the lesion contained mainly neutrophils, with amorphous cellular debris and several small foci of mineralization. Surrounding this was a more granulomatous reaction characterized by significant numbers of Langerhan's type giant cells, macrophages, and lymphocytes (Fig. 1b). Within the giant cells, single acid-fast bacilli were observed on slides stained using the Ziehl Neelson technique. No other etiological agent was observed in slides examined after Gram staining or Grocotts Methenamine Silver staining.

Approximately 1 g of tissue from the lesion was homogenized in 25 ml sterile water. Five milliliters of the homogenate were mixed with an equal volume of 0.15% cetyl-pyridium, incubated at room temperature for 30 minutes, and centrifuged at 3,000 × *g* for 20 minutes. The pellet was swabbed onto 7H11, Stonebrinks, and Stonebrinks with glycerol agar slopes. All tubes were incubated at 37 C in a humid atmosphere for 5 weeks. A single colony type grew on all media.

Initially, targeted polymerase chain reaction (PCR) assays were used in an attempt to identify the organism. The DNA extracts were prepared from the test isolate using a commercial kit[a] according to the manufacturer's instructions. In addition, DNA was extracted from pure cultures of *M. avium* American Type Culture Collection (ATCC) 25291, *M. fortuitum* (local isolate formerly identified by Queensland Health Department), *M. intracellulare* ATCC 13950, and *M. bovis* (isolate obtained from national proficiency testing program) for use as controls.

The PCR assays were conducted on the test isolate DNA and on the appropriate positive controls for *M. bovis* and *M. avium,* as reported previously.[2,15] These PCR assays are routine tests at Oonoonba Veterinary Laboratory for mycobacteria isolated from cattle. Both PCR assays showed no amplification of DNA from the isolate; hence, additional procedures for speciation of the isolate were implemented.

The DNA extracts from *M. avium, M. fortuitum, M. intracellulare,* and the test isolate were analyzed using a modification of a previously described restriction enzyme analysis (REA)–PCR.[9] In brief, reactions consisted of 1× polymerase buffer with ammonium sulfate,[b] 1.5 mM magnesium chloride, 200 μM of each deoxynucleoside triphosphate (dNTP), 0.25 μM of each primer Sp1 and Sp2,[12] 1 U *Taq* polymerase[b] per 50 μl, 5 μl template DNA per 50 μl, and a volume balance of nuclease-free sterile water. Reactions were cycled at 94 C for 5 minutes; 38 × (94 C for 1 minute; 59 C for 30 seconds; and 72 C for 45 seconds). Amplicons were purified using spin columns,[c] and 5 μl aliquots were used for digestions with 2 U of the respective restriction endonuclease. *Hae*III[d] and *Cfo*I[d] were used. All digestions were incubated at 37 C for 2 hours. Digests were resolved using 4% agarose gel electrophoresis and compared with the reported PCR-REA profiles.[9]

This assay amplified products of 220–280 bp. The amplicon sizes from the control DNAs differed according to species and were similar to those described previously.[9] The amplicon sizes are shown in Table 1. *Mycobacterium fortuitum,* used as a control, gave a double product of closely sized fragments. The REA fragment sizes are also shown in Table 1. The 3 species used as controls gave fragment sizes as described previously.[9] The test sample profile matched that described by the same authors for *M. asiaticum.* The PCR and the REA results are shown in Fig. 2.

The PCR was also conducted on DNA from the test isolate using a modified version of a mycobacterial

Table 1. Results for PCR-REA analysis.

Sample	Results from this study			Closest profile described by Roth *et al.*[9]			
	PCR product (~ bp)	*Hae*III digestion fragments (~ bp)	*Cfo*I digestion fragments (~ bp)	PCR product (bp)	*Hae*III digestion fragments (bp)	*Cfo*I digestion fragments (bp)	Identity
M. fortuitum	280, 260	170, 150, 110	260, 200, 80	279, 257	170, 148, 109	257, 200, 79	*M. fortuitum* I
M. intracellulare	220	90[a], 40	none	217–220	92, 85, 42	none	*M. intracellulare* I or *M. scrofulaceum*
M. avium	220	90[a], 40	140, 100	217–220	92, 85, 42	126–144, 91–96	*M. avium* or *M. kansasii*
Test sample	220	160, 60	140, 75	217–220	168, 51	144, 75	*M. asiaticum*

[a] See Fig. 2, this 'fragment' is wider and brighter than others and likely to consist of two or more closely sized fragments that were not discernable through agarose gel electrophoresis.

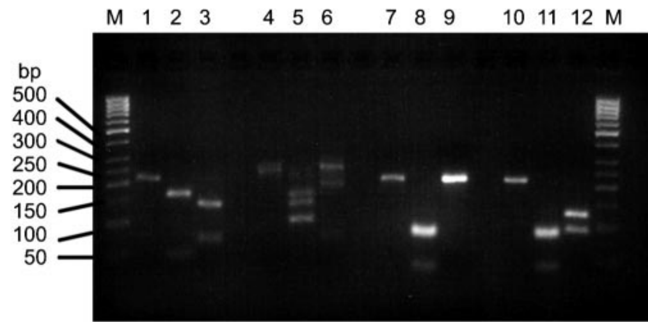

Figure 2. PCR-REA of partial 16–23S spacer regions. M = GeneRuler 50 bp (MBI Fermentas); 1 = PCR, test sample; 2 = *Hae*III digest, test sample; 3 = *Cfo*I digest, test sample; 4 = PCR, *M. fortuitum*; 5 = *Hae*III digest, *M. fortuitum*; 6 = *Cfo*I digest, *M. fortuitum*; 7 = PCR, *M. intracellulare*; 8 = *Hae*III digest, *M. intracellulare*; 9 = *Cfo*I digest, *M. intracellulare*; 10 = PCR, *M. avium*; 11 = *Hae*III digest, *M. avium*; 12 = *Cfo*I digest, *M. avium.*

genus PCR.[15] In brief, reactions consisted of 1× polymerase buffer with ammonium sulfate,[b] 1.5 mM magnesium chloride, 200 μM of each dNTP, 0.2 μM of each primer MYCGEN-F[15] and MYCGEN-R-mod: 5′-TGC ACA CAG GCC ACA AG-3′, 1 U *Taq* polymerase[b] per 50 μl, 5 μl template DNA per 50 μl, and a volume balance of nuclease-free sterile water. Reactions were cycled at 94 C for 5 minutes; 40 × (94 C for 30 seconds; 60 C for 30 seconds; and 72 C for 1 minutes 15 seconds); and 72 C for 5 minutes. This amplified a 1,030 bp (approximate) region of the 16S ribosomal RNA gene.

Amplicons were purified using spin columns[c] and ligated into plasmid vectors,[d] according to the manufacturer's instructions. Ligated plasmids were transformed into competent *Escherichia coli* JM109 cells,[d] and transformants were selected using blue/white screening. Transformants were cloned by growth in Luria Bertani medium containing 100 μg ml^{-1} ampicillin, and plasmids were extracted using a miniprep kit.[d] Extracted plasmids were used as templates for nucleotide sequencing reactions[e] using universal M13 primers.[d] Sequences were analyzed using capillary electrophoresis[f] by the Advanced Analytical Centre, James Cook University, Townsville, Queensland, Australia. Resulting chromatograms were examined using computer software.[g] Flanking vector sequence was discarded, and remaining data were used to form a consensus contiguous sequence from triplicate plasmids. The consensus sequence was compared with all bacterial gene sequence entries in the GenBank database using a Basic Local Alignment Search Tool (BLAST) search (www.ncbi.nlm.nih.gov).

The PCR of the internal transcribed spacer (ITS) region was conducted on DNA from the test isolate using previously described primers[8] with cycle parameters described elsewhere[6] that were found in this lab-

oratory to provide a stronger reaction. In brief, reactions consisted of 1× polymerase buffer with ammonium sulfate,[b] 1.5 mM magnesium chloride, 200 μM of each dNTP, 0.2 μM of each primer Ec16S and Mb23S,[8] 1 U *Taq* polymerase[b] per 50 μl, 5 μl template DNA per 50 μl, and a volume balance of nuclease-free sterile water. Reactions were cycled at 35 × 94 C for 1 minutes; 63 C for 1 minute; and 72 C for 1 minute. This amplified a 480-bp (approximate) region of the 16S–23S ITS sequence. Amplicons were purified, cloned, and sequenced as above.

The consensus sequence from 3 plasmids containing each of the 2 amplicons showed the 16S amplicon to be 1,025 bp and the ITS amplicon to be 480 bp. The sequences have been entered into the Genbank database (www.ncbi.nlm.nih.gov) and assigned accession numbers AY722097 (partial 16S) and AY722098 (partial ITS).

A BLAST search of bacterial DNA sequences in GenBank indicated a 100% match between the obtained 16S sequence and *M. asiaticum* 16S ribosomal DNA accession number AF480595.[11] Other matches, with lower probabilities, included *M. marinarum* (accession number AF456240, 98% similarity), *M. bovis* subsp. *bovis* (accession number BX248338, 98% similarity), and *M. tuberculosis* subsp. *caprae* (accession number AJ131120, 98% similarity). A similar search using the ITS sequence as an input file showed a 98% match with the ITS sequence of *M. asiaticum* accession number AB026703.[6] The next most likely match provided was a 91% match to *M. szulgai* (accession number AB026704).

The test isolate was also submitted to Queensland Mycobacterium Reference Laboratory for identification based on phenotypic characteristics. The colonies were yellow pigmented. The isolate showed mature growth in 11 days on Lowenstein Jenson medium. Growth was observed at 25, 32, and 36 C but not at 44 C. The organism did not reduce nitrate, was highly catalase positive, and hydrolyzed Tween 80 within 5 days and aryl sulfatase after 14 days. This biochemical profile was characteristic of either *M. gordonae* or *M. asiaticum.* However, the isolate produced its yellow pigment only after exposure to light and did not produce pigment in the dark (photochromogenic). Hence, phenotypic characterization was consistent with *M. asiaticum.*

The results obtained from the 4 separate analyses all identified the isolated organism as *M. asiaticum.* To the authors' knowledge, this is the first report of *M. asiaticum* isolated from cattle. In this case, it can be concluded that the organism had caused the pyogranuloma in the mediastinal lymph node. The presence of numerous tumors throughout the carcass suggests that the animal may have been immunocompromised and therefore, as in the reported human cases, predisposed to infection. As with the human cases, the animal had been located in a subtropical region. This is not the first report of *M. asiaticum* from Central Queensland. In 1983, there were at least 2 cases of human pulmonary mycobacteriosis caused by this organism.[1] In addition, 4 of 5 recent (2003–2004) human cases of *M. asiaticum* were from patients who reside in Central Queensland. This observation further suggests a geographical association (Gilpin, personal observation).

Pyogranulomas in bovine lymph nodes are normally associated with bacteria such as *Rhodococcus equi,* or the filamentous *Nocardia*/*Streptomyces* group. Mycobacteria are more commonly associated with a fibrous and caseated granulomatous reaction, and these lesions can normally be differentiated from a pyogranuloma through histological examination.[7] Hence, the pyogranulomatous lesion resulting from infection by *M. asiaticum* described in this report can be described as atypical with respect to mycobacterial reactions. Although mycobacteria may not routinely be sought as an etiogic agent of pyogranulomas, this case indicates that atypical reactions can and do occur.

It can be hypothesized that this animal acquired *M. asiaticum* from its habitual environment, and its possible immunocompromised state resulted in an opportunistic infection. The likelihood that this organism poses a health risk to cattle or persons handling infected cattle in this geographical area cannot be ascertained until more data regarding the frequency and transmission of the organism are available. Although *M. asiaticum* was historically considered to be a rare opportunistic pathogen, the recent increase in isolates associated with human infections in Central Queensland may indicate a rise in the frequency of environmental occurrence. Alternatively, the increase in human isolations may be because of modern identification technologies.

Molecular biology tools continue to play an important role in objective identification of etiological agents as well as the more common use of these tools to target a single species. Their use is of particular benefit for mycobacterial speciation because these bacteria have extremely slow growth rates and conventional test results may take months to obtain. In this study, previously reported research articles have been applied rapidly to a routine diagnostic investigation with results obtained a few days after the initial isolation compared with 3–4 weeks (or a number of months for some species) required for a phenotypic identity.

Sources and manufacturers

a. High Pure PCR template preparation kit, Roche Diagnostics, Castle Hill, Australia.
b. MBI Fermentas, Progen Industries, Archerfield, Australia.

c. QIAquick PCR purification kit, QIAgen, Clifton Hill, Australia.
d. pGEM-T and Wizard SV Plus Miniprep system, Promega, Annandale, Australia
e. ABI BigDye Terminator cycle sequencing mix, Applied Biosystems, Scoresby, Australia.
f. Amersham Biosciences MegaBace system, Castle Hill, Australia.
g. Sequencher™ v. 4.0.5 software, Gene Codes Corporation, Ann Arbor, MI.

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Natural infection of a horse with *Fascioloides magna*

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Abstract. A 25-year-old Quarterhorse mare was euthanized for a variety of medical reasons. At necropsy, 7 liver flukes, identified as *Fascioloides magna,* were recovered from the liver. This is the first report of *F. magna* in a horse.

Key words: Cushing's disease; *Fascioloides magna*; liver flukes; pituitary adenoma.

A 25-year-old Quarterhorse mare was presented at the Large Animal Clinic of the Veterinary Medical Center, University of Minnesota, with a 2-year history of chronic weight loss, quidding, sinusitis, and hirsutism. On admission, the mare was emaciated and had a body condition score of 1.5 (range 1.0–5.0). On the basis of physical examination, diagnoses of Cushing's syndrome, aortic regurgitation, and multiple tooth root abscesses were made. Because of its poor prognosis, the owners elected to have the mare euthanized. At necropsy, lesions unrelated to Cushing's syndrome were observed in the liver. The liver contained numerous necrotic tracts and several multifocal firm nodules (Fig. 1). The nodules were cysts that contained flukes and a dark semiliquid substance. Histopathology revealed hemorrhagic tracts and extensive areas of interstitial fibrosis. The cyst fluid and fibrotic tracts contained an abundance of fluke pigment and fluke eggs. Some eggs were free in the parenchyma, however, many were surrounded by granulomatous inflammation. Seven liver flukes ranging in size from about 3

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