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Short Communication

Applicability of In-House Loop-Mediated Isothermal Amplification for Rapid Identification of *Mycobacterium tuberculosis* Complex Grown on Solid Media

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SUMMARY: A simple, rapid, and low-cost identification method is required in tuberculosis high-burden countries. We report the applicability of in-house loop-mediated isothermal amplification (LAMP) targeting 16S ribosomal RNA for the rapid identification of *Mycobacterium tuberculosis* complex grown on Lowenstein-Jensen media. Eighty acid-fast staining-positive clinical isolates were selected and used to evaluate the LAMP assay in comparison with polymerase chain reaction and conventional culture-based tests. The LAMP assay identified 60 *M. tuberculosis* isolates from 80 clinical isolates using simple heat-extracted DNA directly from the colony suspension. The results were in complete agreement with those obtained using the other methods, and the utility of the direct LAMP assay from a colony was demonstrated. The LAMP assay appears to be a practical and low-cost method that can be used for the rapid identification of *M. tuberculosis* isolates and suitable for endemic low-resource settings.

Tuberculosis (TB) is still the most common deadly infectious disease worldwide. In Thailand, almost 130,000 people suffer from active TB and approximately 11,000 deaths occur annually. The nation ranks 18th on the list of 22 "TB high-burden countries" in the world (1). Although most mycobacterial infections are still caused by *Mycobacterium tuberculosis* complex (MTC), non-tuberculous mycobacteria (NTM) have been documented to cause a number of human pulmonary infections in developed and developing countries (2-3). The increasing incidence of TB and NTM infections caused by the acquired immunodeficiency syndrome (AIDS) epidemic has resulted in the need for rapid and accurate identification of isolates grown on media so that appropriate treatment can be prescribed.

The culture and identification of MTC is still the gold standard for diagnosing TB, although MTC can be directly identified from clinical specimens using genetic methods such as polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP) because these methods cannot differentiate between live and dead TB bacilli. However, conventional identification methods depending on biochemical assays and the phenotypic examination of colony formation on solid media are labor-intensive and time-consuming owing to the slow growth rate of MTC (4). In addition, the results of biochemical tests are sometimes inconclusive because of their low sensitivity and reproducibility. Thus, a rapid means of identifying MTC is essential for enhancing diagnostic services in mycobacteria laboratories and for improving the management of patients. PCR-based methods for the detection of MTC grown on Lowenstein-Jensen (L-J) media have been reported and proposed as an alternative method (5). Till date, the use of traditional nucleic acid amplification appears to be restricted to the laboratory setting, equipment, and technical expertise. The immunochromatography test (ICT) is commercially available and is widely used for the rapid confirmation of *M. tuberculosis* cultures (6-7). Although ICT is an easier method for the detection of MTC from culture samples, adequate growth with prolonged incubation is necessary to avoid false negative results (8). In recent years, several new molecular techniques, including LAMP, have been developed and used for *M. tuberculosis* detection (9-12). Compared with modern molecular methods, which are relatively complex and economically unsuitable for laboratories with limited resources, LAMP is a low-cost

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molecular assay that combines specificity and sensitivity. The advantages of the LAMP technique include its simplicity, i.e., isothermal amplification at a constant temperature of 60°C-65°C without any need for sophisticated equipments such as a thermal cycler, and the direct visual inspection of gene amplification in the reaction tube as opposed to analysis by gel electrophoresis (13-17). The positivity of the reaction can be easily detected by the naked eye as a color change or the observation of a white precipitate (15,17). Because of its simplicity and cost effectiveness, LAMP is a promising molecular technique that could be readily applied to the rapid detection and identification of *M. tuberculosis* in resource-limited settings.

This study aimed to assess the performance and applicability of in-house LAMP, known as TB-LAMP, to the rapid and accurate identification of MTC grown on L-J medium, which is used commonly in conventional mycobacterial culture. Sputum samples were collected, decontaminated by N-acetyl-L-cysteine-NaOH treatment, inoculated onto L-J slants, and examined for growth or contamination at 37°C (4). The bacteria that grew on L-J medium were examined to determine their colony morphology, growth rate, pigmentation, and Ziehl-Neelsen staining results. DNA was extracted from colonies recovered from L-J media that were positive for acid-fast bacilli (AFB) using a simple boiling method. In brief, putative small colonies were picked and suspended in a microcentrifuge tube containing 100 μl distilled water. DNA was extracted by boiling the cell suspension using a dry heat block at 80°C for 10 min. After brief spinning at 2,000 × g for 5 s, an aliquot of cell lysate was added directly to the LAMP reaction mixture as described in previous studies (12,18). In brief, LAMP for MTC was performed in a 20-μl reaction mixture, which contained 2.0 μl of 10 × LAMP buffer [200 mM Tris-HCl (pH 8.8), 100 mM KCl, 100 mM NH_4SO_4, and 1% Triton X-100], 14 mM dNTPs, 0.8 M betaine, 300 mM $MgSO_4$, 30 pmol FIP and BIP primers, 20 pmol of FL and BL primers, 5 pmol F3 and B3 primers, 8 U *Bst* DNA polymerase (New England Biolabs, Inc., Ipswich, Md., USA), 1 μl Fluorescent Detection Reagent (FDR; Eiken Chemical Co., LTD., Tokyo, Japan) and 7 μl cell lysate. The set of 6 primers used in this study was described in our previous studies (12,18). After incubation at 65°C for 1 h in a small heat block, DNA amplification was detected in LAMP reactions as a color change from orange to green using the naked eye (Fig. 1). The results were compared with those of multiplex PCR analysis, which could differentiate between MTC and NTM in a single tube, as well as classical biochemical tests, which comprised niacin accumulation, nitrate reduction, catalase production, and susceptibility to paranitrobenzoic acid (PNB) when heavy growth of each isolate was achieved (4).

The specificity and sensitivity of TB-LAMP were intensively examined in our previous study of direct detection from clinical specimens (12) and liquid culture (18). TB-LAMP was specific to MTC and could directly detect as few as 9 tubercle bacilli in sputum samples. However, the direct detection of MTC from clinical specimen cannot differentiate between living and dead TB bacilli, while liquid culture examination is not sustainable in many developing countries because of its

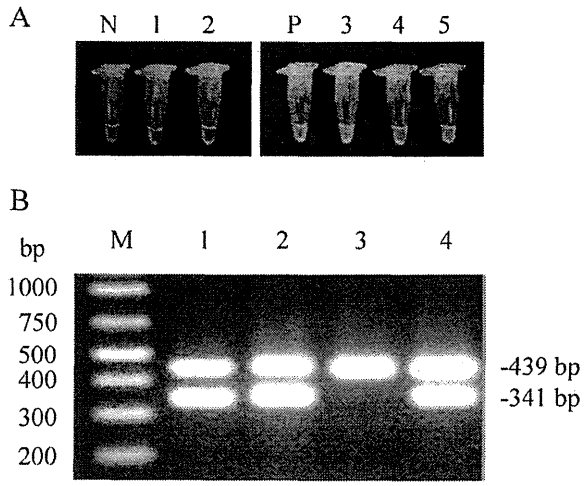

Fig. 1. Identification of *M. tuberculosis* complex isolates by TB-LAMP and multiplex PCR.

DNA extracted by heat lysis from mycobacterial clinical isolates positive for acid-fast bacilli was used for TB-LAMP and multiplex PCR reactions. *M. tuberculosis* complex and non-tuberculous mycobacteria were identified by visual observation of the color change in LAMP and by agarose gel electrophoresis in multiplex PCR. A. Visual appearance of LAMP results showing N: negative control, 1-2: non-tuberculous mycobacterial isolates, P: positive control, and 3-5: *M. tuberculosis* complex isolates. B. Multiplex PCR results analyzed by agarose gel electrophoresis. A 439-bp DNA band specific for *Mycobacterium spp.* reported to contain NTM in the samples. PCR positive for 439 bp specific for *Mycobacterium spp.* and 341 bp specific for MTC reported as containing MTC in the samples. Lane M: DNA marker; Lane, 1, 2 and 4: PCR product from *M. tuberculosis* complex isolates and Lane 3: PCR product from non-tuberculous mycobacterial isolates.

cost, although it is recommended in many settings. Solid culture examination is still considered essential, and it has been used in many developing countries. Thus, we examined the feasibility of TB-LAMP for the detection of MTC in early culture-positive samples. Using TB-LAMP, 60 clinical isolates, which were presumed to be MTC on the basis of visual observations of the colonies, were rapidly identified as MTC by TB-LAMP. As expected, the remaining 20 isolates, which were presumed to be NTM, yielded negative results with TB-LAMP. The TB-LAMP results were in complete agreement with the multiplex PCR results (Fig. 1). In addition, all the isolates with the biochemical characteristics of MTC and susceptibility to PNB exhibited positive results by TB-LAMP (Table 1). There were no false-positive identification results using TB-LAMP among all 20 NTM isolates (Table 1). These concordant identification results confirmed the specificity of TB-LAMP for MTC, and the simple DNA extraction from the tiny colony on L-J medium at the beginning of culture growth made identification by this method much quicker than that by the combination of biochemical tests and susceptibility to PNB. In addition, the overall procedure of the TB-LAMP assay allowed *M. tuberculosis* identification to be completed in less than 2 h without any requirements of expensive or complex instruments. The LAMP system uses a simple aluminum heat block that can be powered by a handy battery, therefore, it can be used in difficult settings that experience power interrup-

Table 1. Concordance of identification results by TB-LAMP with conventional methods

	M. tuberculosis complex*	Non-tuberculous mycobacteria
TB-LAMP		
Positive	60 (100%)	0 (0%)
Negative	0 (0%)	20 (100%)

*Isolates were identified by conventional methods, multiplex PCR, biochemical tests, and PNB susceptibility.

tions. This study clearly demonstrated the utility of TB-LAMP for the rapid identification of MTC grown on L-J medium. Conventional identification has a long delay because of the need for heavy growth and labor-intensive procedures; however, it could be substituted with this alternative nucleic acid isothermal amplification method. The preparatory steps required to extract DNA for the LAMP reaction in a simple heating method also reduced the infection risk and the cost of the test. Conventional nucleic acid amplification tests are not widely used in developing countries because of contamination by carry-over products. An advantage of LAMP is that the results can be observed without any further analysis unlike conventional nucleic acid amplification tests. Naked-eye observation of the color change in the reaction mixture without opening the lid of the reaction tube reduces the risk of cross-contamination.

TB is the most common mycobacterial infection in developing countries; therefore, this simple and economic identification method based on TB-LAMP could be suitable for use in any laboratories that perform mycobacterial culture.

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Conflict of interest None to declare.

Ethics approval Not applicable as this study used clinical isolates.

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MOLECULAR ASPECT

Characterization of extensively drug-resistant *Mycobacterium tuberculosis* in Nepal

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SUMMARY

The emergence of extensively drug-resistant tuberculosis (XDR-TB) has raised public health concern for global control of TB. Although molecular characterization of drug resistance-associated mutations in multidrug-resistant isolates in Nepal has been made, mutations in XDR isolates and their genotypes have not been reported previously. In this study, we identified and characterized 13 XDR *Mycobacterium tuberculosis* isolates from clinical isolates in Nepal. The most prevalent mutations involved in rifampicin, isoniazid, ofloxacin, and kanamycin/capreomycin resistance were Ser531Leu in *rpo*B gene (92.3%), Ser315Thr in *katG* gene (92.3%), Asp94Gly in *gyr*A gene (53.9%) and A1400G in *rrs* gene (61.5%), respectively. Spoligotyping and multilocus sequence typing revealed that 69% belonged to Beijing family, especially modern types. Further typing with 26-loci variable number of tandem repeats suggested the current spread of XDR *M. tuberculosis*. Our result highlights the need to reinforce the TB policy in Nepal with regard to control and detection strategies.

1. Introduction

Worldwide emergence of multi- and extensively drug-resistant tuberculosis (MDR and XDR-TB) has become a major obstacle to TB control. XDR-TB is a form of TB caused by *Mycobacterium tuberculosis* (MTB) strains, which is resistant to isoniazid (INH) and rifampicin (RIF), defined as multidrug-resistant MTB (MDR-MTB), as well as fluoroquinolone (FQ) and any of the second-line anti-TB injectable drugs, amikacin (AMK), kanamycin (KAN), or capreomycin (CAP). By the end of 2010, 68 countries had reported at least one case of XDR-TB.[1] XDR-TB is the result of an adverse treatment outcome of MDR-TB; many cases are never diagnosed due to limitations in laboratory capacity to test for second-line drug resistance.[2] Treatment of XDR-TB patients is more challenging and less successful than that of patients with other types of TB.[3] An extremely high death rate from XDR-TB was reported in patients co-infected with HIV in South Africa.[2,4]

In Nepal, TB is a major public health problem. The incidence of all forms of TB was estimated to be 173/100,000 population while the incidence of new smear-positive cases was at 77/100,000 in 2008. The four surveys conducted between 1996 and 2007 have indicated the fluctuating prevalence of MDR-TB among new cases of between 1.1% and 3.7% (1.1% in 1996, 3.7% in 1999, 1.4% in 2001 and 2.9% in 2007). The latest estimate of MDR-TB is 2.9% and 11.7% among new and recurrent cases, respectively.[1,5,6] Although the prevalence of drug-resistance confirming mutations in MDR-TB isolates in Nepal have been reported recently,[6] to the best of our knowledge, no published data on mutations and genotypes of XDR-MTB strains are currently available from Nepal.

Molecular epidemiological studies of *M. tuberculosis* strains have identified variability in the phylogeography of strains globally.[7,8] Beijing strains are most prevalent globally and also associated with enhanced acquisition of drug resistance; however their resistance patterns varied regionally.[9] Drug resistance in *M. tuberculosis* is commonly caused by mutations in various genes. Previous works have indicated that mutations within 81-bp core region of the RNA polymerase β-subunit gene (*rpo*B) gene are the cause of RIF resistance in more than 90% of cases.[10,11] In contrast, several different loci are known to be involved in INH resistance,

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especially *katG* and *inhA*.[6,10] Mutations in a conserved quinolone resistance-determining region (QRDR) of the *gyrA* or *gyrB* genes encoding DNA gyrase are often involved in fluoroquinolone (FQ) resistance.[12] Resistance to aminoglycosides (KAN and AMK) and CAP is attributed to mutations in 16s rRNA (*rrs*) gene.[13,14]

The present study documents drug resistance-associated mutations in XDR isolates from Nepal. To gain an insight into the epidemiology of these isolates, genotyping by using spoligotyping, multilocus sequence typing (MLST) and variable number of tandem repeats (VNTR) were also performed.

2. Materials and methods

2.1. M. tuberculosis isolates

A total of 109 MDR *M. tuberculosis* clinical isolates were randomly selected from isolates bank at German Nepal Tuberculosis Project (GENETUP), Nepal, collected over a 3-year period from 2007 to 2010. Each isolates were recovered from individual patients with pulmonary TB.

2.2. Antibiotic susceptibility testing

Testing for susceptibility to first- and second-line drugs was carried out at GENETUP using the conventional proportional method on Löwenstein–Jensen medium according to the World Health Organization guidelines[15] with the following critical drug concentrations: INH (Cat No. 2261/0801; Fatol Arzneimittel GmbH, Schiffweiler, Germany); 0.2 μg/ml, RIF (Cat No. 004030; Fatol); 40 μg/ml, streptomycin (STR) (Cat No. S6501; Sigma–Aldrich, St. Louis, MO); 4 μg/ml, ethambutol (EMB) (Cat No. 1237/0806; Fatol); 2 μg/ml, ofloxacin (OFX; Cat No. 08757; Sigma–Aldrich); 2 μg/ml, KAN (Cat No. 60615; Sigma–Aldrich); 30 μg/ml and CAP (Cat No. C4142; Sigma–Aldrich); 40 μg/ml.

2.3. DNA extraction

DNA was prepared for PCR by mechanical disruption, as described previously.[6] Briefly, the colonies were suspended in TE buffer consisting of 10 mM Tris–HCl (pH 8.0) and 1 mM EDTA in a 2 ml screw-cap vial, one-fourth of which was filled with 0.5 g glass beads (0.1 mm) (Bio Spec Products Inc., Bartlesville, OK). Mycobacterial cells were disrupted by shaking with 0.5 ml chloroform on a cell disrupter (Micro smash; Tomy Seiko Co. Ltd., Tokyo, Japan) for 1 min. After centrifugation, the DNA in the upper layer was concentrated by ethanol precipitation and dissolved in 100 μl TE buffer.

2.4. PCR amplification and DNA sequencing of drug resistance-associated genes

PCR reactions were performed in a 20 μl mixture consisted of 0.25 mM each of dNTPs, 0.5 M betaine, 0.5 μM of each primer (Primers for rrs, gyrA and gyrB in Table 1 and those in Poudel A et al.[6] for *rpoB*, *katG* and *inhA* gene segment amplification). One U GoTaq DNA Polymerase (Promega, Madison, WI), GoTaq buffer and 1 μl DNA template. The reactions were carried out in a thermal cycler (Bio-Rad Laboratories, Ipswich, MA) under the following conditions: initial denaturation at 96 °C for 60 s followed by 35 cycles of denaturation at 96 °C for 10 s, annealing at 55 °C for 10 s and extension at 72 °C for 30 s with a final extension at 72 °C for 5 min. PCR products were sequenced according to the manufacturer's instructions with the same primers used for PCR and the Big Dye Terminator v3.1 Cycle Sequencing Kit (Life Technologies Corp., Carlsbad, CA) using an ABI PRISM 3130*xl* Genetic Analyzer (Life Technologies Corp.). The resulting sequences were compared with wild-type sequences of *M. tuberculosis* H37Rv using Bio-Edit software (version 7.0.9) (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

Table 1
Nucleotide sequence of primers used for PCR and sequencing.

Locus	Primer	Nucleotide sequence (5′–3′)	Target region	Product size (bp)
gyrA	TB *gyrA* S	AGCGCAGCTACATCGACTATGCG	220–339	321
	TB *gyrA* AS	CTTCGGTGTACCTCATCGCCGCC		
gyrB	TB gyrB S	CGGCACGTAAGGCACGAGAG	1373–1770	398
	TB *gyrB* AS	GAACCGGAACAACAACGTCAAC		
rrs	TB *rrs* S	AGTCCCGCAACGAGCGCAACCC	1350–1550	665
	TB *rrs* AS	GATGCTCGCAACCACTATCCA		

2.5. Phylogenetic markers

Spoligotyping was performed according to the standard protocol.[16] and the spoligotype in the binary format was compared with the SpolDB4 database.[17] Another molecular epidemiological investigation was performed by PCR amplification of the 26 variable *M. tuberculosis* microsatellites and assigned an allele number based on the number of repeats as described previously.[18] A combined spoligotype-VNTR UPGMA3 dendrogram was computed and drawn using Bionumerics 6.0 version software (Applied Maths, Sint-Marten-Latems, Belgium). MLST targeting 10 chromosomal positions were performed according to Filliol et al.[19]

3. Results

3.1. Drug-susceptibility patterns

Among 109 MDR-MTB isolates obtained, 13 were found to be XDR (Table 2). Three of the patients having XDR-TBs (84, 90 and 123) were naive for MDR treatment. Of the remaining 96 isolates, 41, 1, and 1 were mono-resistant to OFX, KAN, and CAP, respectively, and categorized as pre-XDR-MTB.

3.2. Geographical distribution of XDR M. tuberculosis isolates

The XDR-MTB isolates were originated from patients living in five main cities of Nepal (Figure 1): Kathmandu ($n = 7$), Pokhara ($n = 3$), Butwal ($n = 1$), Bhairahawa ($n = 1$) and Dhangadhi ($n = 1$). The number of XDR-TB in Kathmandu correlates well with its high population.

3.3. Mutations identified in the rpoB, katG, inhA, gyrA, gyrB and rrs genes

Sequence analysis identified the most frequent mutations conferring Ser to Leu amino acid substitution at position 531 (Ser531Leu) in *rpo*B (12/13), Ser315Thr in *katG* (12/13), Asp94Gly in *gyrA* (7/13), and a mutation from A to G at nucleotide position 1400 (A1400G) in *rrs* (9/13). Other mutations with lower rates were seen in *rpo*B (Asp516Val; 1/13), *inhA* regulatory region (C-15T; 1/13), *gyrA* (Ser91Pro; 1/13, Asp94Ala; 2/13, Asp94Asn; 1/13, Asp94His; 1/13, and Asp94Tyr; 1/13), and *rrs* (C1401T and G1483T; two each), while none had mutations in the quinolone resistance-determining region of *gyr*B (Table 2).

3.4. Spoligotyping and MLST

Among XDR-TB isolates, Spoligotyping revealed the predominance of Beijing family strains (9/13). In addition, 1 strain of CAS

Table 2
Antimicrobial susceptibility profile and mutation pattern of the different drug-target genes or regions among XDR isolates.

Strain no.	Drug susceptibility profile*							Mutation pattern in different drug-target genes or regions†					Spoligotype based clade with ST	Geographical location	Age of patient
	RFP	INH	STR	EMB	OFX	KAN	CAP	*rpoB*	*katG*	*inhA* regulatory region	*gyrA*	*gyrB*	*rrs*			
84	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt§	Asp94Gly	wt	A1400G	Beijing (Modern)	Kathmandu	21
86	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Gly	wt	C1401T	Beijing (Modern)	Kathmandu	16
90	R	R	R	R	R	R	R	Asp516Val	Ser315Thr	wt	Ser91Pro	wt	A1400G	Beijing (Ancient)	Kathmandu	26
103	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Gly	wt	C1401T	Beijing (Modern)	Kathmandu	24
108	R	R	S	S	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Gly	wt	A1400G	CAS	Kathmandu	40
118	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Gly	wt	A1400G	Beijing (Modern)	Kathmandu	25
123	R	R	R	R	R	R	R	Ser531Leu	wt	C-15T	Asp94Asn	wt	G1483T	Beijing (Modern)	Kathmandu	21
139	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Ala	wt	A1400G	Beijing (Modern)	Pokhara	25
140	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Ala	wt	A1400G	T2	Pokhara	33
142	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Tyr	wt	G1483T	New	Pokhara	45
151	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94His	wt	A1400G	T1	Bhairahawa	40
155	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Gly	wt	A1400G	Beijing (Modern)	Butwal	18
161	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Gly	wt	A1400G	Beijing (Modern)	Dhangadhi	32

* INH, isoniazid; RFP, rifampicin; STR, streptomycin; EMB, ethambutol; OFX, ofloxacin; KAN, kanamycin; CAP, capreomycin; R, resistant; S, susceptible.
† Mutations in rpoB, katG and gyrA are presented as amino acid changes with codon position; mutations in rrs gene and inhA promoter region are presented as nucleotide changes with mutation position.
§ WT, wild type.

family, 2 strains of T family (T1 and T2) and 1 strain of undefined type were also identified. MLST confirmed 8 isolates with Beijing spoligotype belonged to modern types (Table 2).

3.5. Cluster analysis by VNTR

VNTR typing grouped the isolates into seven unique patterns and two clusters (Figure 2). Each cluster contained three isolates of the Beijing family. Among the clustered isolates, 86 and 103 in a cluster (cluster 1) had the same profile of drug resistance-associated mutations (*rpoB*-Ser531Leu, *katG*-Ser315Thr, *gyrA*-Asp94Gly, and *rrs*-C1401T), whereas 84 carried a distinct mutation in *rrs* (A1400G). Similarly, 118 and 161 in another cluster (cluster 2) had the same profile of drug resistance-associated mutations (*rpoB*-Ser531Leu, *katG*-Ser315Thr, *gyrA*-Asp94Gly, and *rrs*-A1400G) and 123 showed a distinct mutation pattern (C-15T at *inhA* regulatory region instead of *katG*-Ser315Thr for INH resistance, *gyrA*-Asp94Asn for FQ resistance, and *rrs*-G1483T for KAN/CAP resistance).

4. Discussion

In this study, we investigated drug resistance-associated mutations and genotypes of XDR-MTB isolates in Nepal. This study also raises concerns over the high proportion of pre-XDR-TB in Nepal. The high rate of pre-XDR-MTB isolates implied the inappropriate usage of drugs, especially FQs, including OFX. OFX is the most commonly prescribed antibiotic for respiratory tract infection in Nepal and this might lead to the emergence of pre-XDR-TB with resistance to OFX. As drug resistance in *M. tuberculosis* is due to the stepwise accumulation of mutations in the genome, this pool of pre-XDR-MTB isolates are always at the risk of developing XDR-TB.

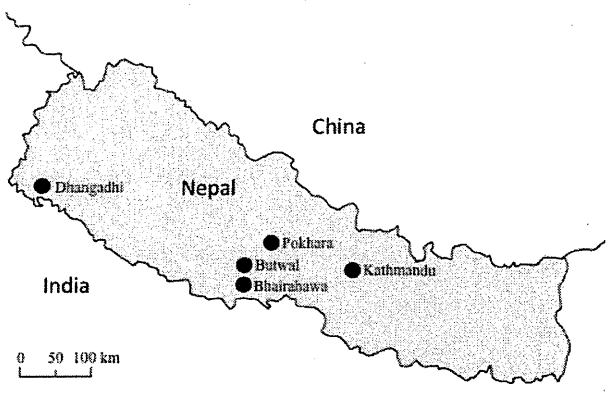

Figure 1. Geographical location of XDR-TB isolation. Cities where XDR-MTB has been isolated are indicated by a closed circle.

Sequence analysis of the hot spot regions of various genetic loci showed that the most common mutations among XDR isolates were Ser531Leu of *rpoB*, Ser315Thr of *katG*, Asp94Gly of *gyrA* and A1400G of *rrs* for RIF, INH, OFX and KAN/CAP resistance, respectively. Other studies have also reported similar mutations among XDR-TB isolates from different countries.[20–23] As mutations such conferring amino acid substitutions, Ser531Leu in *rpoB* and Ser315Thr in *katG* with low fitness costs are known to dominate the drug-resistant isolates.[24]

Genotyping of the isolate by spoligotyping and MLST pointed out the predominance of strains belonging to the modern type Beijing genotypes. The similar involvement of XDR-MTB by modern type Beijing genotypes has been reported from South Africa,[4] India,[20] and China,[23] while the ancient type Beijing family predominates in Japan.[25] Over-representation of Beijing genotype in XDR-MTB in this study compared to the lower prevalence of this genotype in non-MDR and MDR isolates (33 and 51%, respectively; data not shown) supported the previous study that this genotype has been associated with drug resistance,[4,26] because of its higher mutation rates and lower fitness costs with specific mutations.[24] The significantly low average age of patients suffering from Beijing genotype MTB compared to patients suffering from MTB with other genotypes (23.1 ± 4.8 vs 39.5 ± 4.9 years old; Table 2) may suggest the higher transmissibility of Beijing genotype XDR-MTB among the young generation because of their frequent movement.[27]

Although the numbers of isolates were small, complete matches of VNTR, including three hypervariable loci (QUB 11a, QUB 3232, QUB 3336) and drug resistance-associated mutations between two isolates in each cluster, suggested the possible transmission of XDR-TB in Nepal. MDR treatment of a patient who was the source of strain No. 103 started 3rd, April 2006 and the duration of MDR treatment was 12 month. In contrast, that of strain No. 86 started 29th, October 2009 and the duration of MDR treatment was 1 month. By these facts, we arrived at the idea that patient with strain No. 103 might be a source of transmission of XDR-TB and that with

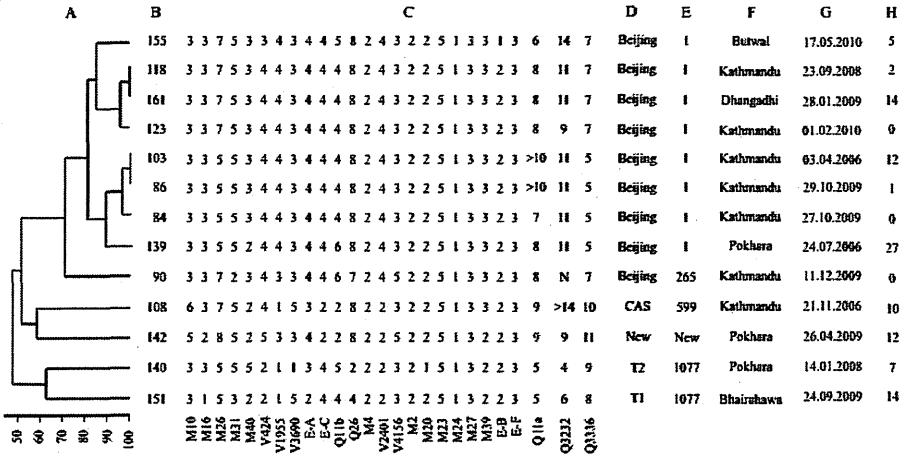

Figure 2. Dendrogram and schematic representation of VNTR typing and spoligotyping results obtained with 13 XDR-TB isolates in Nepal. Column A: dendrogram (UPGMA method, distance matrix average of spoligotyping-based and VNTR) built with Bionumerics version 6, B: strain identification, C: 26 loci VNTR results, D: spoligotyping-based defined clades; E: spoligotyping international type, F: geographical location, G: MDR treatment start date and H: duration of MDR treatment.

strain No. 86 might be a recipient. Alternatively, there might be common transmission source(s) to these patients. Situation was different in another cluster. MDR treatment of a patient who was the source of strain No. 118 started 23rd, September 2008 and the duration of MDR treatment was 2 month and primary XDR-TB was suspected. In contrast, that of strain No. 161 started 28th, January 2009 and the duration of MDR treatment was 14 month. The existence of common infection source of these strains was supposed. The transmission of XDR-TB was also speculated from the fact that three patients (from whom strain No. 84, 90 and 123 were isolated) were naive for MDR-TB treatment. It is interesting that transmission of XDR-TB were speculated not only within Kathmandu but also between Kathmandu and Dhangadhi, apart more than 650 km (Figure 1). As Kathmandu is the capital of Nepal and people come and go frequently from different parts of Nepal, transmission between people living in Kathmandu and those living far from Kathmandu might be possible. Indeed, the patient from whom strain No. 161 was isolated has a history of traveling to Kathmandu. The possibility of transmission of XDR-TB seemed to be high, especially in cluster 1 (including strains No. 86 and 103), because the *rrs*-C1401T mutation carried by both strain No. 86 and 106 was rare between KAN/CAP-resistant isolates.[10,13] On the other hand, care should be taken when concluding XDR-TB transmission in cluster 2 (including strains No. 118 and 161) as both of the mutations, *gyrA*-Asp94Gly and *rrs*-A1400G, have been reported to be rather common in OFX- and KAN/CAP-resistant MTB, respectively, and the distance between the two cities is great. The high rate of pre-XDR-TB in MDR-TB might suggest the acquisition of XDR phenotype during successive transmission as these strains belong to the Beijing family, known to have higher mutation-acquiring capacity. The high number of MDR-TB patients who stop treatment in Nepal could also explain this high drug resistance acquisition rate.[5] Both the possibility of direct transmission and acquired resistance should be considered equally for XDR-TB in Nepal.

5. Conclusion

The majority of XDR-MTB isolates in this study belonged to the Beijing family. Infections of this family were more common among younger generation than those belonging to other spoligotype families. In addition, the identical pattern of VNTR and drug resistance-associated mutations suggested the possible transmission of Beijing genotype XDR-MTB among people in Nepal. Our findings emphasize the urgent need to identify patients suffering from XDR-TB with Beijing genotype MTB and to treat them in isolated wards for a better control program to prevent the spread of this incurable disease.

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◆特集／肉芽腫のすべて

肉芽腫症としてのハンセン病の病態と臨床

四津里英*　石井則久**

Key words：組織球性肉芽腫，マクロファージ(macrophage)，らい菌(*Mycobacterium leprae*)，類上皮細胞性肉芽腫，Ridley-Jopling 分類

Abstract　ハンセン病は，*Mycobacterium leprae* を病原菌とし，肉芽腫を形成する慢性感染症である．皮膚と末梢神経を主に侵し，それらの症状が診断のポイントとなる．らい菌自体に毒力はほとんどなく，生体からもらい菌を排除する際のらい菌特異的免疫機能がその臨床像および病理組織像に深く関わる．従って，病理組織所見を観察することは重要で，個体のらい菌特異的免疫機能の差異により組織球性肉芽腫像と類上皮細胞性肉芽腫がみられる．前者ではらい菌が多く存在，後者ではらい菌の存在を証明することは困難である．病理組織像を含めて，皮疹やらい菌の検出，末梢神経の病像を総合してハンセン病と診断する．治療は，リファンピシン，ダプソン，クロファジミンによる多剤併用療法が行われる．

治療薬がない時代には，顔面・四肢の変形，失明など多くの後遺症を残した疾患である．日本では，強制隔離政策が 1930 年代～1996 年まで行われていたことから，社会的問題としても重要な疾患である．

はじめに

ハンセン病は抗酸菌の一種であるらい菌(*Mycobacterium leprae*；*M. leprae*)による慢性感染症である[1]．臨床症状は多彩で，これはらい菌に対する生体の免疫反応が多様であることによる．菌の生体に対する直接障害よりも宿主の免疫・炎症による組織障害，特に末梢神経障害とその後遺症が大きな問題となり，偏見・差別にもつながってきた．病理学的には肉芽腫の形成が特徴的である．

ハンセン病の病態の理解には免疫応答と，肉芽腫形成の機序を解明することが必要である．

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感染源と感染経路

ハンセン病の病原菌であるらい菌は発育至適温度が 30～33℃である．毒力はほとんどなく，感染力は極めて弱いため，乳幼児期にらい菌に大量・頻回に曝露されることが発症に結びつく(呼吸器感染)．鼻粘膜である程度増殖した菌は，血中に入り，温度の比較的低い皮膚および表在末梢神経に定着し細胞内に寄生する．しかし，発症には宿主となるヒトの免疫状態も関係するため，必ずしも感染があったからといって発症はしない．また潜伏期間は数年～数十年と長い．

症状と病型分類

宿主のらい菌に対する特異的な免疫状態により症状は多彩である[2][3]．この免疫状態の違いに基づいて分類を行ったものが Ridley-Jopling 分類である(表 1，図 1)．

らい菌に対する細胞性免疫がほとんど欠如して

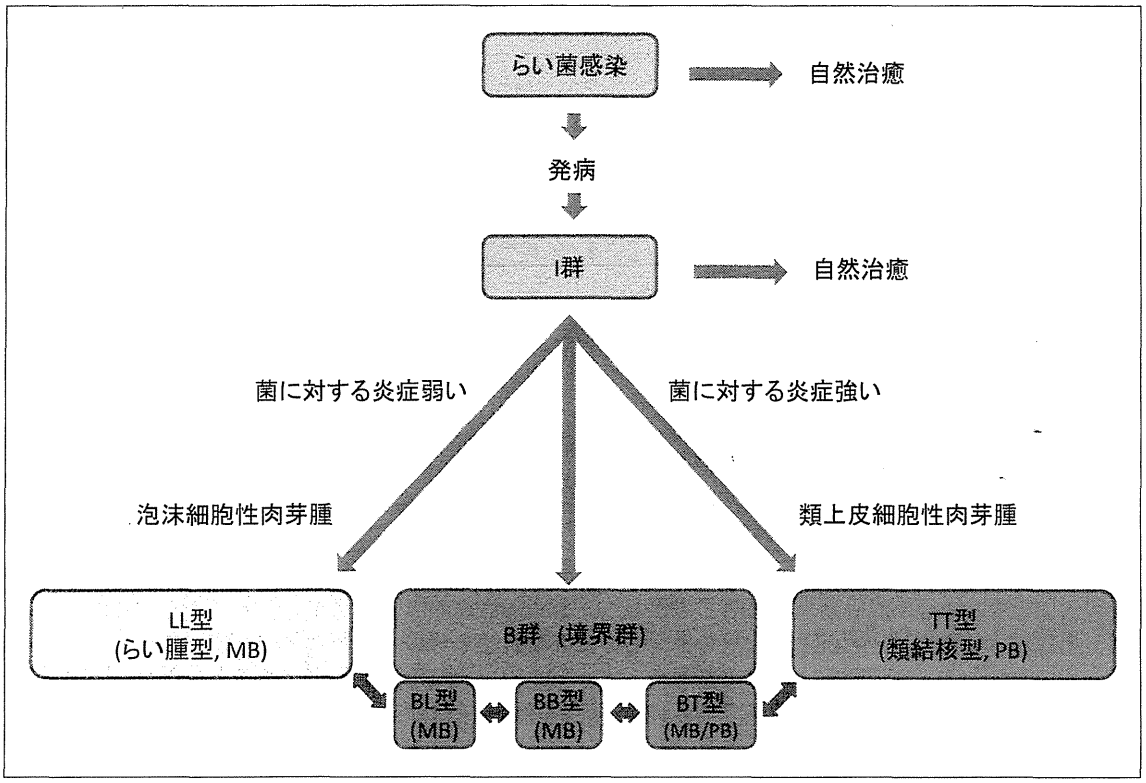

図 1. ハンセン病の発症と病型

表 1. ハンセン病の病型分類

菌数による分類	少菌型 (paucibacillary : PB)	多菌型 (multibacillary : MB)
免疫学的分類 (Ridley-Jopling 分類)	(I 群) TT 型	B 群 (BT 型 BB 型 BL 型) LL 型
らい菌に対する細胞性免疫能	良好	低下/なし
皮膚スメア検査	陰性	陽性
らい菌	少数/発見しがたい	多数
皮疹の数	少数	多数
皮疹の分布	左右非対称性	左右対称性
皮疹の性状	斑(環状斑)	紅斑(環状斑), 丘疹, 結節
皮疹の表面	乾燥性, 無毛	光沢, 平滑
皮疹部の知覚障害	高度 (触覚, 痛覚, 温度覚)	軽度/正常
病理所見	類上皮細胞性肉芽腫 巨細胞, 神経への細胞浸潤	組織球性肉芽腫 組織球の泡沫状変化
病理でのらい菌	陰性	陽性
主たる診断根拠	皮疹部の知覚障害	皮膚スメア検査などでのらい菌の証明
治療(multidrug therapy : MDT)	WHO/MDT/PB 6 か月間 リファンピシン, DDS	WHO/MDT/MB 1~3 年間 リファンピシン, DDS, クロファジミン

いるらい腫型(lepromatous leprosy type ; LL)では, 左右対称性の結節や紅斑が主症状である(図2). 末梢神経は初期には目立たないが, 徐々に左右対称性に知覚(触・痛・温度覚), 運動覚が障害されていく.

類結核型(tuberculoid type ; TT)では, 細胞性免疫の一部が残っており, 左右非対称, 皮疹は数個, 境界明瞭な環状紅斑や脱色素斑を主症状とし, 多くの場合皮疹にほぼ一致して末梢神経障害の合併がある(図3).

その両方の特徴を持ち合わせるのが境界群(borderline group)であり, LL 型に近いかあるいは TT 型に近いかで BL 型(図4), BB 型, BT 型(図5)に分類される. なお, 発症初期は未定型群(indeterminate group ; I)と分類される.

一方で, 途上国の現場で診断・治療選択を容易にするための分類として WHO 分類がある. この分類では, 病変部からの菌数により多菌型(multibacillary ; MB)と少菌型(paucibacillary ; PB)に分類する. MB は Ridley-Jopling 分類の LL, BL, BB, 一部の BT に, PB は I, TT, 一部の BT にそれぞれ該当する(表1).

ハンセン病とマクロファージ

病気の原因はらい菌であるが, 病態あるいは病像は宿主(個々人)の免疫能を反映しているため,

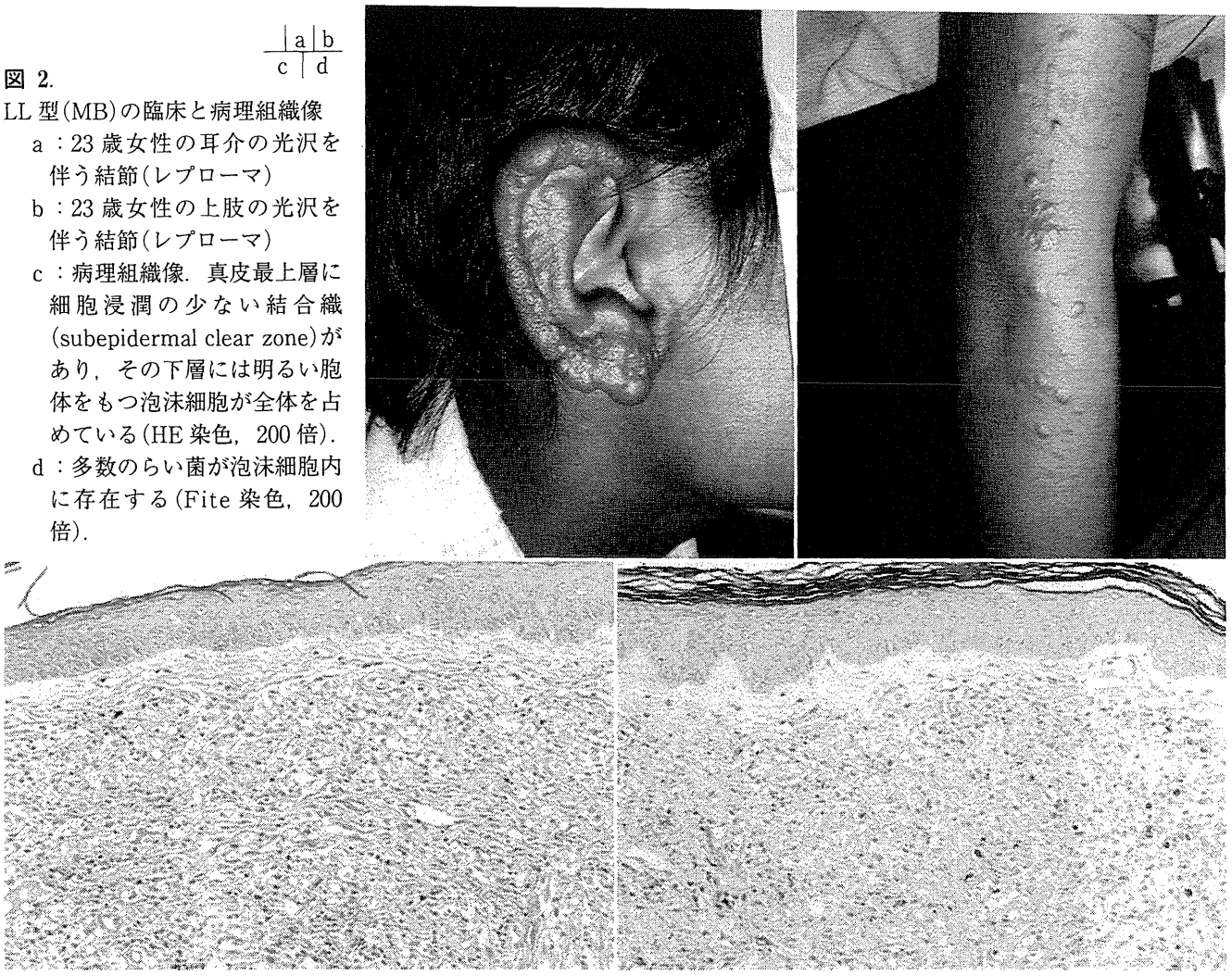

図 2.
LL 型(MB)の臨床と病理組織像
a：23 歳女性の耳介の光沢を伴う結節(レプローマ)
b：23 歳女性の上肢の光沢を伴う結節(レプローマ)
c：病理組織像．真皮最上層に細胞浸潤の少ない結合織(subepidermal clear zone)があり，その下層には明るい胞体をもつ泡沫細胞が全体を占めている(HE 染色，200 倍)．
d：多数のらい菌が泡沫細胞内に存在する(Fite 染色，200 倍)．

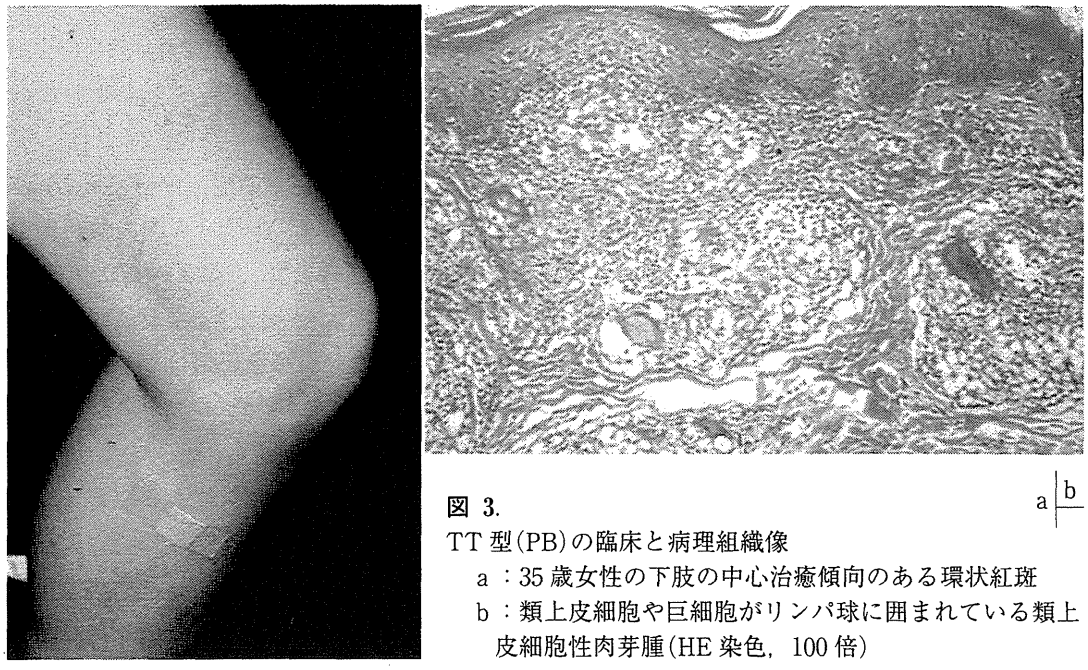

図 3.
TT 型(PB)の臨床と病理組織像
a：35 歳女性の下肢の中心治癒傾向のある環状紅斑
b：類上皮細胞や巨細胞がリンパ球に囲まれている類上皮細胞性肉芽腫(HE 染色，100 倍)

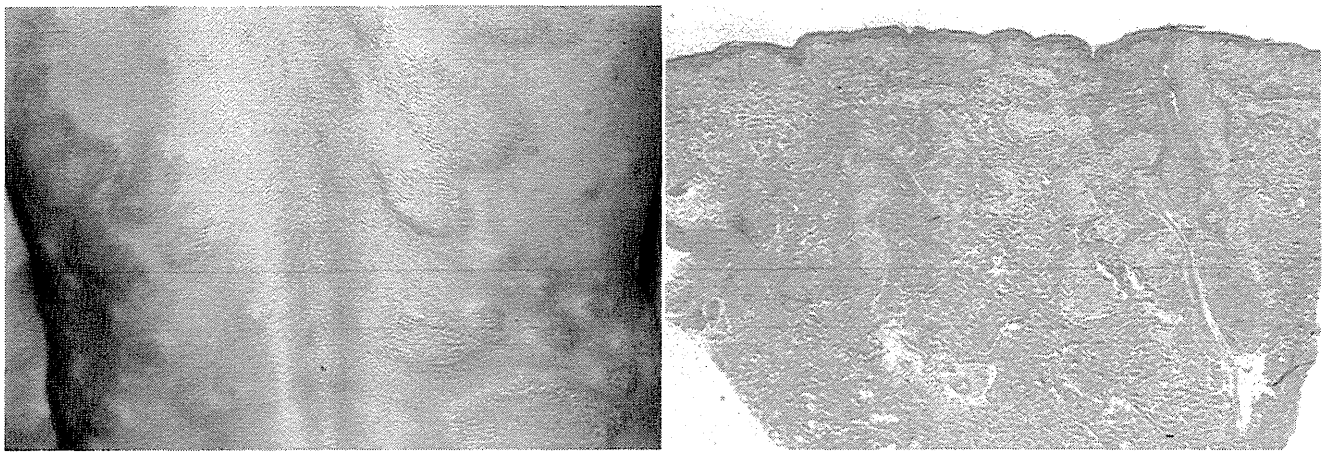

図 4. BL 型(MB)の臨床と組織像 a|b

a：48 歳男性の背部．左右対称性で湿潤をふれる紅斑局面．一部は中心治癒傾向がある．

b：泡沫細胞が巣状に散在する(HE 染色，100 倍)．

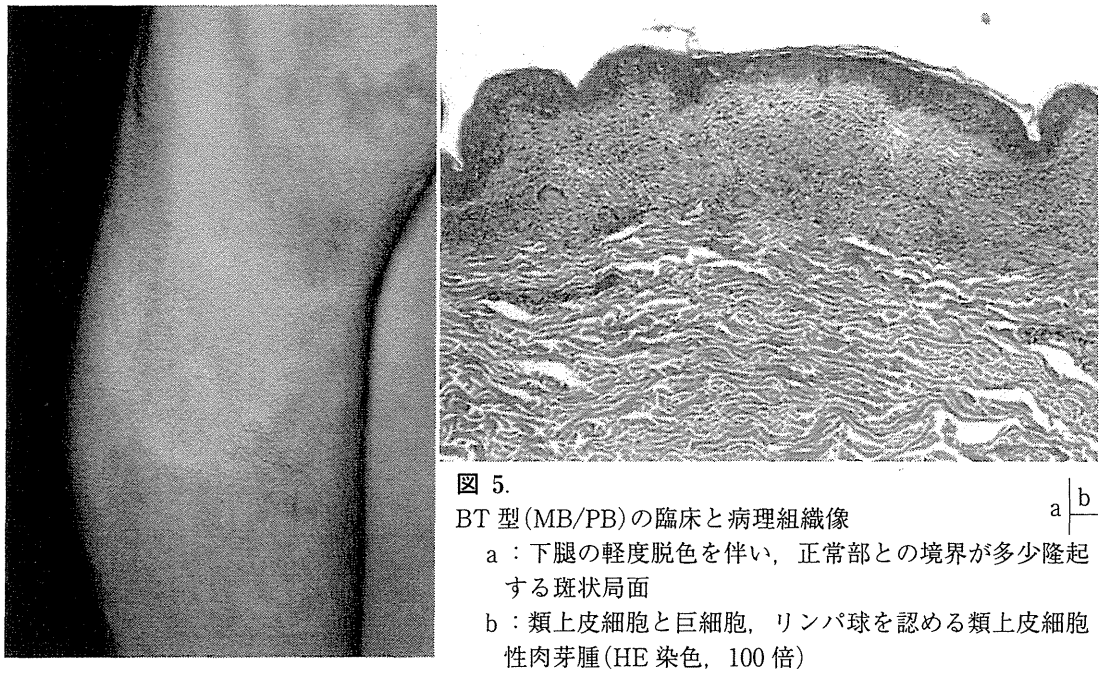

図 5.

BT 型(MB/PB)の臨床と病理組織像 a|b

a：下腿の軽度脱色を伴い，正常部との境界が多少隆起する斑状局面

b：類上皮細胞と巨細胞，リンパ球を認める類上皮細胞性肉芽腫(HE 染色，100 倍)

ハンセン病は「免疫病」ととらえることもできる．らい菌の排除には自然免疫と獲得免疫の双方が関与している．

1．自然免疫

生体に入ったらい菌は，まずマクロファージに取り込まれる(図 6)[4)~6)]．その際，マクロファージの細胞膜上に発現している toll-like receptor2 (TLR2)によって，らい菌膜表面の脂質成分を認識し，炎症性サイトカインやインターフェロンなどが放出される．これに対して，マクロファージの細胞膜の裏打ち蛋白である coronin，actin binding protein，1A(CORO1A)は，貪食されたらい菌を取り込んだファゴソームの膜に移行し，種々の消化酵素をもつライソゾームとの融合を阻止する．そのため，らい菌はプロセシングを受けにくい状態になり，マクロファージ内での増殖が可能となる．LL 型患者では，この病態が持続・慢性化し，特徴的な臨床像および後述する病理組織像へと進展する．

自然免疫反応において，らい菌の殺菌に関与する TLR2 と，マクロファージ内生存をサポートする CORO1A の相互関係によって，らい菌の動態が左右されると考えられるが，それを制御している因子については不明である．

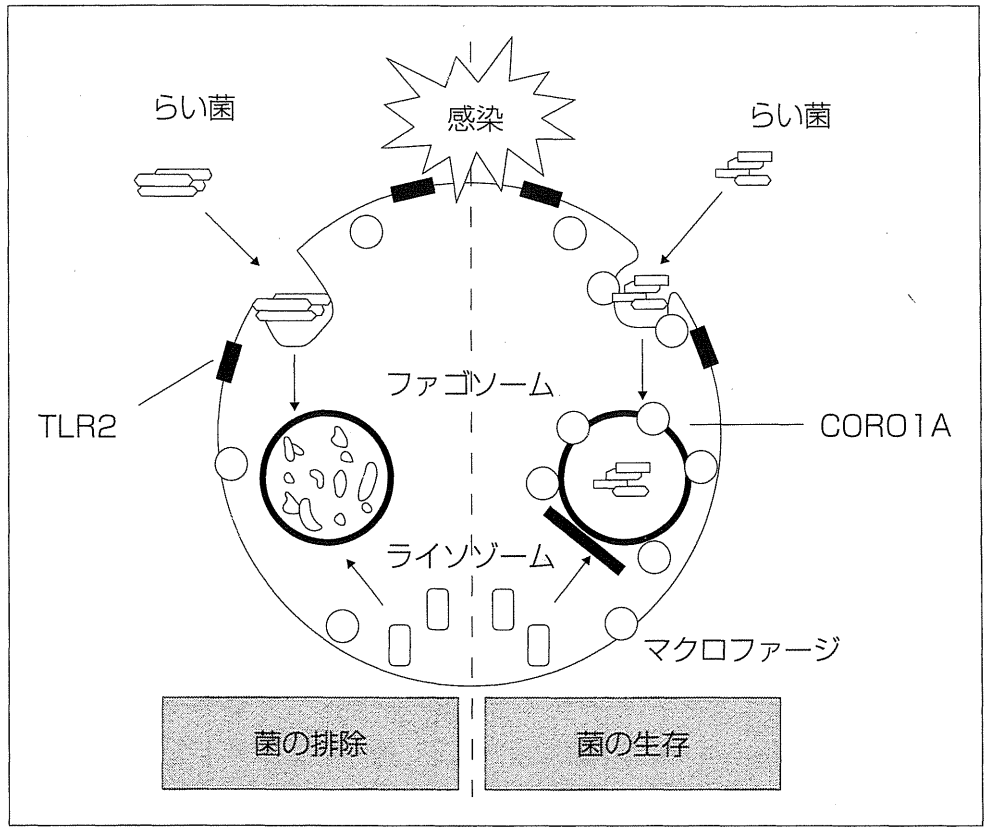

図 6.
らい菌のマクロファージ内での動態(日ハンセン病会誌, 77：57, 2008 より引用)

2．獲得免疫

マクロファージ内に取り込まれたらい菌はエンドゾームに入り，プロセシングの過程で細分化され，主要組織適合抗原(major histocompatibility complex；MHC，ヒトでは human leukocyte antigen；HLA)と結合して，マクロファージ細胞表面に発現されると考えられる．HLA-A(あるいは B，C)と結合し細分化されたらい菌由来抗原は，$CD8^+$ T 細胞を活性化させ，キラー T 細胞としてらい菌排除に働く．一方，HLA-DP(あるいは DQ，DR)と結合して細分化されたらい菌由来抗原エピトープ(HLA-A などと結合する成分とは異なる)は，$CD4^+$ T 細胞を活性化させ，他の T 細胞の機能を活性化させるヘルパー T 細胞として働く．

さらに，らい菌の存在により一部のマクロファージ類似(貪食活性がほとんどない)の CD1a 陽性の樹状細胞(dendritic cells)が活性化し，上記のマクロファージの活性化と同様に，$CD4^+$ T 細胞および $CD8^+$ T 細胞が活性化され，らい菌に対する免疫反応を惹起する．

マクロファージによる自然免疫はどの病型でも共通する免疫応答反応だが，通常この獲得免疫は，TT 型や B 群内の TT 型により近縁の病型(BT 型など)の患者において認められる反応である．

免疫反応からみたハンセン病

遺伝素因にある程度の関連があったうえで，らい菌が生体に侵入すると，まず自然免疫が働き，多くの場合でらい菌は排除される(自然治癒)．排除されないらい菌については獲得免疫で排除される．ただし，なんらかの関与(遺伝的あるいは宿主免疫能など)で CD1a 陽性の樹状細胞が活性化されない場合，獲得免疫能が不完全になり，ハンセン病を発症し，その程度で病型が決定されるのではないかと推測されている[5)7)]．これら獲得免疫は T 細胞を中心とした免疫応答であることから，ハンセン病の病態発現には，細胞性免疫が重要な役割を果たしていると言える．この各個人のらい菌に対する細胞性免疫能の程度で，ハンセン病にもいくつかの病型を認めることができ，それを分類したのが前述した Ridley-Jopling 分類に当たる(表 1，図 1)．なお，らい菌に対する個々人の免疫能の程度が，感受性遺伝子に規定されるの

か，あるいは他の因子によるかは不明である．

TT 型の皮疹部では Th1 細胞および I 型サイトカイン（IFN-γ，IL-2，IL-12 など）が優位である[8)9)]．活性化したマクロファージや組織球はらい菌を貪食し，殺菌・消化し，さらに貪食しきれなかった細胞を取り囲み，互いに密着し，肉芽腫を形成する．これは Th1 サイトカインと細胞性免疫によって強化される肉芽腫反応であり，結核菌を代表とする抗酸菌などの非消化性異物に対する代表的な，肉芽腫を形成することにより病原体を封じ込めようとする生体防御反応である[10)]．TT 型ハンセン病でみられる類上皮細胞性肉芽腫は，結核結節，サルコイド結節（明らかな病因は不明だが，なにかしらの病原体が関与している可能性が指摘されている[11)]），異物肉芽種などでみられる類上皮細胞とほぼ同じ細胞構成成分からなり，完成度が極めて高い．単球やマクロファージと類上皮細胞によりらい菌処理が進めば炎症は収束する．病変部はしだいに線維性組織になり，瘢痕治癒の形をとるが，線維化による組織障害の一部は不可逆的である．

一方，LL 型の皮疹部では Th2 細胞，$CD8^+$T 細胞，II 型サイトカイン（IL-4，IL-5，IL-10 など）が優位である[12)]．そのため細胞性免疫は抑制され，phenolic glycolipid（PGL）などの液性免疫が増強しているとされる．らい菌に対する抗体産生が TT 型ではほとんど認められないのが，LL 型ではこれが認められる病態を示す証拠の一つだが，残念なことにここで産生された液性抗体はらい菌の排除に働かない．そのため，らい菌を多数細胞内に取り込んだマクロファージが増加し，びまん性に拡大浸潤していく組織球性肉芽腫像となる．

病理組織検査

病変部皮膚の肉芽腫の状態や，らい菌との関係を検討する．

1）I 群（PB）：特異的な像はなく，真皮の血管周囲や神経，付属器の周囲にリンパ球などの軽度非特異的な細胞浸潤が認められ，肉芽腫もらい菌も認めない．

2）LL 型（MB）：らい菌を多数取り込んだマクロファージが泡沫細胞として認められ（組織球性肉芽腫），リンパ球はほとんど認めない（図 2）．多数の泡沫細胞が真皮全体を占める場合もあるが，真皮の最上層が細胞浸潤のない結合織の層（subepidermal clear zone）として残る（図 2-c）．らい菌を多数認め，泡沫細胞内には多数のらい菌がいる（globi）（図 2-d）．

3）TT 型（PB）：真皮に類上皮細胞や巨細胞が，多数のリンパ球に取り囲まれて存在する類上皮細胞性肉芽腫であるが，乾酪壊死を認めることは稀である（図 3）．末梢神経にも浸潤細胞を認める．らい菌は認めない．

4）BL 型：泡沫状ないし空胞状の組織球を比較的多数認め，リンパ球も少数認める．らい菌は多数認める（図 4）．

5）BT 型（MB/PB）：TT 型に類似する．リンパ球浸潤が軽度，巨細胞が少ないなどが，鑑別点である．らい菌を認める場合と認めない場合がある（図 5）．

診断と治療

ハンセン病の診断は ① 知覚低下した皮疹，② 末梢神経の障害（知覚低下，運動障害，神経肥厚など），③ らい菌の検出（皮膚スメア検査，病理組織抗酸菌染色，PCR 法），④ 病理組織所見，を総合して診断する．

治療は WHO が提唱する多剤併用療法（multidrug therapy；MDT）を参考に行う．リファンピシン（RFP），ジアフェニルスルホン（DDS，ダプソン），クロファジミン（CLF）が主であるが，その他クラリスロマイシン（CAM），ミノサイクリン（MINO），キノロンなども有効である[13)]．

さいごに

ハンセン病は，世界的にみるといまだ毎年約 22 万人の新規発症のある肉芽腫を呈する疾患であり，世界的な現場に行った際，皮膚科医として本

疾患の基礎から臨床，さらに社会的な問題などの知識を有していることは必須である[14]．また，日本においては歴史的に決して忘れてはならない疾患であること，また今後も在日外国人患者をみる機会があるので，ポイントを押さえて覚えておいて頂きたい．

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◆特集／必読！　知っておくべき感染症

抗酸菌感染症

常深祐一郎*　石井則久**

Key words：抗酸菌(acid fast bacillus)，結核(tuberculosis)，非結核性抗酸菌症(non-tuberculous mycobacteriosis)，ハンセン病(Hansen's disease)，BCG(Bacille Calmette-Guérin)，ブルーリ潰瘍(Buruli ulcer)，クオンティフェロン(QuantiFERON®)

Abstract　抗酸菌感染症には結核，非結核性抗酸菌症，ハンセン病が含まれる．抗酸菌感染症は臨床症状が多岐にわたり，臨床像だけからの診断は困難である．そのため，疑わなければ検査をせず，検査をしなければ診断はつかない．必ず鑑別診断のなかに入れておかなければならない疾患群である．本稿では抗酸菌感染症を疑う臨床像とその際に行う検査について述べる．さらに最近のトピックスとして，BCG接種後副反応と接種時期の問題，難治性潰瘍を呈する抗酸菌感染症であるブルーリ潰瘍，新しい結核診断法のクオンティフェロンの原理と解釈について解説する．抗酸菌感染症を頭の片隅にいつも置いておき，見慣れない臨床像，難治な皮膚病変に遭遇したときには思い出せるようにしておきたい．

はじめに

抗酸菌感染症に含まれる範囲は広く，結核，非結核性抗酸菌症，ハンセン病からなる[1)~3)]．広く浅くすべてを述べることは本稿の意図するところではないため，まず最も基本となる抗酸菌感染症を疑う臨床像と検査方法について述べ，加えて最近のトピックスを取り上げる．

抗酸菌感染症を疑う臨床像

皮膚結核では，赤褐色丘疹，鱗屑・痂皮を伴う中央萎縮性の局面，疣状の局面，結節，潰瘍，膿瘍，瘻孔などがみられる．非結核性抗酸菌症では，紅色丘疹，結節，膿瘍，潰瘍，瘻孔，皮下結節，疣状局面を呈し，リンパ管に沿った配列をとることがある．ハンセン病では，境界明瞭または不鮮明な紅斑，環状の紅斑，浸潤性紅斑，丘疹，結節，脱色素斑，知覚低下を伴う皮疹，神経肥厚がみられる．神経障害の徴候として繰り返す熱傷や外傷も大切である．また，皮疹の急激な発赤，浮腫や末梢神経麻痺の増悪，結節性紅斑にも注意する(らい反応)[4)]．ただし極言すると抗酸菌感染症に特異的な皮疹はないため，常に鑑別診断に入れておくことが重要である．筆者の経験した抗酸菌感染症の臨床像を列挙するが，これをみてもさまざまな臨床像を呈することが分かる(図1～7)．難治な丘疹・結節や潰瘍，通常の抗菌薬に反応しない発赤腫脹，熱感を伴わない膿瘍などは要注意である．

抗酸菌感染症を診断するための検査[1)~4)]

抗酸菌感染症を疑った場合に行う検査について述べる(図8)．特殊な検査も含まれるため，検体を採取する前に，検査室や検査会社，依頼先の専門施設などにあらかじめ問い合わせて，採取・保存条件や輸送容器などの情報を確認しておくことが大切である．

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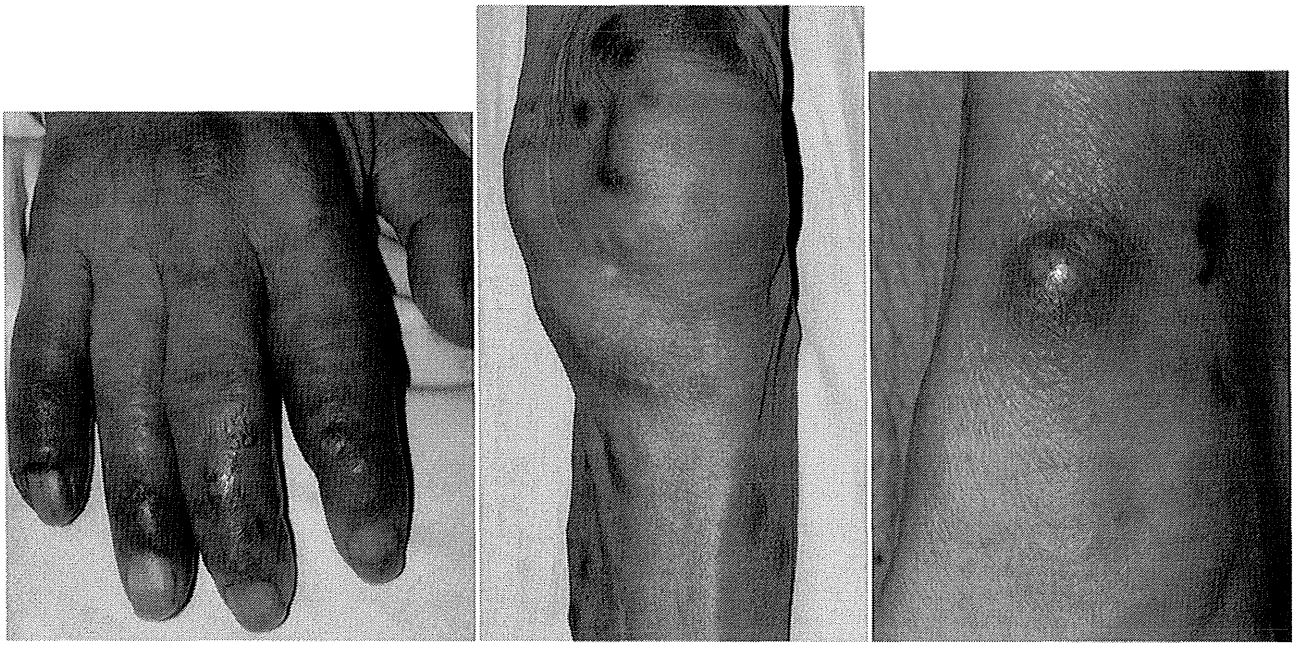


図 1. *Mycobacterium chelonae* 感染症
発赤を伴う大小の結節や膿瘍が多発している．（今井千恵，常深祐一郎，川嶋智彦ほか：血液透析患者に生じた *Mycobacterium chelonae* による播種状皮膚非結核性抗酸菌症．皮膚科の臨床，51(4)：473-476，2009 の図 1 より転載）

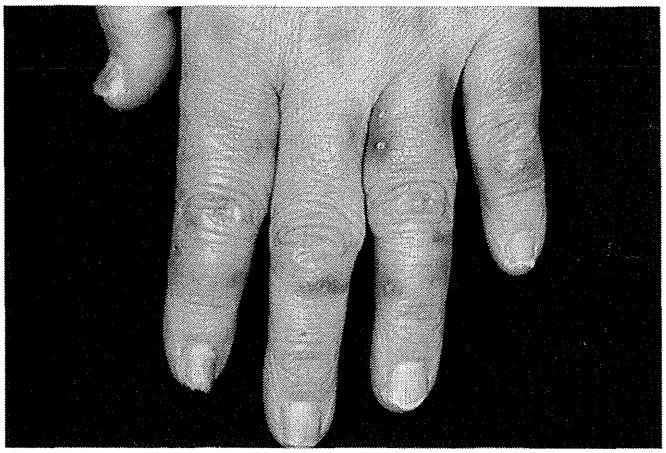
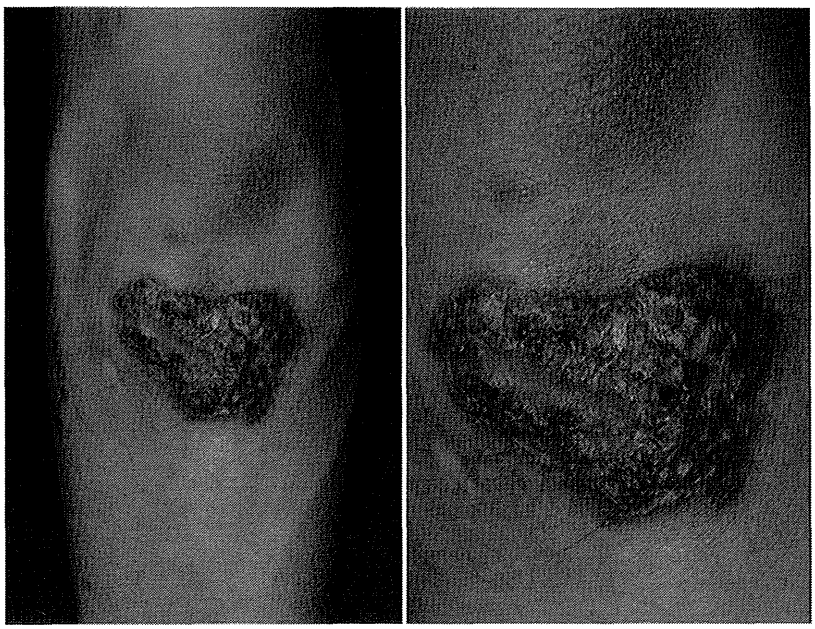
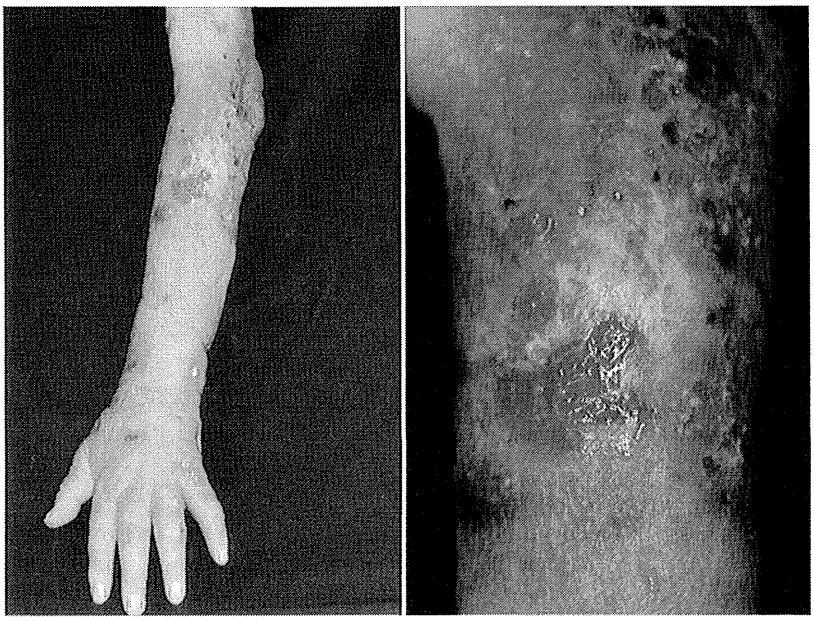


図 2.
Mycobacterium chelonae 感染症
手指に褐色の小結節が散在している．一部は中央に痂皮をつけている．



a | b

図 3.
Mycobacterium marinum 感染症
表面に痂皮と潰瘍を伴い扁平隆起した紅褐色結節（b は a の拡大図）（a は，宮本明栄，常深祐一郎，菊池かな子ほか：皮膚 *Mycobacterium marinum* 感染症．皮膚科の臨床，53(1)：3-4，145-148，2011 のクリニカラー 1 図より転載）



a | b

図 4. *Mycobacterium marinum* 感染症
手指から上腕遠位にかけての広範囲に大小の潰瘍が多発している(b は a の拡大図).

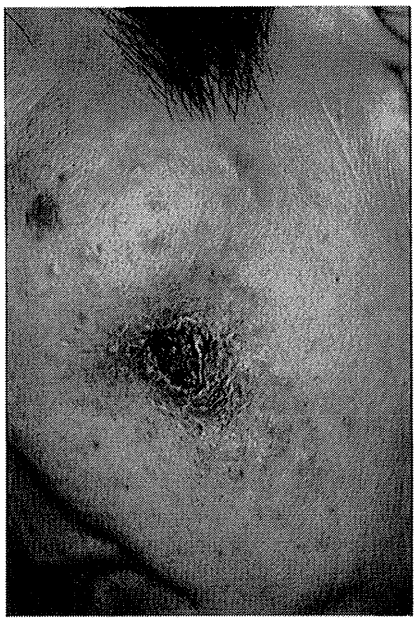
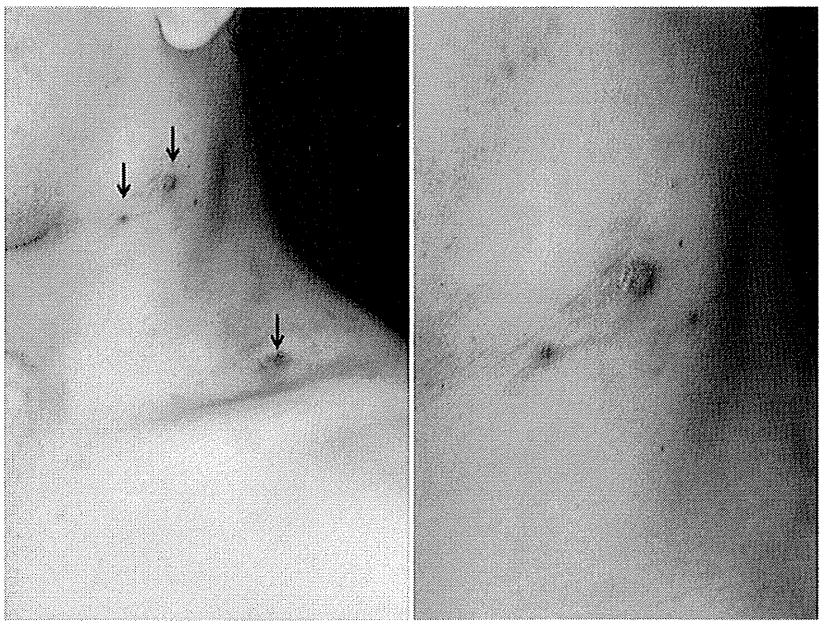
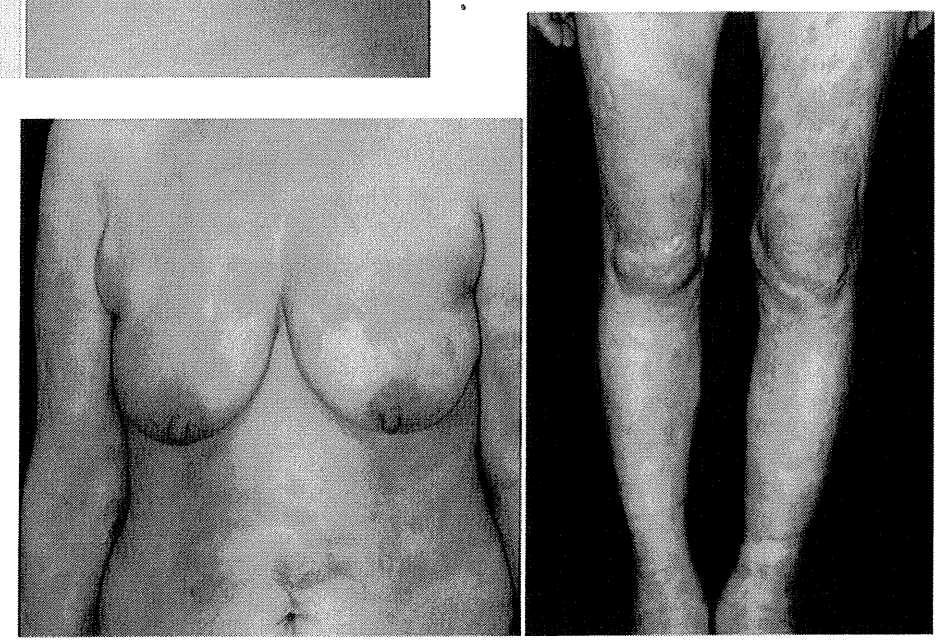


図 5. 尋常性狼瘡
紅色丘疹が多発し，中央で癒合して浸潤を触れる局面を形成している．痂皮をつけた潰瘍を伴う．



a | b

◀図 6.
皮膚腺病
頸部の 3 か所に皮下にまで触れる紅褐色結節があり，そこに瘻孔が形成されている(b は a の拡大像).



a | b

図 7.
ハンセン病(らい反応)
体幹四肢に浮腫性紅斑が多発している．四肢末梢は感覚低下を伴っている.