Diagnostics of non-tuberculous mycobacteria

Bruijnesteijn van Coppenraet, L.E.S.

Chapter 5.

Application of real-time PCR to recognize atypical mycobacteria in archival skin biopsies: High prevalence of *Mycobacterium haemophilum*.

Lesla ES Bruijnesteijn van Coppenraet[1], Vincent THBM Smit[2], Kate E Templeton[1], Eric CJ Claas[1] and Edward J Kuijper[1].

Departments of [1]Medical Microbiology and [2]Pathology, Leiden University Medical Center, The Netherlands. Supported by a grant from the Foundation Microbiology Leiden.

Diagnostic Molecular Pathology. 2007 issue 16, pages 81-6.

Abstract.

Atypical mycobacterial skin infections are difficult to diagnose owing to their aspecific histopathologic presentations and to the presence of few bacteria. Therefore, these infections are often not recognized. Molecular detection of mycobacterial DNA has proven to be useful in clinical samples. The aim of this study was to investigate the incidence of mycobacterial involvement in skin biopsies showing granulomatous inflammation, using real-time polymerase chain reaction (PCR).
Real-time PCR specific for the genus *Mycobacterium* and the species *Mycobacterium avium* and *Mycobacterium haemophilum* was performed on formalin-fixed/paraffin-embedded biopsies from patients with granulomatous inflammation of the skin, from the period 1984 to 2004. A control group was assembled from patients with proven basal cell carcinoma. Amplicons of all positive reactions were sequenced to confirm or identify the mycobacterial species.
Of 30 patients, 13 (43%) were found to be positive for mycobacterial infection, of whom only 5 patients had been previously diagnosed with a mycobacterial disease. *M. haemophilum* was identified as the most common species (n=7). The other identified species were *Mycobacterium malmoense, Mycobacterium gordonae*, and *Mycobacterium marinum*. The results show that real-time PCR is useful in detecting mycobacterial infections in undiagnosed formalin-fixed / paraffin-embedded skin samples and that the application of molecular approaches would improve the diagnoses of mycobacterial skin infections.

Introduction.

Nontuberculous mycobacteria (NTM) cause a variety of diseases mostly in immunocompromised patients in lungs, skin, lymph nodes, bone, joints, and intestines. The incidence of NTM-infections has increased over the last few years, mainly in immunocompromised patients but also in immunocompetent. The increase may be related to the development of new diagnostic technologies (1). However, the true incidence is still probably many times higher than that clinically diagnosed.
Mycobacterium leprae (leprosy) and *Mycobacterium* ulcerans (Buruli ulcer) are the most notorious pathogens causing specific cutaneous infections, but other NTM like *Mycobacterium marinum*, *Mycobacterium avium*, and *Mycobacterium haemophilum* have highly variable clinical presentations. They can manifest as pustuls, hyperkeratotic papels, suppurative noduli, ulcerations, cellulitis, or erythematous plaques. The histologic picture is often a granulomatous lesion that can show variable forms of cellular infiltration (2,3). Owing to the aspecific presentation, clinical and histologic recognition of mycobacterial involvement is difficult. The conventional diagnostics for a mycobacterial infection in formalin-fixed/paraffin-embedded (FF/PE) materials is mostly restricted to acid-fast staining but this has an unsatisfying sensitivity. Therefore, a molecular approach is considered to be a valuable alternative method.

Recently, a real-time polymerase chain reaction (PCR) assay, specific for the genus *Mycobacterium*, which could detect the slow-growing species and the species *M. avium* and *M. haemophilum*, was developed in our hospital. Application of this test on cervical lymph node biopsies in comparison with acid-fast stain and culture revealed that the molecular approach had a significantly higher sensitivity (4). This assay helped to elucidate the incidence of slow-growing mycobacterial species-associated lymphadenitis in The Netherlands (5).
The aim of this study was to retrospectively apply the real-time PCR assay on FF/PE skin biopsies with histologic evidences of granulomatous inflammation and to compare the results with the earlier diagnostic examinations and clinical information.

Materials and Methods.

Patient materials.

A digital search was performed in the database of the department of pathology from our hospital. The database was searched from 1984 to 2004 for cases that consisted of a histologic diagnosis of granulomatous inflammation of the skin. Forty-two cases were identified; 12 of the 42 were excluded as either there was insufficient material or they could not be found in the archives. The clinical and histologic specifications of the remaining 30 patients who were included are listed in Table 2. The collection included materials from 14 male and 16 female patients. The patients' ages ranged from 14 to 81 with a medium age of 47 years. A control group was assembled from archival skin biopsies of 30 patients with diagnosed basal cell carcinoma (BCC). The patients were matched for age and the biopsies were matched for year of investigation and body location.

Conventional mycobacterial diagnostics.

In 5 cases, mycobacterial diagnostics had been requested at the time of the case presentations (Table 2). On the original biopsy materials, Ziehl-Neelsen (Zn) staining was performed and a culture at 35°C on solid Löwenstein-Jensen (LJ) medium, and, after the year 2000, also in liquid MGIT medium (Mycobacterial Growth Indicator Tube, Becton Dickinson). No *M. haemophilum* specific culture conditions were applied until 2004.

DNA-extraction.

The FF/PE biopsies were at least 1 mm^2 in size. Five to 10 sections of 10 µm were collected in sterile tubes for DNA extraction. The sections were cut after removal of the outer parts of the paraffin blocks. To avoid cross contamination, the microtome block was wiped with xylene and the blade was shifted or discarded with each new block. With every 8 samples, a negative control was added, which consisted of a paraffin block without tissue. Deparaffinization and tissue digestion was performed according to an in-house method using an overnight incubation at 56°C with proteinase K (Invitrogen, Breda, The Netherlands) (add per sample: 0.5 mg proteinase K, dissolved in 400 µL buffer solution with 0.01M Tris-HCl and

0.45% Tween 20). Then the paraffin was separated from the tissue cells by increasing the temperature to 95°C for 5 minutes, centrifuging, cooling at 4°C for 5 minutes. This allows the paraffin to coagulate and the transfer of the approximately 350 µL of the liquid volume to a clean tube. Subsequently, a second incubation with fresh proteinase K (add per sample: 0.2 mg proteinase K, dissolved in 50 µL buffer solution with 0.01M Tris-HCl and 0.45% Tween 20) was performed for 1 hour at 60°C for an extra rigorous digestion of the acid-fast wall of the intracellular bacilli. The total volume of 400 µL tissue cells and buffer was then used for DNA extraction. For 1 patient (patient 30), only sections on glass slides were available. These were scraped off the glass slide and were processed similarly for DNA extraction. The DNA-extraction protocol described by Boom et al (6) was followed. DNA was eluted in 50 µL TE and stored at 4°C before using in the PCR.

Table 1: sequences of primers and probes.

oligo	**sequence (5'-3') with attached fluorophore and quencher for probes**	**product size**
16S forward primer [a]	TAACACATGCAAGTCGAACG	ca 323 bp
16S reverse primer [a]	CCCATTGTGCAATATTCCCC	
ITS forward primer [b]	GGGGTGTGGTGTTTGAG	ca 170 bp
ITS reverse primer [b]	CTCCCACGTCCTTCATC	
genus-specific Taqman-probe [c]	FAM -TGGATAGTGGTTGCGAGCATC- Tamra	
M. avium specific molecular beacon [c]	FAM-CCCACCGGCCGGCGTTCATCGAAATGGTGGG-DabCyl	
M. haemophilum specific MinorGrooveBinder-probe [c]	VIC –ACGCCACCATTACT- MGB	

[a] 16S PCR used in early molecular identification of culture in patient 10.

[b] Genus and species specific real-time PCR retrospectively used for all specimens.

[c] Probes applied in combination with ITS forward and reverse primer.

Real-time PCR and nucleotide sequence analysis.

Real-time PCR was performed as described previously (4,7). Oligos used for real-time PCRs and for the sequencing of the internal transcribed spacer (ITS) and 16S rRNA gene are described in Table 1. The genus-specific PCR was performed twice for each sample, the *M. avium* and *M. haemophilum*-specific PCRs only once because of the limited amount of DNA extract.

When a positive signal was found in the real-time PCR, the amplicon was sequenced to determine the involved species or to confirm a species-specific signal. All positive PCR reactions were analyzed with gel-electrophoresis. Bands between 150 and 200 bp were subsequently cut out and purified with the Qiagen gel-extraction kit (Qiagen, Venlo, The Netherlands). These bands represent the amplified region of the mycobacterial ITS. Sequence reactions have been performed with the PCR primers using the Qiagen Big Dye terminator ready reaction kit (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and have been analyzed on an ABI 3100 automatic sequencer (Applied Biosystems).

Results.

Archival FF-PE materials from 30 patients were collected from the archives of the pathology department. All the available clinical and histologic information on these patients was collected and the data revealed 14/30 immunocompromised patients (Table 1). Overall, the materials from 13 of the 30 (43%) patients contained mycobacteria (or mycobacterial DNA), diagnosed by conventional methods or by real-time PCR. Table 3 describes the diagnostic results of 15 samples from the 13 positive patients. Fourteen samples from 12 patients showed a positive signal in the genus-specific PCR.

Amplicons formed in real-time PCR were sequenced to confirm or determine the species. All the amplicons but 1 (patient 3) were successfully sequenced and were recognizable as mycobacteria. The materials of 7 patients were positive for *M. haemophilum*, of which 3 were also directly identified in the *M. haemophilum*-specific PCR. No variation was found between the *M. haemophilum* sequences and 100% concordance was found with *M. haemophilum*-specific sequences in the NCBI BLAST database. The latter differentiated this species clearly from others. Other identified species were *Mycobacterium malmoense* (n=1), *Mycobacterium gordonae* (n=2), and *M. marinum* (n=1). Two sequences did not match any known sequences.

For several samples only 1 of the duplicate genus-specific reactions was positive. All of these samples had high Threshold cycle (Ct) values (Ct between 40 and 47). For the samples for which duplicate positive reactions were obtained, the Ct-values were lower (Ct between 32 and 41). The sequences of the positive duplicate reactions were identical.

Conventional mycobacterial diagnostics was requested for only 5 patient materials. All the 5 patients were subsequently diagnosed with a mycobacterial infection. Two were only positive in Zn staining (patients 8 and 28) and 3 were both Zn stain and culture positive (patients 7, 9, and 10). Discrepancies to the current molecular identification were found in the latter 3 patients: 2 patients (patients 7 and 10) were previously diagnosed with a *M. marinum* infection, but the real-time PCR assay identified *M. haemophilum* in both cases. One patient (patient 9) previously had a positive culture for *M. marinum* but yielded no positive signal in real-time PCR. Fresh sections were cut from the materials from these 3 patients and processed again. The new PCR results confirmed our initial PCR results. From 1 patient, 3 different materials were collected. The first sample dated from 1987 and 2 dated from 1993 but were from different locations of the body. All the 3 samples yielded positive signals in PCR but all with different sequences. The sample from 1987 yielded a *M. haemophilum*, 1 from 1993 resembled an *M. gordonae* and 1 sequence did not match any known sequence (Table 3).

A control group was assembled from the biopsies of 30 patients with histologic proven BCC. Of these, 2 materials yielded positive signals for the genus *Mycobacterium* (Ct values: 42.1 and 39.0) in one of the duplicate tests. From these 2 materials paraffin sections were prepared again and tested a second time in the real-time PCRs. Now only 1 sample yielded positive signals, twice in the genus-PCR and once in the *M. haemophilum*-specific PCR (Ct values: genus-specific PCR: 38.9 and 39.3, *M. haemophilum*-specific PCR: 41.1). The other

sample now remained consequently negative in all PCRs performed and could not be confirmed. However, it was only weakly positive the first time round (Ct value: 42.1). No positive reactions were obtained in the *M. avium*-specific PCR. Negative controls (paraffin blocks) remained negative in all reactions.

Discussion.

In the last few years, several molecular assays for the detection of mycobacteria in clinical materials have been developed. Especially, the diagnosis of *Mycobacterium tuberculosis* infection has improved considerably, mainly because of the ability of PCR to deliver results in a clinically relevant timeframe and the resulting improved sensitivity (8–12). For the detection of atypical mycobacteria, several PCR assays have been developed (4,13–17). Also for the detection of mycobacteria in FF/PE materials, molecular assays have been applied to improve the sensitivity of the diagnostics. However, a real-time PCR assay for the direct recognition of nontuberculous mycobacteria in FF/PE specimens has not been evaluated, as described previously.

From the 14 materials that yielded positive real-time PCR signals, 13 were successfully identified as mycobacteria by sequencing of the PCR amplicon. The obtained sequences of *M. haemophilum*, *M. malmoense*, and *M. marinum* were 100% identical to sequences in the NCBI database and were, therefore, clearly identifiable. The sequences of *M. gordonae* showed 99% and 97% homology to known sequences. Because the variability of the ITS sequences in this species is considerable, the obtained sequences can be considered representative for *M. gordonae*. For 2 patient materials, however, the obtained ITS sequences did not yield species identification. Both were recognizable as mycobacteria but could not be ascribed to 1 species. They both resembled the species *M. tuberculosis* and *Mycobacterium asiaticum* with 90% to 94% homology. Probably, as there were few bacteria available to be detected in the samples, several of the cases were on the detection limit of the assay (shown by the high Ct-values) and hence only positive in 1 PCR. However, comparison with the results from the control group shows that these results are associated with granulomatous inflammation. Moreover, a more sensitive assay would require a multi-copy target, which is not available for the detection and differentiation of slow-growing mycobacteria.

The risk of PCR inhibition has not been completely excluded. An internal control could be of benefit, but it could also affect the sensitivity of the assay and has, therefore, not been applied here. The sensitivity of the assay (both genus and species specific) has been determined previously to be 27 CFU per reaction in spiked pus (4). This explains the high Ct values encountered in several samples for which a more sensitive assay would provide stronger PCR reactions.

As the diagnosis of rapid-growers is less difficult, the assay applied here is mainly designed for slow-growers. The detection limit for several rapid-growing species like *Mycobacterium chelonae* can be completely different owing to sequence variability.

Several discrepancies were examined in more detail. In 2 patients *M. haemophilum* was identified by ITS sequencing; whereas, previously *M. marinum* infection had been diagnosed using conventional cultures not suitable for the recovery of *M. haemophilum*. In the first patient (patient 7), the species *M. marinum* was identified biochemically. For the second patient (patient 10) 16S sequencing of the cultured isolate revealed *M. marinum*. Both 16S and ITS are sufficiently discriminative. A possible explanation for these discrepancies is the presence of a mixed infection. Dual infections have been described before and it is suggested that superinfections of previously established granulomas can occur (18–21). For another patient a positive culture for *M. marinum* had originally been obtained but real-time PCR remained repeatedly negative, although it was suitable for the detection of this species. This could be caused by the degrading of DNA, a sensitivity problem or nonhomogenous distribution of the bacteria in the sample.

The prevention of laboratory contamination with mycobacterial bacilli or DNA has been carefully taken into account and precautionary measures have been applied as much as possible. Although it cannot be excluded, it is not expected that positive reactions, obtained during this study, are the result of previous contaminating handling of the materials because the sections that were cut out of the paraffin blocks had all been cleared from the outer parts. However, as no other diagnostic tool can distinguish between contaminant and true positive sample, this assay cannot either.

The control group was assembled from patients with diagnosed BCC. Because mycobacterial infections are not linked to specific histopathologic patterns (22), it is very difficult to exclude mycobacterial involvement in samples of a control group. Our aim of testing this control group was to confirm that the mycobacteria are not a contamination owing to the handling of the sample but are indeed present within the tissue. Two materials from the control group gave positive real-time PCR signals. This could indicate carriership or colonization. However, these patients have conclusive diagnoses of BCC, but seemed to have severe and variable skin disease. In the histologic description of the biopsies inflammatory infiltrates were found besides evidence of BCC. This could be indicative for an additional infection by atypical mycobacteria.

Mycobacterial infection has often been described in immunocompetent patients although the pathogenic properties of most atypical mycobacteria are unclear. In this test group 6 patients were found positive for mycobacterial skin involvement, which had no known underlying disease at the time of diagnosing the skin infection, but it remains possible that they had a genetic disorder predisposing for mycobacterial infections (23, 24) or a yet unrecognized underlying disease. Interestingly, such an immunocompromising condition was recognized in 2 patients after the onset of the skin inflammation.

Striking is the high prevalence of *M. haemophilum* in these patient materials. In 7 of 14 positive biopsies *M. haemophilum* was identified. This species is an important pathogen in cervicofacial lymphadenitis (5,7), has been described as cause of pneumonia (25,26), arthritis (25,26), and bacteremia (27) and is probably underestimated in skin infections as well. *M. haemophilum* was first described as the cause of skin inflammation in 1978 (28). Since then approximately 100 cases of *M. haemophilum* associated skin infection have been

described worldwide. In The Netherlands the first skin infection owing to *M. haemophilum* was described in 1997 (29). Other *M. haemophilum* infections occurred only sporadically until a series of *M. haemophilum*-associated lymphadenitis was recently discovered (7). The histologic manifestation of *M. haemophilum* is generally granulomas with giant cells, lymphocytes, and histiocytes. Necrosis can be absent. The number of bacteria is mostly low. Several publications suggest this species to be underdiagnosed (25,30,31), which is confirmed by the results described here. Although increasing number of laboratories are applying specific culturing methods suitable for the recovery of *M. haemophilum*, some laboratories still do not. In some cases when the culture becomes positive at 30 °C, it is assumed that *M. marinum* is involved without proper species identification (32). It is shown here that *M. haemophilum*-infections were present in The Netherlands years before the first diagnosis.

In conclusion, the application of molecular diagnostics can be useful to diagnose atypical mycobacterial skin infections in FF/PE patient materials. A higher awareness about mycobacterial involvement and the diagnostic possibilities should be raised by both clinicians and pathologists.

Acknowledgement.

The authors thank Dr P. Petit and P. Buijtels from the Vlietland Hospital, Schiedam, The Netherlands for paraffin sections and patient details.

Table 2: description of archival tissue samples and the available diagnostic data.

patient no.	year of biopsy	location biopsy	clinical diagnosis (previous) [a]	diagnosis pathology [b]	underlying disease [c]
1	1998	skin eyelid	furuncle	Zn-negative granulomatous inflammation	-
2	1987/1993	skin calf	recurrent nodular nonsuppurative panniculitis	Zn negative gran inflamm, dd TB or sarcoidosis	Weber-Christians Disease
3	1995	skin cervix	cystadenoma multiplex, -mucinose	granulomatous inflammation	-
4	1995	skin leg	vasculitis?	Zn-negative granulomatous inflammation	RA / Methrotrexaat
5	1995	skin arm	gran annulare, BCC, atyp myc?	Zn-negative granulomatous inflammation	osteoporose
6	1988	skin biopsy	exanthema, viral reaction.	Zn-negative granulomatous inflammation	-
7	1994	skin elbow	granuloma	granulomatous inflammation	-
8	1993	skin hand	chronic inflammation of the skin	gran inflamm, ZN positive fitting TB	-
9	2002	skin hand	swimming pool granuloma	swimming pool granuloma	DM II + kindeyfailure
10	2001	skin hand	mycosis or swimming pool granuloma	Zn-neg atyp gran inflamm, swimming pool granuloma?	-
11	1997	skin face	BCC	BCC, one lesion granulomatous inflamm	-
12	1997	skin cheek	dermatomycosis, CDLE??	Zn-neg, sarcoidosis/ leprosy/ gran inflamm	-
13	1999	skin back	leukemicskin infiltration	gran dermatitis	CLL
14	2000	skin cheek	naevus	Zn-neg, granulomatous dermatitis	-
15	1984	skin leg	vasculitis, panniculitis	mycosis	AML in 1993
16	1987	skin biopsy	epithial cyst	granulomatous inflammation	endometrium carcinoma in 1995
17	1994	skin cheek	actinic keratosis, eczema	granulomatous inflammation	-
18	1991	skin arm	kerato acanthoma	ulcerating granulomatous inflammation	kidney transplant
19	1988	skin arm	pseudolymphoma	gran inflammation reactive but secundary?	-
20	2001	skin wrist	chronic inflammation of the skin	Zn-neg, gran inflamm/ sarcoidosis	-
21	1996	skin side	vasculitis	chronic inflammation, persistent insect-bite	SLE
22	1986	skin biopsy	lichen nitidus/striatus	chronic inflammation	-
23	1992	skin eyelid	chalazion	chalazion	-
24	1989	skin eyelid	granuloma	chalazion (zn-neg)	-
25	1999	skin eyelid	granuloma	granulomatous inflammation (corpus alienum)	-
26	1998	skin eyelid	-	granulomatous inflammation (chalazion?)	-
27	1996	skin eyelid	chalazion	chalazion	RA
28	2002	skin arm/leg	chronic inflammation, sarcoidosis/ mycobacterial skin infection	Zn positive granulomatous inflammation	RA + lymphoedema / Methrotrex + prednisone
29	2004	skin arm/leg	vasculitis, sarcoidosis, infectious ??	ulcerating chronic and granulomatous inflammation	DM I
30	2004	skin hand	cellulitis + arthritis		RA / Methrotrexaat

[a] clinical diagnosis is given by a dermatologist and confirmation is requested from the pathologist. [b] diagnosis is determined by a pathologist by means of microscopical examination of the biopsy. [c] any given information about possible immune-influencing disease or treatment.

Table 3: description of results.

	previously performed conventional diagnostics [a]		retrospectively performed real-time PCR and sequencing of the obtained amplicons [b].			
patient no.	**Zn-stain**	**mycobacterial culture (and species identified)**	**PCR genus 1**	**PCR genus 2**	**PCR *M. haemophilum***	**sequencing of PCR product and BLAST results.**
2 (3 biopsies)	-	n.r.	-	38,3	-	*M. haemophilum:* 178/178 (100%) AY579400.1
	-	n.r.	38,9	38,7	-	*M. gordonae:* 134/138 (97%) AB026692.1
	-	n.r.	-	37,9	-	143/155 (92%) AY722098.1 *M. asiaticum*; 103/106 (97%) Z17211.1 *M. gastri*; 156/173 (90%) X58890.1 *M. tuberculosis*
3	n.r.	n.r.	47	-	-	no recognisable *M. spp*
7	+	+ (*M.marinum*)	41,9	40	-	*M. haemophilum:* 141/141 (100%) AY579400.1
8	+	-	-	45,5	-	*M. marinum:* 168/168 (100%) AJ315573.1
9	+	+ (*M.marinum*)	-	-	-	-
10	+	+ *(M.marinum*)	38,1	-	36,6	*M. haemophilum:* 110/110 (100%) AY579400.1
11	n.r.	n.r.	-	41	-	146/155 (94%) AY722098.1 *M. asiaticum*; 162/175 (92%) AE000516.2 *M. tuberculosis*
15	n.r.	n.r.	41,5	-	-	*M. haemophilum:* 178/178 (100%) AY579400.1
16	n.r.	n.r.	45,0	39,6	-	*M. malmoense:* 171/171 (100%) AF367025.1
25	n.r.	n.r.	-	36,8	-	*M. gordonae:* 161/162 (99%) AY604571.1
28	+	-	37,1	36	35,4	100% *M. haemophilum:* 130/130 (100%) AY579400.1
29	n.r.	n.r.	41,2	39,2	-	100% *M. haemophilum:* 144/144 (100%) AY579400.1
30	n.r.	n.r.	-	39,5	36,6	100% *M. haemophilum:* 140/140 (100%) AY579400.1

[a] conventional mycobacterial diagnostics performed by the department of pathology or medical microbiology. n.r. = no mycobacterial diagnostics requested.

[b] ITS directed genus-specific (performed twice) or *M. haemophilum*-specific real-time PCR result. Either negative or noted in Ct-value (the higher the Ct-value, the weaker the PCR reaction). Blast results are noted in length of successfully obtained bi-directional sequence and percentage of homology with the most likely Blast hits.

References: Chapter 5.

1. Wagner D, Young LS. Nontuberculous mycobacterial infections: a clinical review. Infection. 2004;32:257-70.
2. Bartralot R, Garcia-Patos V, Sitjas D, Rodriguez-Cano L, Mollet J, Martin-Casabona N, Coll P, Castells A, Pujol RM.Clinical patterns of cutaneous nontuberculous mycobacterial infections. Br J Dermatol. 2005;152:727-34.
3. Escalonilla P, Esteban J, Soriano ML, Farina MC, Piqu E, Grilli R, Ramirez JR, Barat A, Martin L, Requena L. Cutaneous manifestations of infection by nontuberculous mycobacteria. Clin Exp Dermatol. 1998;23:214-21.
4. Bruijnesteijn Van Coppenraet ES, Lindeboom JA, Prins JM, Peeters MF, Claas EC, Kuijper EJ. Real-time PCR assay using fine-needle aspirates and tissue biopsy specimens for rapid diagnosis of mycobacterial lymphadenitis in children. J Clin Microbiol 2004;42:2644-50.
5. Lindeboom JA, Prins JM, Bruijnesteijn van Coppenraet ES, Lindeboom R, Kuijper EJ. Cervicofacial lymphadenitis in children caused by *Mycobacterium haemophilum*. Clin Infect Dis. 2005;41:1569-75.
6. Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J. Rapid and simple method for purification of nucleic acids. J Clin Microbiol. 1990;28:495-503.
7. Bruijnesteijn van Coppenraet LE, Kuijper EJ, Lindeboom JA, Prins JM, Claas EC. *Mycobacterium haemophilum* and lymphadenitis in children. Emerg Infect Dis 2005; 11:62-8.
8. Shamputa IC, Rigouts And L, Portaels F. Molecular genetic methods for diagnosis and antibiotic resistance detection of mycobacteria from clinical specimens. APMIS. 2004;112:728-52. Review.
9. Palomino JC. Nonconventional and new methods in the diagnosis of tuberculosis: feasibility and applicability in the field. Eur Respir J. 2005;26:339-50.
10. Hazbon MH. Recent advances in molecular methods for early diagnosis of tuberculosis and drug-resistant tuberculosis. Biomedica. 2004;24 Supp 1:149-62. Review.
11. Hsiao PF, Tzen CY, Chen HC, Su HY. Polymerase chain reaction based detection of *Mycobacterium tuberculosis* in tissues showing granulomatous inflammation without demonstrable acid-fast bacilli. Int J Dermatol. 2003;42:281-6.
12. Van der Zanden AGM, Hoentjen AH, Heilmann FGC, Weltevreden EF, Schouls LM, van Embden JDA. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* complex in paraffin embedded tissues and in stained microscopic preparations. J Clin Pathol Mol Pathol 1998:51;209-214.
13. Stauffer F, Haber H, Rieger A, Mutschlechner R, Hasenberger P, Tevere VJ, Young KK. Genus level identification of mycobacteria from clinical specimens by using an easy-to-handle *Mycobacterium*-specific PCR assay. J Clin Microbiol. 1998;36:614-7.
14. Park H, Jang H, Kim C, Chung B, Chang CL, Park SK, Song S. Detection and identification of mycobacteria by amplification of the internal transcribed spacer regions with genus- and species-specific PCR primers. J Clin Microbiol. 2000 Nov;38(11):4080-5. Erratum in: J Clin Microbiol 2001;39:828.
15. Schulz S, Cabras AD, Kremer M, Weirich G, Miethke T, Bosmuller HC, Hofler H, Werner M, Fend F. Species identification of mycobacteria in paraffin-embedded tissues: frequent detection of nontuberculous mycobacteria. Mod Pathol. 2005;18:274-82.
16. Totsch M, Bocker W, Brommelkamp E, Fille M, Kreczy A, Ofner D, Schmid KW, Dockhorn-Dworniczak B. Diagnostic value of different PCR assays for the detection of mycobacterial DNA in granulomatous lymphadenopathy. J Pathol. 1996;178:221-6.
17. Bascunana CR, Belak K. Detection and identification of mycobacteria in formalin-fixed, paraffin-embedded tissues by nested PCR and restriction enzyme analysis. J Clin Microbiol. 1996;34:2351-5.
18. Mahaisavariya P, Chaiprasert A, Khemngern S, Manonukul J, Gengviniij N, Ubol PN, Pinitugsorn S. Nontuberculous mycobacterial skin infections: clinical and bacteriological studies. J Med Assoc Thai. 2003;86:52-60.
19. Hawrot AC, Carter EL. Simultaneous chronic cutaneous infection with *Mycobacterium* marinum and Nocardia asteroides. J Am Acad Dermatol. 2005;52:703-4.

20. Cosma CL, Humbert O, Ramakrishnan L. Superinfecting mycobacteria home to established tuberculous granulomas. Nat Immunol. 2004;5:828-35.
21. Ganesan S, Thirlwall A, Brewis C, Grant HR, Novelli VM. Dual infection with atypical mycobacteria and *Mycobacterium* tuberculosis causing cervical lymphadenopathy in a child. J Laryngol Otol. 2000;114:649-51.
22. Busam KJ, Kiehn TE, Salob SP, Myskowski PL. Histologic reactions to cutaneous infections by *Mycobacterium haemophilum*. Am J Surg Pathol. 1999;23:1379-85.
23. Ottenhoff TH, Verreck FA, Hoeve MA, van de Vosse E. Control of human host immunity to mycobacteria. Tuberculosis (Edinb). 2005;85:53-64.
24. de Jong R, Altare F, Haagen IA, Elferink DG, Boer T, van Breda Vriesman PJ, Kabel PJ, Draaisma JM, van Dissel JT, Kroon FP, Casanova JL, Ottenhoff TH. Severe mycobacterial and Salmonella infections in interleukin-12 receptor-deficient patients Science. 1998;280:1435-8.
25. Shah MK, Sebti A, Kiehn TE, Massarella SA, Sepkowitz KA. *Mycobacterium haemophilum* in immunocompromised patients. Clin Infect Dis. 2001;33:330-7.
26. Saubolle MA, Kiehn TE, White MH, Rudinsky MF, Armstrong D. *Mycobacterium haemophilum*: microbiology and expanding clinical and geographic spectra of disease in humans. Clin Microbiol Rev. 1996;9:435-47. Review.
27. Sturenburg EE, Horstkotte MA, Aberle J, Meyer K, Richter E, Laufs R, Sobottka I. Disseminated *Mycobacterium haemophilum* infection as initial manifestation of AIDS. Tuberculosis (Edinb). 2004;84:341-5.
28. Sompolinsky D, Lazgiel A, Naveh D, et al. *Mycobacterium haemophilum* sp. nov., a new pathogen of humans. Int J Syst Bacteriol 1978;28:67 75.
29. Kuijper EJ, Wit FW, Veenstra J, Bottger E. Recovery of *Mycobacterium haemophilum* from a skin infection in an HIV-1-infected patient after the start of antiretroviral triple therapy. Clin Microbiol Infect.1997;3:584-585.
30. Sampaio JL, Alves VA, Leao SC, De Magalhaes VD, Martino MD, Mendes CM, Misiara AC, Miyashiro K, Pasternak J, Rodrigues E, Rozenbaum R, Filho CA, Teixeira SR, Xavier AC, Figueiredo MS, Leite JP. *Mycobacterium haemophilum*: emerging or underdiagnosed in Brazil? Emerg Infect Dis. 2002;8:1359-60.
31. Dobos KM, Quinn FD, Ashford DA, Horsburgh CR, King CH. Emergence of a unique group of necrotizing mycobacterial diseases. Emerg Infect Dis. 1999;5:367–78.
32. Ang P, Rattana-Apiromyakij N, Goh CL. Retrospective study of *Mycobacterium* marinum skin infections. Int J Dermatol. 2000;39:343-7.