Molecular identification of *Mycobacterium avium* subspecies *paratuberculosis* in an Italian patient with Hashimoto's thyroiditis and Melkersson–Rosenthal syndrome

Mycobacterium avium subspecies *paratuberculosis* (MAP) causes Johne's disease, a chronic granulomatous intestinal condition that affects ruminants, including cattle, sheep, goats and farmed deer (Naser *et al.*, 2004). Water and milk supplies have both been suggested as vehicles of MAP transmission between cattle and humans (Pickup *et al.*, 2006; Groenendaal & Zagmutt, 2008). Milk may be contaminated directly within the udder or indirectly as a result of faecal contamination (Giese & Ahrens, 2000). This bacterium has attracted considerable interest owing to the rapidly growing body of scientific evidence suggesting similarities between Johne's disease in ruminants and Crohn's disease in humans, which has led to speculation that MAP might be one of the causative agents in Crohn's disease (Naser *et al.*, 2004). However, because of the complex nature of human Crohn's disease, as well as the conflicting experimental evidence, a definitive link between MAP and Crohn's disease can neither be confirmed nor excluded at present. Some authors believe there is no evidence that a single pathogen, including MAP, is involved in the pathogenesis of inflammatory bowel diseases (Packey & Sartor, 2009), while others have reported that MAP may asymptotically colonize apparently healthy individuals (Juste *et al.*, 2008). Therefore, no causal link has been scientifically established between MAP and Crohn's disease, although it is important to note that much evidence supports the possibility of such a relationship. The MAP debate has gathered momentum in recent years because of the more frequent detection of MAP in the blood and tissues of Crohn's disease patients as compared with controls. The over-representation of MAP DNA in Crohn's disease patients suggests a probable role for MAP in the aetiology

of this pathological condition (Bentley *et al.*, 2008).

There is still much to be learned about MAP and the diseases it may cause in humans. Recent findings by Sechi and colleagues and by Dow link diabetes to Crohn's disease, since they found MAP bacteraemia involved with autoimmune diabetes (Sechi *et al.*, 2005, 2008; Dow, 2008). Consumption of cows' milk early in life is a recognized risk factor in the development of these diseases, and environmental micro-organisms are thought to trigger autoimmune responses in genetically susceptible individuals (Dow, 2008).

Recently, we described the case of a 52-year-old woman with non-caseating granulomatous inflammation in the oral and facial regions, accompanied by facial nerve palsy, diagnosed as Melkersson–Rosenthal syndrome (MRS), a condition of unknown aetiology. In addition, the patient has suffered from Hashimoto's thyroiditis for the previous 4 years as confirmed by an endocrinological investigation and laboratory tests (Scagliusi *et al.*, 2008).

In view of the literature supporting the involvement of MAP in autoimmune disease (Sechi *et al.*, 2005, 2008; Dow, 2008), and a possible autoimmune aetiology for MRS (Gottwald, 1981), an analysis of the patient's family and personal history and lifestyle, led us to hypothesize the presence of a MAP infection. The patient had lived since childhood on a farm with cattle, in an Italian region where MAP is widespread in cattle-breeding farms. For a long period of her life, she drank cows' milk produced directly at her farm and her diet was still largely based on dairy products. In addition, to the above-described clinical picture she had suffered for many years from bowel disorders. For these reasons,

we performed blood tests for the presence of MAP.

RT-PCR was used to reveal the presence of viable MAP using mRNA extracted from peripheral blood mononuclear cells (PBMCs) and amplifying IS900 sequences unique to MAP (Green *et al.*, 1989). We investigated four samples collected at 1 month intervals. A 20 ml aliquot of the patient's peripheral blood was transferred into tubes containing EDTA (disodium salt) anticoagulant. The samples were immediately processed to obtain PBMCs. Samples were diluted with an equal volume of PBS and overlaid on Ficoll–Paque solution (Amersham Biosciences). The subsequent Ficoll gradient centrifugation was performed at room temperature. The PBMC interphase was transferred to 30 ml PBS and centrifuged (500 g, 10 min). The supernatant was discarded and cell pellets were resuspended in 20 ml PBS. Cells were counted with trypan blue and centrifuged (500 g, 5 min). After removing the supernatant, total RNA was extracted from cells using TRIzol reagent (Invitrogen) (1 ml TRIzol for 1×10^7 cells). Cells were lysed by repetitive pipetting and incubated for 5 min at ambient temperature to permit complete dissociation of nucleoprotein complexes. We used DNase I [$10 \mu\text{l}$ DNase I (ml sample) $^{-1}$ at 37 °C for 30 min] (Applied Biosystems) to remove contaminating DNA. DNase I was removed from the reaction prior to PCR by the use of heat (70 °C for 5 min). DNase-treated RNA (2 μg) was reverse transcribed to cDNA using the SuperScript first-strand cDNA synthesis kit (Invitrogen), according to the manufacturer's instructions. A primary and a secondary round of PCR were done. Oligonucleotide primers were derived from the DNA insertion sequence IS900. The primers P90 (5'-GTTCGGGGCCG-TCGCTTAGG-3') and P91 (5'-GAGGTC-

GATCGCCACGTTGA-3') were used for the first round to amplify a unique 398 bp fragment of the IS900 gene. Sensitivity and specificity for the amplified MAP DNA fragment were achieved by the use of AV1 (5'-ATGTGGTTGCTGTGTTGGATGG-3') and AV2 (5'-CCGCCGCAATCAACTCCAG-3) oligonucleotide primers in the second round to reamplify a 298 bp internal nucleotide sequence of the 398 bp template. The PCR mixture consisted of 40 µl PCR buffer, 5 mM MgCl₂, 0.2 mM dNTP, 6 % DMSO, 2 µM primers, and 2.5 U Platinum *Taq* polymerase (Invitrogen) and 10 µl DNA template. The PCR mixture in the second round of the PCR was the same, except that 5 µl PCR product from the first round was used as a template and the AV1 and AV2 oligonucleotide primers were substituted. The PCR cycling conditions were: 95 °C for 5 min, 34 cycles of 95 °C for 1 min, 58 °C for 1.5 min and 72 °C for 1.5 min, and a final extension of 10 min at 72 °C. The amplification product size was assessed on a 2 % agarose gel. The positive MAP control was ATCC 19698 DNA, and a band co-migrating with the ATCC 19698 DNA at the predicted amplicon size of 298 bp was considered positive. As negative control a MAP-negative subject was used. Bands obtained from four samples were purified and sequenced (Primm). The sequence identity of the final 298 bp amplicon was compared with the GenBank accession number EF514830 sequence for MAP IS900 using the International Nucleotide Sequence Database (National Institutes of Health – International Nucleotide Sequence Database Collaboration, <http://www.ncbi.nlm.nih.gov>) and sequence alignment analyses. The gel of PCR products (Fig. 1) showed a band that co-migrated with the positive control at the predicted 298 bp amplicon size. The sequenced DNA obtained from four samples showed 100 % identity with MAP IS900.

There is current evidence that a percentage of healthy individuals have MAP DNA in their blood, the significance of which remains to be determined (Collins *et al.*, 2000; Juste *et al.*, 2008). However, there are specific states of immune dysfunction or genetic predisposition that could promote mycobacterial infections. Identification of MAP in the blood of this Hashimoto's

thyroiditis patient could be more than a coincidence. It is common knowledge that human pathogens often express proteins with a high antigenic potential and strong homologies with human proteins. Evolutionary pressures based upon the necessity to escape host-specific immune responses may have determined this phenomenon, known as 'molecular mimicry'. It is reasonable to assume that certain individuals can develop abnormal immune responses upon contact with an antigen that mimics a self-protein. The human heat-shock protein Hsp60, for example, with a high degree of homology with Hsp65 of MAP, shares a sequence homology with a wide range of autoantigens including those of Hashimoto's thyroiditis (Jones *et al.*, 1993). Furthermore, recent findings report a possible *Mycobacterium tuberculosis* aetiology for MRS (Apaydin *et al.*, 2000).

To the best of our knowledge, this report is the first observation of a MAP infection in a patient with MRS. The findings are consistent with early exposure to dietary cows' milk and underline the possibility that milk from cattle with Johne's disease could be a potential transmission vehicle for MAP to humans.

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Fig. 1. Agarose electrophoresis gel of amplified IS900 fragments. M, molecular marker; lane 1, MAP-positive control; lane 2, patient sample; lane 3, distilled water negative control; lane 4, MAP-negative control subject; 5, RT-PCR of internal control GADPH.

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