鈴木幸一、 中村和昭、 谷川和也、 川島　晃、 Huhehasi Wu、 赤間　剛、 林もゆる、 関村　慎、 Pham Dang Bang、 石井則久	らい菌ゲノム由来RNA発現の網羅的解析とその意味するもの	日本ハンセン病学会誌	78	61-65	2009
石井則久、 森　修一、 永岡　譲、 鈴木幸一	WHO 第9回ハンセン病制圧のための技術勧告（Technical Advisory Group: TAG）会議報告	日本ハンセン病学会誌	78	75-88	2009
永岡　譲、 石田　裕、 鈴木幸一、 石井則久	ミャンマーにおけるハンセン病制圧記念行事（2009）の報告	日本ハンセン病学会誌	78	251-253	2009
北見由季、 北見 周、 飯島正文、 石井則久	ネパール人男性に生じたハンセン病(BL型)の1例	皮膚科の臨床	51	483-486	2009
森山一隆、 菊池一郎、 石井則久	ハンセン病患者から生まれた子供たち - 奄美大島における妊娠・出産・保育・養育のシステムの軌跡 -	日本ハンセン病学会誌	78	231-250	2009
金澤伸雄、 三木田直哉、 李 洪錦、 中谷友美、 尾崎元昭、 小坂眞紀、 石井則久、 西村泰行、 古川福実。	日本人のハンセン病発症における細菌センサー分子の遺伝子多型の関与	日本ハンセン病学会誌	78	255-261	2009
石井則久	Hansen病の皮膚症状	皮膚病診療	31	1366-1370	2009
石井則久	ハンセン病の現況	日本皮膚科学会雑誌	119	2573-2575	2009

Ⅲ. 研究成果の刊行物・別刷

Chapter 3

Microbiology and Experimental Leprosy

Masanori Matsuoka

3.1 INTRODUCTION

Hansen's disease was revealed to be an infectious disease by *Mycobacterium leprae* in 1873 by Gerhard Henrik Armauer Hansen of Norway. He reported in a Norwegian medical journal that "Spedalskhedens Åarsager" or rod-shaped bodies, were present in unstained samples prepared from leproma (Pallmary, 1955). Today, the infectious disease caused by *M. leprae* is termed Hansen's disease on account of the discoverer. Microbiological research on *M. leprae* has been hampered considerably by the unfeasibility of cultivation *in vitro*. Nevertheless, based on achievements such as the development of animal models (Shepard, 1960; Kirchheimer, 1971; Kohsaka, 1976) and whole genome sequencing (Cole *et al*, 2001), new findings have been accumulated fast, especially in terms of genetic diagnoses of drug resistance, in genotyping and its application to epidemiological analyses, and microbiological characteristics —once presumed impossible.

3.2 CLASSIFICATION

According to "Bergey's Manual of Systematic bacteriology," *M. leprae* is described in Section 6 as a species of the genus *Mycobacterium* in the Mycobacteria family Mycobacteriacae. *M. leprae* is closely related to *Mycobacterium tuberculosis*.

3.3 LOCALIZATION

M. leprae is found numerously in skin leporma, nasal mucosa, visceral organs, peripheral nerve trunks, and bone marrow in lepromatous leprosy case. Sometimes more than 10^9 of the bacteria exist per gram of a nodule. The bacteria are also observed in skin lesion in the borderline type of leprosy, although they are detected only slightly in the tuberculoid type (Riley & Jopling, 1966). The amount of bacilli in the lesion is called the bacterial index (B.I.) which is used as a criterion for the classification of disease types and for monitoring the therapeutic effects. Bacilli also exists other than patients. Animals of naturally acquired case identified in nine-banded armadillos (Walsh *et al*, 1975), mangabey monkeys (Meyers *et al*, 1985), and chimpanzees (Donham & Leininger, 1977) also harbor the bacilli. Taking these finding into consideration, leprosy is regarded as a zoonosis. Detecting *M. leprae* specific DNA by PCR on nasal mucosa of most residents in areas, where Hansen's disease is prevalent, has revealed contentious contamination by bacteria and suggesting the existence of *M. leprae* in the natural environment (van Beers *et al*, 1994; Matsuoka *et al*, 1999).

A new *Mycobacterium* species, which could not be able to grow artificial media, was discovered from the patients with Lucio phenomenon. As to 20 genes and some pseudo genes showed an overall 90.9% match with *M. leprae*. The isolate is termed *M. lepromatosis* (Han *et al*, 2008, Han *et al*, 2009). Pathogenicity of this bacteria for diffuse lepromatous leprosy should be studied further.

3.4 MICROBIOLOGICAL CHARACTERISTICS

3.4.1 Morphology and structure

M. leprae is rod-shaped bacterium with round ends. of 1-8 μm length and 0.3 μm in diameter (Fig. 3.1). It produces no spores, nor does it have flagella. The bacterial cells' outermost layer has a pseudocapsule structure mainly consisted of lipids phthiocerol dimycocerosate (PDMI) and phenolic glycolipid-I (PGL-I). The bacillus is gram-positive

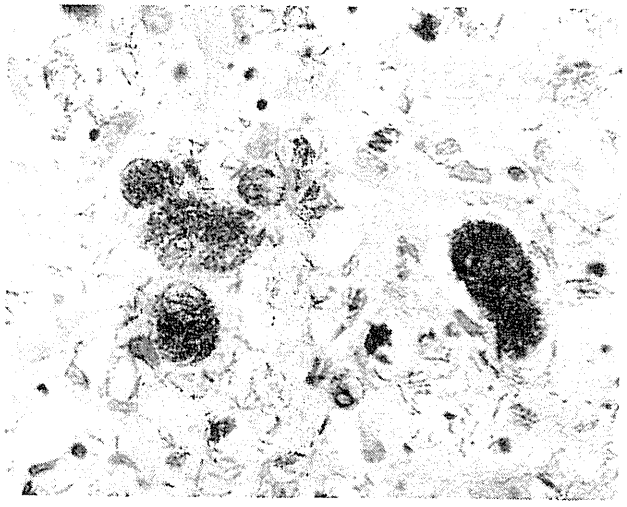


Figure 3.1 *M. leprae* in the leproma of LL patient. (Photo by Matsuoka M)

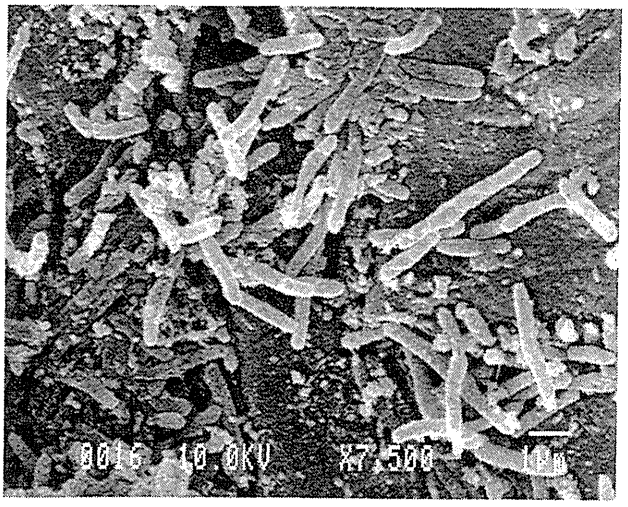


Figure 3.2 Scanning electron microscopic feature of *M. leprae*. (Photo by Amakao K)

and is stained uniformly red by the Ziehl-Neelsen method. Such bacteria are called solid bacteria; some, designated as non-solid, are granular, segmented, short rods, or are extremely elongated. Non-solid bacteria are regarded as those that have lost viability. Ratio of non-solid bacteria is presumed to be increased when the bacilli are inactivated by therapeutic effects. The ratio of the two forms of the bacteria, solid vs non-solid, is designated as the morphological index (M.I.). When treatment effects are achieved, B.I. decreases by about one per year, whereas M.I. shows more rapid change; for that reason, M.I. is sometimes used as an indicator for monitoring therapeutic effects.

M. leprae proliferates in histiocyte. It is arranged in nodes to resemble palisades or cigars; further proliferation results in the formation of globular bacterial masses once called "globi" (Fig.3.2). Neuro-tropism of the bacilli is one of the characteristics and this character is account for neuropathy in leprosy.

Electron microscopic observation shows an electron-transparent layer around the bacterial surface (Takeda *et al*, 2003) (Fig.3.3 and 3.4). This part is presumed to be related to the capsule like structure mainly consisted of PDMI and PGL-I (See Figures in chapter 4). The carbohydrate end of PGL-I is specific structure to *M. leprae*, it is used in serodiagnosis as an antigen of antibody reaction specific to *M. leprae* (Izumi *et al*, 1990). It also has been shown that PGL-I might be involved in the neurotropism of *M. leprae* by first binding to laminin 2 of the Schwann cell and then to the nerve via its receptor dystroglycan (Ng *et al*, 2000).

On the bacteria surface, "paired fibrils" and a "band structure" are observed similarly to other acid-fast bacilli. Many "paired fibrils" are present in the longitudinal direction of the cell. One to several "band structures" circularly surround the cell. A peptidoglycan layer exists on the outer periphery of the cytoplasmic membrane. Its outer layer consists of arabinogalactan and mycolic acid, constituting the 22-nm-thick cell wall. Lipoarabinomannan (LAM) sticks out of the cell membrane with phosphatidylinositol mannoside (PIM) as an anchor. The cell membrane comprises a phospholipid bilayer and various proteins, similarly to other acid-fast bacilli. It has two major membrane-bound proteins: 35-kDa (MMP-I) and 22 kDa (MMP-II).

M. leprae possesses a dopa (3,4-dehydroxyphenylalanine) oxidase that is not found in other acid-fast bacilli, which is useful for identification of the bacteria. Furthermore, *M. leprae* has superoxide dismutase activity, although its *katG* gene is shown to be a pseudogene (Nakata *et al*, 1997). Consequently, it has no catalase activity and the bacilli are resistant to INH.

3.4.2 Genetics

The *M. leprae* genome size is 3.3 Mb; it is smaller than the 4.4 Mb of *M. tuberculosis* (Cole *et al*, 2001). The *M. leprae* genome includes 1614 genes and 1133 pseudogenes, although the *M. tuberculosis* genome contains only 6 pseudogenes. The existence of numerous pseudo genes is supposed as the reason that *M. leprae* has not been grown in any artificial media. Polymerase Chain Reaction (PCR) for *M. leprae* was exploited based on the sequencing data (Woods & Cole 1989; Plikaytis *et al*, 1994) and PCR is applied widely for leprosy study such

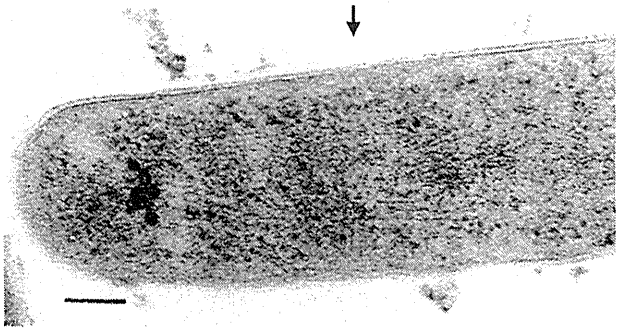


Figure. 3.3 *M. leprae* observed by transparent electron microscope. Arrow indicates electron-transparent layer. Scale bar is 100nm. (Reprint of Takeda A *et al*, 2003)

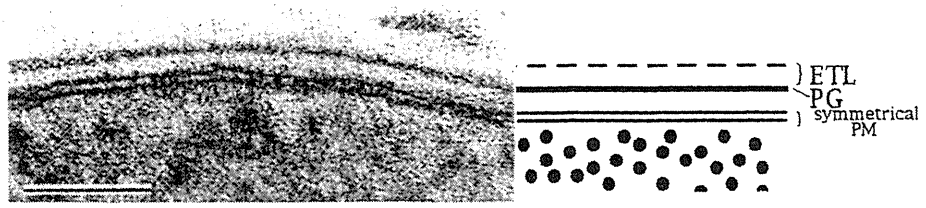


Figure 3.4 Structure of cell wall. Scale bar is 50nm. ETL; Electron-transparent layer, PG; Peptidoglycan, PM; Plasma membrane. (Reprint of Takeda A *et al*, 2003)

as molecular epidemiology (Cole *et al*, 2001, Matsuoka, 2009), drug resistance, (Williams & Gillis, 2004; Matsuoka *et al*, 2007), and specific detection of DNA from clinical samples and ancient skeletal (van Beers *et al*, 1994; Hass *et al*, 2000).

3.4.3 Growth

Although many reports have described *in vitro* culture of *M. leprae*, its cultivation has never been confirmed. Currently, *M. leprae* for laboratory use are obtained from the bacilli inoculated to the susceptible animals and grown (Kirchheimer *et al*, 1971; Kohsaka *et al*, 1976). The bacteria grown in large quantities in armadillos are utilized for purification and analyses of cell components of the bacilli. *M. leprae* grown in nude mice are useful for the experiments as for genetic analysis which could be done by small amount of bacilli.

The generation time of *M. leprae* in the mouse footpad in the logarithmic growth phase is 12-13 days (Shepard & MacRae, 1965) or 11.1±1.92 days (Levy, 1976).

Preferability of lower temperatures for the growth of *M. leprae* is known based on the findings that the favorite sites for lesions in humans are parts of lower temperature such as skin and nasal mucosa, and infection experiments in animals: in the *M. leprae* growth study in the mouse footpad done by Shepard, the growth of the bacteria was most fast when the room temperature was 20°C and the footpad temperature was 30°C. At room temperature of 10 or 30°C (footpad temperature of 25 or 36°C, respectively) bacterial growth was delayed. The relation between the site of *M. leprae* growth and the body temperature in nude mice also showed that sites at lower temperatures such as the footpad, the tail, the eyelid, and the auricle are suitable for the growth of *M. leprae* (Kohsaka *et al*, 1978). In addition, ATP contents and PGL-synthesis are lower at 37°C than at 33°C; the ability for oxidative degradation of palmitic acid, as measured using the Buddemeyer method, is highest at 31-32°C (Fukutomi *et al*, 2004).

3.4.4 Stability of viability

Viability of *M. leprae* out of the body is examined using the mouse footpad method. Results showed survival for 9 days in a dried condition, at 24-33°C; 5 months in the dark at 28-44% humidity; 60 days in saline left at room temperature or in the Hanks' solution in a refrigerator; and 7 days with exposure to sunlight for 3 hours per day (Desikan *et al*, 1995). Additionally, the bacteria survived for 7 days in nasal discharge that had been discharged from a patient and dried.

The bacteria retained infectivity to mice after storage for 12 months in broth (Bacto TB Broth; Difco Laboratories) containing 7.5% dimethyl sulfoxide in liquid nitrogen (−196°C). There was no decrease in viability for 2 weeks in Hanks' solution containing 0.1% bovine serum albumin (0°C). The decreased viability was reduced further through addition of 10% glycerin. In each case, slow freezing and rapid thawing were necessary for better maintenance of viability. *M. leprae* that had been suspended in 10% skim milk and lyophilized were able to proliferate in nude mice even after 4 years.

Nakamura's method for decontamination and

purification using 0.05% trypsin and 1% NaOH is highly effective while with viability loss of *M. leprae* is low (Nakamura, 1994). The bacterial suspension prepared by Nakmura's method and suspended in Hanks' Balance Salt Solution containing 10% glycerin followed by keeping at −84°C retained proliferative capacity in the nude mouse footpad for more than 10 years.

M. leprae is inactivated partially after one hour and completely after two hours by chlorhexidine treatment. Infectivity of the bacilli to mice was lost with single dose of 600, 900 and 1,200 mg rifampicin (Levy *et al*, 1976).

Viability or proportion of viable *M. leprae* is determined by proliferation in the mouse footpad, oxidizing palmitic acid with the release CO_2 measured using the Buddemeyer method (Franzblau, 1988), chemoluminescence measurement of the ATP amount by luciferin/luciferase reaction, and the FDA/EB fluorescent staining method. Application of RT-PCR is also used in some cases (Phenitsuksri *et al*, 2006; Martinez *et al*, 2009). Reverse transcription PCR followed by Real time PCR targeting 16s rRNA showed decline in viability during the course of multidrug therapy (Martinez *et al*, 2009).

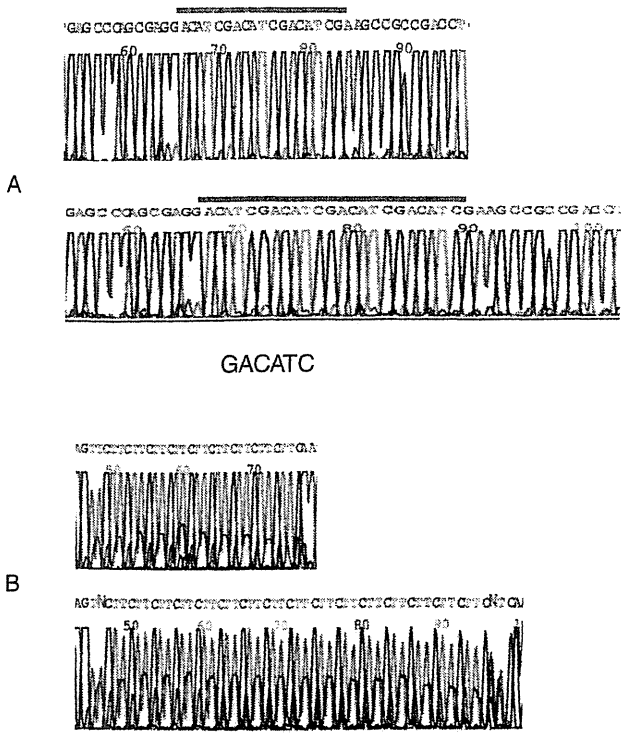


Figure 3.5 Polymorphism of variable number tandem repeats. A; Tandem repeat of 6 bp minisatellite in the *rpoT* gene, B; Tandem repeat of TTC microsatellite. (Figure by Matsuoka M)

3.5 GENETICS AND APPLICATION FOR MOLECULAR EPIDEMIOLOGY

3.5.1 Genome and application

The *M. leprae* genome size is 3.3 Mbp; it is smaller than the 4.4 Mbp of *M. tuberculosis* (Cole *et al*, 2001). The *M. leprae* genome includes 1614 genes and 1133 pseudogenes, although the *M. tuberculosis* genome contains only 6 pseudogenes. The existence of numerous pseudogenes is supposed as the reason that *M. leprae* has not been grown in any artificial media. PCR for *M. leprae* was exploited based on the sequencing data (Woods & Cole 1989; Plikaytis *et al*, 1994) and PCR is applied widely for leprosy study such as molecular epidemiology (Cole *et al*, 2001, Matsuoka 2009), drug resistance (Williams & Gillis 2004; Matsuoka *et al*, 2007), and specific detection of DNA from clinical samples and ancient skeletal (van Beers *et al*, 1994; Hass *et al*, 2000). Comparative genome analysis identified *M. leprae* specific peptides or proteins and some of them specifically react with T cell derived from leprosy patients. Those peptides are potentially eligible for developing rapid diagnostic test for the early detection of *M. leprae* infection (Spencer *et al*, 2005; Geluk *et al*, 2009).

3.5.2 Genomic polymorphism

Typing of isolates is the essential tool for epidemiological analysis as to trace infection route of infectious diseases. However, regarding the phenotypic diversity among *M. leprae* strains, only the difference in the mouse footpad growth manner is known. According to Shepard, growth in the mouse footpad differs by strains of *M. leprae*, i.e., some strains, termed fast strain, reach the higher plateau level sooner, on the contrary, some strains that reach the lower plateau level later with longer generation time. This characteristic is apparently not related to the disease type of the patient from whom the bacteria were derived, and did not change after passage in mice (Shepard & MacRea, 1971).

Genetic polymorphism of *M. leprae* was considered to be extremely limited. Isolates originated in various sources showed no polymorphism by restriction fragment length polymorphism (RFLP), which has been applied for epidemiological analysis of tuberculosis. In fact, RFLP shows no distinguishable polymorphism among *M. leprae* strains from various areas in the world, or from humans, armadillos, and mangabey monkeys (Williams,

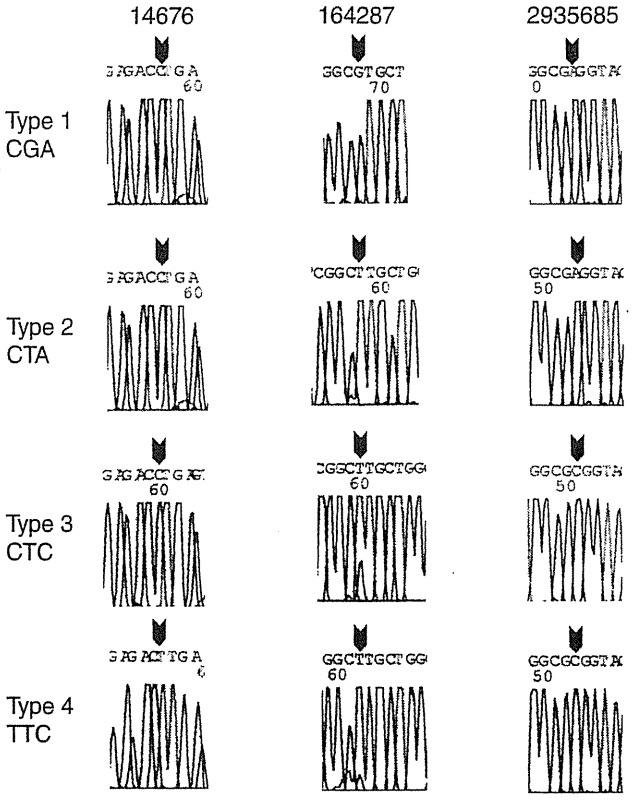


Figure 3.6 Single nucleotide polymorphism. SNP type 1 reveals GCA, SNP type 2 reveals CTA , SNP type 3 reveals CT (Picture by Matsuoka M)C, and SNP type 4 reveals TTC at the position 1467, 1642875, and 2935683 respectively. (Figure by Matsuoka M)

1990). Furthermore, no differences between isolated strains were found in the spacer regions between 16S and 23S ribosomal RNA genes (de Wit *et al*, 1994).

3.5.3 Variable number tandem repeats

Genetic identification first became possible by the discovery that strains of *M. leprae* having either 3 or 4 tandem repeats of 6 nucleotides in the *rpoT* gene (Fig.3.5). Each of the two genotypes shows a characteristic geographical distribution: strains with four repeats is predominance in eastern Asia including Honshu area of Japan; whereas almost all strains had three repeats in the other areas of the world including Okinawa (Matsuoka *et al*, 2000). Genotype of *M. leprae* in Latin America showed that most strains in Mexico had four repeats, indicating that the origin of the disease in Mexico was different from other Latin American countries examined (Matsuoka *et al*, 2005). The global geographical distribution of the respective *rpoT* genotypes in *M. leprae* is presumed to be formed by the prehistoric migration of humankind (Matsuoka *et al*, 2005).

Variable copy number of tandem repeat of three nucleotides TTC was detected in non-coding region of the *M. leprae* (Shin *et al*, 2000) (Fig. 3.5). Isolates from the Philippines showed variable repeats ranging from 10 to 37. Using this variable number of tandem repeats (VNTRS), genotype of *M. leprae* from nasal mucosa of residents and patients at the high prevalent area in Indonesia were compared. Results showed family cases in which different *M. leprae* genotypes were detected from patients and their families who resided together. In addition, patients with different TTC genotype strain were found in multifamily cases (Matsuoka *et al*, 2004). This result disagreed with the conventional conception that a heavy house hold contact with multibacillary patient is the mode of infection and suggests existence of infectious source other than patient. This supposition agreed well with idea derived from seroepidemiology (Abe *et al*, 1990). Existence of many microsatellites of up to five nucleotides or mini-satellites of more nucleotides, which is useful for genotyping was shown by in silico analysis (Groathouse *et al*, 2004). Their application is useful in the elucidation of the infection mode of Hansen's disease based on more detailed genotyping and existence of infectious source other than patient is deduced (Zhang *et al*, 2004; Matsuoka, 2009).

3.5.4 Single nucleotide polymorphisms

M. leprae isolates were classified into 4 types singlenucleotide polymorphisms (SNPs) at position 14676, 1642875, and 2935685 in genomic DNA (Fig.3.6) (Monot *et al*, 2005). The frequency of single nucleotide polymorphisms (SNPs) in the *M. leprae* genome has been shown to be one per 28 kb, which is extremely low compared to other bacteria, suggesting that *M. leprae* distributed worldwide are derived from limited clones (Monot *et al*, 2005). Based on geographic distribution of each SNPS type, type 1 to type 4, in globally different area, it is presumed that the disease originated in east Africa or the Indian subcontinent and spread worldwide with the movement of humankind. Genotyping by SNPs and polymorphism is suitable for the analysis of global transfer of leprosy (Matsuoka *et al*, 2006; Monot *et al*, 2009).

3.6 DRUG RESISTANCE

3.6.1 Drugs used in multidrug therapy and resistance

Promin was introduced into the treatment of

Hansen's disease in the 1940s. In 1953, a clinical case had already been reported in which resistance to dapsone was suspected. The resistance was subsequently proved in 1964 using the mouse footpad method. Currently, many cases of resistance have been reported for dapson, and rifampicin which are key components for the WHO multidrug therapy (MDT) (Maeda *et al*, 2001). Resistance to quinolone has also been reported in a few cases. Not only single drug resistance, multidrug resistance has been demonstrated (Cambau *et al*, 1997; Maeda *et al*, 2001; Matsuoka *et al*, 2003). Although three reports describe resistance to clofazimine; none has been confirmed by a replication study. No reports in the relevant literature have described resistance to minocycline.

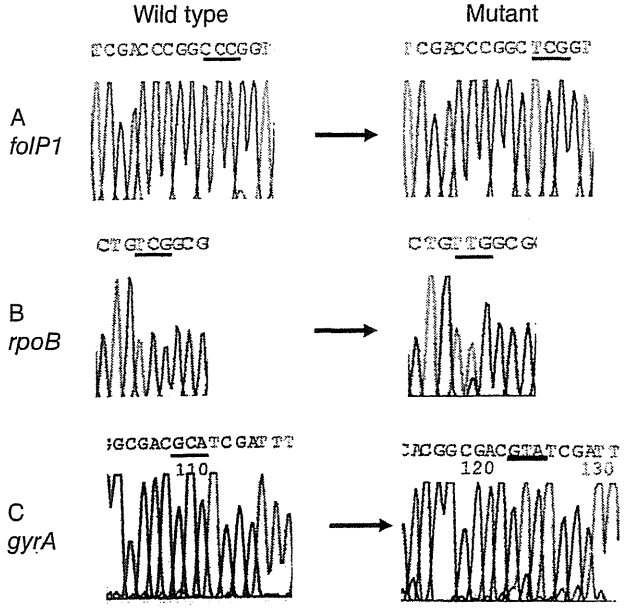


Figure 3.7 Mutation in the *folP1*, *rpoB*, and *gyrA* gene of *M. leprae* which is resistant to dapsone, rifampicin and quinolone. A; *folP1*: 55 (CCC: Pro → CTC: Leu), B; *rpoB*: 425 (Ser: TCG → TTG: Leu), C; gyrA: 91 (Ala: GCA → Val: GTA). (Figure by Matsuoka M)

3.6.2 Mouse footpad method

Drug susceptibility of *M. leprae* has been tested traditionally using the mouse footpad method since 1960s. Five thousand *M. leprae* were inoculated in the footpads of mice. The mice were given feed containing 0.01%, 0.001%, or 0.0001% dapsone; 0.01% rifampicin; 0.001% clofazimine; 0.15% ofloxacin; 0.02% sparfloxacin; or 0.08% minocycline. After 25-30 weeks, susceptibility was determined by the *M. leprae* growth in the footpads (Ji, 1987; Matsuoka *et al*, 2003).

3.6.3 Molecular biological method for detecting drug resistance

Drug resistance to dapsone, rifampicin, and quinolone was recently revealed to be occurred by amino acid substitution at each drug's target site (Williams & Gillis, 2004; Matsuoka *et al*, 2007). The genomic region that codes amino acids conferring resistance is designated as the drug resistance determining region (DRDR).

Dapsone exerts a bacteriostatic effect by competitive binding as the analog of p-aminobenzoic acid (PABA) to dihydropteroate synthase (DHPS) and thus inhibit the synthesis of folic acid. Single-nucleotide mutations in the triplets of the threonine at position 53 and of the proline at position 55 of the *folP1* gene coding DHPS result in amino acid substitutions, causing dapsone to be unable to bind to DHPS, leading to the acquisition of dapsune resistance (Fig.3.7 A) (Kai *et al*, 1999; Williams & Gillis, 2004).

Rifampicin inhibits mRNA transcription by binding to the beta subunit of RNA polymerase. A single-nucleotide mutation in DRDR of the *rpoB* gene coding the beta subunit causes resistance to rifampicin. About 70% of the rifampicin resistance of *M. leprae* is attributable to mutation at position 425 from TCG (Serine) to TTG (Leucine) (Fig. 3.7 B). Furthermore, amino acid substitutions at positions 407, 410, 420, 425 and 427 are involved in the acquisition of rifampicin resistance (Honoré & Cole, 1993; Williams & Gillis, 2004).

New quinolones inhibit DNA replication by binding to the A subunit of DNA gyrase. Amino acid, valine, substitutions at positions 91 in *gyrA* coding the A subunit have been reported for resistant strains (Cambau *et al*, 2001; Williams & Gillis, 2004) (Fig. 3.7 C). Mutations at 89, 92, and 95 are supposed to also confer quinolone resistance according to the analogy to quinolone resistance in *M. tuberculosis*.

Correlation between drug resistance and gene mutation in *M. leprae* has been examined in limited isolates because of the tedious procedure for the mouse footpad method, i.e., it has been examined in less than 180 strains including both susceptible and resistant strains. More data are necessary in the future to enhance the accuracy of the determination of resistance by the search for gene mutations, and to develop simple test methods.

3.6.4 Level of dug resistance

Relapse after the completion of MDT or after symptoms subsided has been observed. Resistant bacteria were detected at high percentages in re-

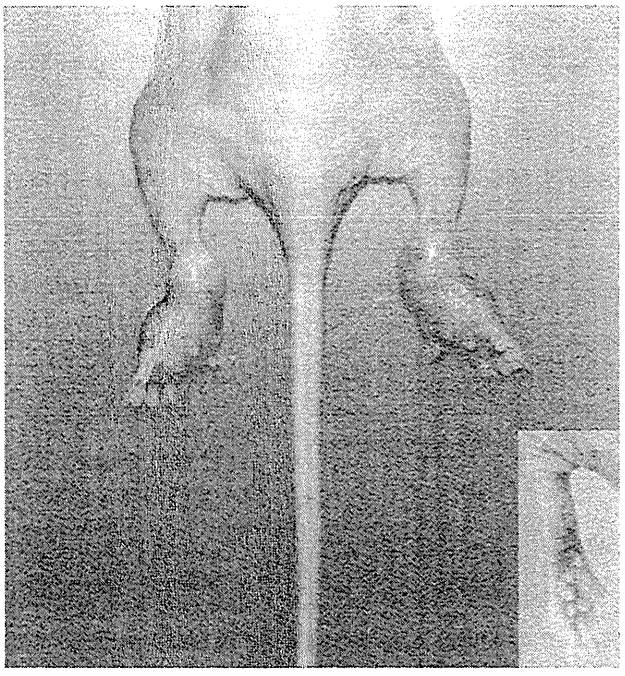


Figure 3.8 Nude mouse footpad swollen. The nude mouse was inoculated 10^7 bacilli into the footpad 11 months ago. Photo at right side is normal nude mouse footpad. (Photo by Matsuoka M)

rapsed or intractable cases in Japan (Maeda *et al*, 2001). On the other hand, follow-up for 10 years after completion of MDT in Cebu Island, Philippines showed recurrence in 10.28/100 patients/year, of which 1 of 15 cases was dapson resistant, whereas the other strains were susceptible to dapsone and rifampicin (Cellona *et al*, 2003). Comparative examination by genotypes of strains is necessary to determine whether these cases resulted from reinfection or relapse. The strain is designated as persistent, a persister, if these are caused by a susceptible strain remaining after therapy (Matsuoka *et al*, 2007). The mechanism of relapse by a persister is an important concern from the perspective of measuring Hansen's disease mainly based on chemotherapy in the future. In this context, global leprosy control of WHO launched out sentinel surveillance on 2008 to monitor the level of drug.

3.7 ANIMAL MODELS

Animal models of Hansen's disease are intended for use as pathological models as well as a means to propagate bacteria for use in experiments. Various means of inoculation to many animal species have been attempted since just after the discovery of *M. leprae* (Johnstone, 1987). Nevertheless, no reproducible experimental transmission was reported until the successful study of limited growth in mice footpads (Shepard, 1960).

3.7.1 Mice

Mice inoculated with 10^3-10^4 *M. leprae* into a footpad show limited growth of 10^5-10^6 at after about 6-8 months, although no changes recognized by naked eye exists. Therefore, bacillary growth is determined by counting the bacilli in smears of footpad homogenates (Sheparad & McRea, 1968). Real-time PCR might be applicable to quantification of the bacilli instead of counting bacilli in smear (Truman *et al*, 2008). Histopathological futures correspond to the BL type of leprosy in humans. This test system is widely applied to the isolation of *M. leprae*, screening of anti leprosy drugs, examination of drug resistance of *M. leprae*, and the like.

3.7.2 Immunocompromised mice

Based on the supposition that the limited growth of the bacteria in footpads of normal mice results from the development of immunity, susceptibility was examined in mice that were immunocompromised by thymectomy and X ray-irradiation (Rees, 1966). At 12 months after inoculation, the bacillary number reached 10^8-10^9 associated with marked swelling in the footpad. Bacteria were also detected in various parts of the body including auricles and the nose, confirming disseminated systemic infection. This model was used in experiments of infection via nasal mucosa, proving that *M. leprae* infects hosts via nasal mucosa. However, it became comparatively disused after reports of higher susceptibility of nude mice.

3.7.3 Nude mice

When the thymus' function as a tissue for lymphocyte maturation site was clarified, and when the importance of cellular immunity in the prevention of *M. leprae* infection became known, the infection experiment of *M. leprae* to athymic nude mice was conducted and succeeded in causing systemic disseminated lepromatous lesions (Fig.3.8) (Kohsaka *et al*, 1976; Chehl *et al*, 1985). Bacillary number in the footpad inoculated with 10^7 bacilli reach to 10^{11} bacilli per footpad after 11 to 12 months. In nude mice, bacterial proliferation in lower temperature parts such as the eyelid, nose, tail, testis, and auricle is extremely high (Kohsaka *et al*, 1978). This model is used for screening of therapeutics for Hansen's disease, or for the isolation of the bacteria. In addition, armadillos cannot be bred in captivity. For that reason, wild armadillos are captured for laboratory

use. They might have natural infection or might be contaminated by other acid-fast bacteria, but *M. leprae* grown in nude mice are useful as a good source of bacteria. Although total bacillary number in the footpad amounts to about 10^{12}/footpad and shows large swelling of footpad at 12 months post infection,these badilli are not suitable for experiments, since bacilli with highest viability could be obtained from moderately enlarged nude mouse footpad (Truman & Krahenbuhl, 2001). The Leprosy Research Center of the National Institute of Infectious Diseases of Japan provides the bacilli to meet the demands of researchers.

3.7.4 Armadillos

Nine-banded armadillos (Fig.3.9), which inhabit the southern US to Latin American countries, normally have body temperature as low as 30-35°C. For that reason, their susceptibility to *M. leprae* was examined, revealing extremely high susceptibility (Kirchheimer, 1971). Intravenous inoculation of a large quantity (10^9 or more) of *M. leprae* develops the disease in 90% or more of the animals. Lesions correspond to the LL type of leprosy in humans. In armadillos, unlike humans, numerous *M. leprae* are detected in almost all organs, and 10^{10} or more *M. leprae* per gram exist in the liver and spleen. The obtained bacteria are used for various experiments requiring large amounts of bacterial components.

Cases of natural infection have been identified in armadillos (Walsh *et al*, 1975). It has been reported that the infection rate in Louisiana is 10% on average (about 30% maximum). No cases of naturally acquired case of infection have been reported previously for the armadillos inhabiting in eastern states as Florida, but spreading to eastward is indicated recently (Loughry *et al*, 2009). Together with naturally acquired infection in monkeys, Hansen's disease is considered to be a zoonosis (Walsh, 1981), and infection of humans, possibly from armadillos, has also been reported (Douglas *et al*, 1987).

3.7.5 Primates

Transmission of *M. leprae* to monkeys has long been conducted. Although results in chimpanzees and in white-handed gibbons (*Hylobates ler*)appear promising, these did not attract much attention. Later, naturally acquired infection was found in mangabey monkeys (Meyers *et al*, 1985), chimpanzees (Donham & Leininger, 1977), and cynomolgus monkeys (Valverde *et al*, 1998) drawing attention to the susceptibility of primates to *M. leprae*. Additionally, rhesus monkeys and African green monkeys have been shown to be susceptible to *M. leprae* by experimental infection (Wolf *et al*, 1985). In cases of infected primates, various lesions resembling those in human disease types are observed, including neuritis and erythema nodosum leprosum (ENL) (Gormos *et al*, 1995). These are considered to be models that are especially suitable for neuropathy studies.

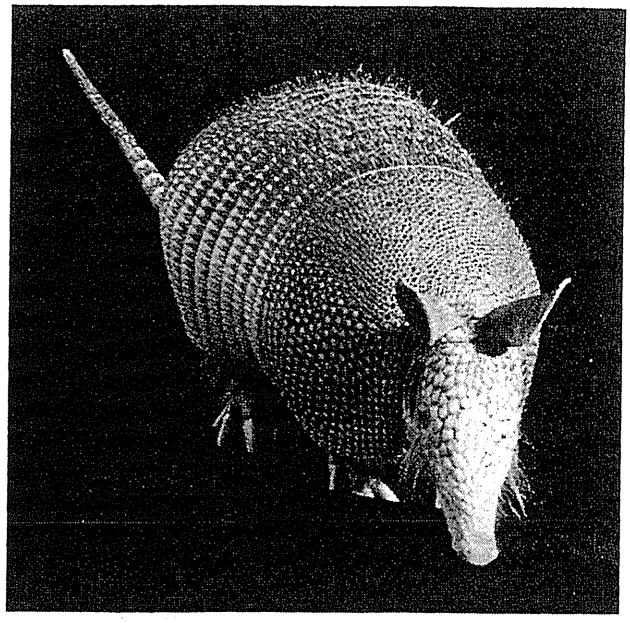


Figure 3.9 Nine banded armadillo (*dasypus novemcinctus*). (Photo by Truman R)

自然 発病例

3.8 INFECTION

3.8.1 Source of infection

The source of infection is presumed to be untreated patients because an untreated patient with the LL type sheds 2×10^8 cells per day from nasal mucosa. Results of many epidemiological studies show that the incidence rate is high in people in contact with LL patients (Noordeen, 1978). On the other hand, many findings as follows indicate sources of infection other than untreated patients: in areas with a high prevalence rate of the disease, *M. leprae* exists in nasal mucosa of many residents, who are positive for the specific anti-*M. leprae* antibody (Saeki *et al*, 2000; van Beers *et al*, 1994); over 16% of residents were antibody positive in an area where had been no new patients for several years (Abe *et al*, 1990); in some cases, the genotypes do not match between a patient who is presumed to be the source of infection and a cohabit-

ing family patient (Matsuoka *et al*, 2004). These facts suggest the possibility of direct infection by sheding from multibacillary patients or inapparent infection and of indirect infection by *M. leprae* that survive in environment after dischange (Desikan & Sreeratsa, 1995).

3.8.2 Infection route

Invasion of *M. leprae* into the body is presumed to be usually via nasal mucosa based on observations: lesions are already apparent in the nasal cavity before dermal lepromatous lesions are observed (Barton, 1974), and the *M. leprae* genes are sometimes detected in nasal mucosa of residents other than patients, including both contact and non-contact (van Beers *et al*, 1994; Saeki *et al*, 2000). In addition, infection established via nasal mucosa in immunocompromised mice was shown (Rees *et al*, 1966). These findings strongly support infection establishment via nasal mucosa.

3.8.3 Pathogenicity

Abe *et al* examined the antibody titer in residents of Okinawa prefecture using fluorescent leprosy antibody absorption (FLA-ABS). Results showed that 22% of residents were positive (Abe *et al*, 1990). Seroepidemiological results obtained in Indonesia also showed that many residents were infected (van Beers *et al*, 1994; Saeki *et al*, 2000). These observations indicate that *M. leprae* posseses adeguate infectivity. On the other hand, based on the discrepancy between the high antibody positive rate and the prevalence rate, it is considered that the virulence of *M. leprae* is not high and that some infected individuals develop the disease because of some immunodeficiency to the bacteria. Hansen's disease is well known for its family accumulation, in which genetic factors of characteristics to define immunological competence might play certain roles.

3.9 MICROBIOLOGICAL DIAGNOSIS

In areas where it is prevalent, Hansen's disease is diagnosed by observations of skin or neural lesions, and classified as either multibacillary or paucilbacillary type for treatment. Microbiological tests in the area of high prevalence are limited to the B.I. test by microscopy.

For definitive diagnosis, the blood antibody value against PGL-I is measured using ELISA or lateral flow test or leprosy-specific gelatin agglutination to determine the existence of infection. The lepromin reaction is used for determination of the disease type: positive is the tuberculoid type and negative is the lepromatous type.

Microbiological identification methods include: 1) amplification of specific DNA regions using PCR (Woods & Cole, 1989; Plikaytis *et al*, 1990; Kurabachew *et al*, 1998) ; 2) acid-fastness determination by Ziehl-Neelsen staining and disappearance of acid-fastness by pyridine treatment; 3) immunostaining using various monoclonal antibodies that recognize epitopes specific to *M. leprae*; 4) limited growth in the mouse footpad; and 5) non-proliferation in all artificial media including media for *M. tuberculosis*.

REFERENCES

Abe M, Ozawa T, Minagawa T & Yoshino Y (1990). Immunoepidemiological studies on substantial infection in leprosy II. Geographical distribution of seropositive responders with special reference to their possible source of infection. *Jpn J Lepr* 59: 162-1681.

Barton R. P. E (1974). A clinical study of the nose in lepromatous leprosy. *Lepr Rev* 45: 135-144.

Cambau E, Perani E, Guillemin I, Jamet P & Ji B (1997). multidrug-resistance to dapsone, rifampicin, and ofloxacin *in Mycobacterium leprae. lancet* 349: 103-104 .

Cambau E, Bonnafous P, Perani E, Sougakoff W, Ji B & Jarlier V (2001). Molecular detection of rifampicin and ofloxacin resistance for patients who experience relapse of multibacillary leprosy. *Clin Infect Dis* 34: 39-45.

Cellona R. V, Balagon M. V. F, dela Cruz E. D, Brugos J. A, Abalos R. M, Walsh G. P, Topolski R, Gelber R. H & Walsh D. S (2003). Long-term efficacy of 2 year WHO multidrug therapy (MDT) in multibacillary (MB) patients. *Int J Lepr Other Mycobact Dis* 71: 308-319.

Chehl S, Ruby J, Job C. K & Hastings R. C (1985). Transmission of leprosy in nude mice. *Am J Trop Med Hyg* 34: 1161-1166.

Cole S. T, Eiglmeier K, Parkhill J, James K. D, Thompson N. R, Wheeler P. R, Honore N, Garnier T, Churcher C, Harris D, Mungall K, Basham D, Brown D, Chillingworth T, Connor R, Davis R. M, Devlin K, Duthoy S, Feltwell T, Fraser A, Hamlin N, Holroyd S, Hornsby T, Jagels K, Lacroix C, Maclean J, Moule S, Murphy L, Oliver K, Quail M. A, Rajandream M. A, Rutherford K. M, Rutter S, Seeger K, Simon S, Simmonds M, Skelton J, Squares R, Squares S, Stevens K, Taylor K, Whitehead S, Woodward J. R & Barrel B. G (2001). Massive gene decay in the leprosy bacillus. *Nature* 409: 1007-1011.

Desikan K.V & Sreevatsa (1995). Extended studies on the viability of *Mycobacterium leprae* outside the human body. *Lepr Rev* 66: 287-295.

de Wit M. Y & Klatser P. R (1994). *Mycobacterium leprae* isolate from different sources have identical sequences

of the spacer region between the 16S and 23S ribosomal RNA genes. *Microbiol* 140: 1983-1987.

Donham K. J & Leininger J. R (1977). Spontaneous leprosy–like disease in a chimpanzee. *J Infect Dis* 136: 132-136.

Douglas A. T, Mines J. S, Duncan C. T, Mack T. M & Rea T. H (1987). Armadillo exposure among Mexican-born patients with lepromatous leprosy. *J Infect Dis* 156: 990-992.

Franzblau S. G (1988). Oxidation of palmitic acid by *Mycobacterium leprae* in an axenic medium. *J Clin Microbiol* 26:18-21.

Fukutomi Y, Matsuoka M, Minagawa F, Toratani S, McCormic G & Krahenbuhl J (2004). Il-10 treatment of macrophages bolsters intracellular survival of Mycobacterium leprae. *Int J Lepr Other Mycobact Dis* 72: 16-25.

Geluk A, Spencer JS, Bobasha K, Pessolani M. C, Pereira G. M, Banu S, Honoré N, Reece S. T, MacDonald M, Sapkota B. R, Ranjit C, Franken K. L, Zewdie M, Aseffa A, Hussain R, Stefani M. M, Cho S. N, Oskam L, Brennan P. J, Dockrel H. M; IDEAL Consortium (2009). From genome-based in silico prediction s to ex vivo verification of leprosy diagnosis. *Clin Vaccine Immunol* 16: 352-359.

Gormus G.P, Xu K, Baskin L.N, Bohm R.P, Blanchard J.L, Mack P.A, Ratterree M.S, McClure H.M, Meyers W.M & Walsh G.P (1995). Experimental leprosy in monkeys. I. Sooty mangabey monkeys: transmission, susceptibility, clinical and pathological findings. *Lepr Rev* 66: 96-104.

Groathouse N. A, Rivoire B, Kim H, Lee H, Cho S. N, Brennan P. J & Vissa V. D (2004). Multiple polymorphic loci for molecular typing of strains of *Mycobacterium leprae. J Clin Microbiol* 42: 1666-1672.

Han X. Y, Soe Y. H, Sizer K. C, Schoberle T, May G. S, Soencer J. S, Li W & Naier R. G (2008). A new Mycobacterium species causing diffuse lepromatous leprosy. *Am J Clin Pathol* 130: 856-864.

Han X. Y, Sizer K. C, Thompson E. J, Kabanja J, Li J, Hu P, Gomez-Valero L & Silva F. J (2009). Comparative sequence analysis of *Mycobacterium leprae* and the new leprosy causing *Mycobacteirum lepromatosis. J Bacteriol* 191: 6067-6074.

Hass C. J, Zink A, Palfi G, Szeimies U & Nerlich A. G (2000). Detection of leprosy in ancient human skeletal remains by molecular identification of *Mycobacterium leprae. Microbiol Infect Dis* 114: 428- 436.

Honoré N & Cole S. T (1993). Molecular basis of rifamipin resistance in *Mycobacterium leprae. Antimicrob Agents Chemother* 37: 414-418.

Izumi S, Fujiwara T, Ikeda M, Nishimura Y, Sugiyama K & Kawatsu K (1990). Novel gelatin particle agglutination test for serodiagnosis of leprosy in the field. *J Clin Microbiol* 28: 525-529.

Ji B (1987). Drug susceptibility testing of *Mycobacterium leprae. Int J Lepr Other Mycobact Dis* 55: 830-835.

Johnstone A. S (1987). The search for animal models of leprosy. *Int J Lepr Other Mycobact Dis* 55: 535-547.

Kai M, Matsuoka M, Nakata N, Maeda S, Gidoh M, Maeda Y, Hashimoto K, Kobayashi K & Kashiwabara Y (1999). Diaminodiphenilsulphone resistance of *Mycobacterium leprae* due to mutation in the dihydropoteroate syntethase gene. *FEMS Microbiol lett* 177: 231-235.

Kirchheimer W. F & Storrs E. E (1971) Attempts to establish the armadillo (*Dasypus novemcinctus* Linn.) as a model for the study of leprosy, I. Report of lepromatoid leprosy in an experimentally infected armadillo. *Int J Lepr Other Mycobact Dis* 39: 693-702.

Kohsaka K, Mori T & Ito T (1976). Lepromatoid lesion developed in the nude mice inoculated with *Mycobacterium leprae. La lepro* 45: 177-187.

Kohsaka K, Makino M, Mori T & Ito T (1978). Establishment of experimental leprosy in nude mice. *Jpn J Bacteriol* 33: 389-394. (in Japanese)

Kurabachew M, Wondimu A & Ryon J. J (1998). Reverse transcription-PCR detection of *Mycobacterium leprae* in clinical specimens. *J Clin Microbiol* 36: 1352-1356.

Levy L (1976). Studies on the mouse footpad technique for cultivation of *Mycobacterium leprae* 3. Doubling time during logarithmic multiplication. *Lepr Rev* 47: 103-106.

Levy L, Shepard C. C & Fasal P (1976). The bactericidal effect of rifampin on *M. leprae* in man: (a) single dose of 600, 900, 1200 mg: and (b) daily doses of 300mg. *Int J Lepr Other Mycobact Dis* 44: 183-187.

Loughry W. J, Truman R. W, McDounough C. M, Tilak M-K, Garnier S, & Delusuc F (2009). Is leprosy spreading among nine-banded armadillos in the Southeastern United State? *J Wildlife Dis* 45: 144-152.

Martinez A. M, Lahili R, PittmenT. L, Scollard D, Truman R, Moraes M. O & Williams D. L (2009). Molecular detection of *Mycobacterium leprae* viability by use of Real-Time PCR. *J Clin Microbiol* 47: 2142-2130.

Maeda S, Matsuoka M, Nakata N, Kai M, Maeda Y, Hashimoto K, Kimura H, Kobayashi K & Kashiwabara Y (2001). Multidrug resistant *Mycobacterium leprae* from Patients with leprosy. *Antmicrob Agents Chemother* 45: 3635-3639.

Matsuoka M, Izumi S, Budiawan T, Nakata N & Saeki K (1999). Mycobacterium leprae DNA in daily using water as a possible source of leprosy infection. *Indian J Lepr* 71: 61-67.

Matsuoka M, Maeda S, Kai M, Nakata N, Chae G-T, Gillis T. P, Kobayashi K, Izumi S & Kashiwabara Y (2000). *Mycobacterium leprae* typing by genomic diversity and global distribution of genotypes. *Int J Lepr Other Mycobact Dis* 68: 121-128.

Matsuoka M, Kashiwabara Y, Zhang L, Gotoh M & Kitajima S (2003). A second case of multidrug resistant *Mycobacterium leprae* isolated from a Japanese patient with relapsed lepromatous leprosy. *Int J Lepr Other Mycobact Dis* 71: 240-243.

Matsuoka M, Zhang L, Budiawan T, Saeki K & Izumi S (2004). Genotyping of *Mycobacterium leprae* on the basis of polymorphism of TTC repeats for analysis of leprosy transmission. *J Clin Microbiol* 42: 741-745.

Matsuoka M, Zhang L, Fafutis M, Legua P & Wiens C (2005). Polymorphism in the *rpoT* gene in *Mycobacterium leprae* isolates obtained from Latin American countries and its possible correlation with the spread of leprosy. *FEMS Microbial Letter* 243: 311-315.

Matsuoka M, Lopez R, Budiawan T, Kyaw K & Chae G-T (2006). Genotypic analysis of *Mycobacterium leprae* isolates from Japan and other Asian countries reveals global transmission pattern of leprosy. *FEMS Microbiol Letter* 261: 150-154.

Matsuoka M, Teky B, Khin S, Tan E. V, dela Cruz E, Gelber R, Saunderson P, Balagon M. A & Pannikar V (2007). The frequency of drug resistance mutation in *Mycobacterium leprae* isolates in untreated and relapsed leprosy patients from Myanmar, Indonesia, and the Philippines. *Lepr Rev* 78: 343-352.

Matsuoka M (2009). Recent advances in the molecular epidemiology of leprosy. *Jpn J Lepr* 78: 67-73.

Matsuoka M (2010). Drug resistance in Leprosy. *Jpn J Infec Dis*. 63: 1-7.

Meyers W. M, Walsh G. R, Brown H. L, Binford C. H, Imes G. D, Hadfield T. L, Schlagel C. J, Fukunishi Y, Gerone P. J, Wolf R. H, Gormus B. J, Martin L. N, Harboe M & Imaeda T (1985). Leprosy in mangabey monkey – Naturally acquired infection. *Int J Lepr Other Mycobact Dis* 53: 1-14.

Monot M, Honore N, Garnier T, Araoz R, Copee J-Y, Lacroix C, Sow S, Spencer J. S, Truman R. W, Williams D. L, Gelber R, Virmond M, Flageul B, Cho S, Ji B, Paniz-Mondolfi A, Convit J, Young S, Fine P, Rasolofo V, Brennan P. J & Cole S. T (2005). On the origin of leprosy. *Science* 308: 1040-1042.

Monot M, Honoré N, Garnier T, Zidane N, Sherafi D, Paniz-Mondolfi A, Matsuoka M, Taylor G. M, Donoghue H. D, Bouwman., Mays, Watson C, Lockwood D, Khamispour A, Dowlati Y, Jianping S, Rea T. H, Vera-Cabrera L, Stefani M. M, Banu S, Macdonald M, Sapkota B. R, Spencer J, Thomas J, Harshman K, Singh P, Busso P, Gattiker A, Rougemont J, Brennan P. J & Cole S. T (2009). Comparative genomic and phylogeographic analysis of *Mycobacterium leprae*. *Nature Genetics* 41: 1282-1289.

Nakamura M (1994). Elimination of cantaminats in a homogenate of nude-mouse footpad experimentally infected with *Mycobacterium leprae. Jpn J lepr* 64: 47-50.

Nakata N, Matsuoka M, Kashiwabara Y, Okada N & Sasakawa C (1997). Nucleotide sequence of the *Mycobacterium leprae katG* region. *J Bacteriol* 179: 3053-3057.

Ng V, Zanazzi G, Timpl R, Tals J. F, Salzer J. L, Brennan P. J. & Rambukkana A (2000). Role of the cell wall phenolic glycolipid-1 in the peripheral nerve predilection of *Mycobacterium leprae. Cell* 103: 511-524.

Noordeen S. K (1978). Infectivity of leprosy. In: Chattarjee, B. R. (ed) A window on leprosy. Chandi Memorial Leprosy Foundation, New Delhi, pp. 59-63.

Pallamary P (1955). Translation of "spedalskhedens Årsager" (causes of leprosy) by G. Armauer Hansen, from Norsk Magazine for Laegervidenskaben 4: 76-79. *Int J Lepr Other Mycobact Dis* 23: 307-309.

Phenitsuksri B, Rudeeneksin J, Supapkul P, Wacchapong S. Mahotan K & Brennan P. J (2006). A simple reverse transcriptase PCR for rapid detection of *Mycobacterium leprae* in skin specimens. *FEMS Immunol Med Microbiol* 48: 319-328.

Plikaytis B. B, Gelber R. H & Shinnick T. M (1990). Rapid and sensitive detection of *Mycobacterium leprae* using a nested-primer gene amplification assay. *J Clin Microbiol* 28: 1913-1917.

Rees R. J. W (1966). Enhanced susceptibility of thymectomized and irradiated mice to infection with *Mycobacterium leprae. Nature* 211: 657-658.

Ridley D. S & Jopling W. H (1966). Classification of leprosy according to immunity – a five group system. *Int J Lepr Other Mycobact Dis* 54: 255-273.

Saeki K, Budiawan T, Matsuoka M & Izumi S (2000). Epidemiological significance of *M. leprae* in the residential environment: Detection of *Mycobacterium leprae* on the surface of nasal cavity of inhabitants in a leprosy endemic area using polymerase chain reaction. *Jpn J Dermator* 110: 153-160. (in Japnese)

Shepard C. C (1960). The experimental disease that follows the injection of human leprosy bacilli into foot-pad of mice. *J Exp Med* 112: 445-454.

Shepard C. C & MacRea D. H (1965). *Mycobacterium leprae* in mice: Minimal infectious dose, relationship between staining quality and infectivity and effect of *cortizone. J Bacteriol* 89: 365-372.

Shepard C. C & MacRea D. H (1968). A method for counting acid –fast bacteria. *Int J Lepr Other Mycobact Dis* 36: 78-82.

Shepard C. C & MacRae D. H (1971). Hereditary characteristic that varies among isolates of *Mycobacterium leprae. Infect Immun* 3: 121-126.

Shin Y. C, Lee H, Walsh G. P, Kim J. D & Cho S. N (2000). Variable numbers of TTC repeats in *Mycobacterium leprae* DNA from leprosy patients and use in train differentiation. *J Clin Microbiol* 38: 4543-4538.

Spencer J. S, Dockrell H. M, Kim J. H, Marques M. A, Williams D. L, Martins M. V, Martins M. V, Lima M. C, Serno E. N, Pereira G. M, Matos H, Fonseca L. S, Sampaio E. P, Ottenfoff T. H, Geluk A, Cho S. N, Stoker N. G, Cole S. T, Brennan P. J, & Pessolani M. C (2005). Identification of specific proteins and peptide in *Mycobacterium leprae* suitable for the selective diagnosis of leprosy. *J immonol* 175: 7930-7938.

Takeda A, Umeda A, Matsuoka M, Yoshida S, Nakamura M & Amako K (2003). Comparative study of the cell structure of *Mycobacterium leprae* and *M. tuberculosis* using the electron microscopy freeze-substitution technique. *Microbiol Immunol* 47: 265-270.

Truman R. W & Krahenbuhl J. L (2001). Viable *M. leprae* as a research reagent. *Int J Lepr Other Mycobact Dis* 69: 1-12.

Truman R. W, Andrews P. K, Robbins N. Y, Adams L. B, James L, Krahenbuhl J. L & Gillis T. P (2008). Enumeration of *Mycobacterium leprae* using Real-Time PCR. *PLOS Negl Trop Dis* 2: e238.

Valverde C. R, Canfield D, Tarara R, Esteves M. I & Gormus B. J (1998). Spontaneous leprosy in a wild-caught cynomologus macaque. *Int J Lepr Other Mycobact Dis* 66: 140-148.

van Beers S, Izumi S, Madjid B, Maeda Y, Day R & Klatser P. R (1994). An epidemiological study of leprosy infection by serology and polymerase chain reaction. *Int J Lepr Other Mycobact Dis* 62: 1-9.

Walsh G. P, Storrs E. E, Burchfield H. P, Cottrell E. H, Vid-

rine M. F & Binford C. H (1975). Leprosy-like disease occurring naturally in armadillos. *J Reticuloendothel Soc* 18: 347-351.

Walsh G. P, Meyers W. M, Binford C. H, Gerone P. J, Wolf R. H & Leininger J. R (1981). Leprosy - a zoonosis. *Lepr Rev* 52: 77-83.

Williams D. L, Gillis T. P & Portaels F (1990). Geographically distinct isolates of *Mycobacterium leprae* exhibit no genotypic diversity by restriction fragment-length polymorphism analysis. *Mol Microbiol* 4: 1653-1659.

Williams D. L & Gillis T. P (2004). Molecular detection of drug resistance in *Mycobacterium leprae. Lepr Rev* 75: 118-130.

Wolf R. H, Gormus B. J, Martin L. N & Baskin G. B (1985). Experimental leprosy in three species of monkeys. *Science* 237: 529-531.

Woods S. A & Cole S. T (1989). A rapid method for the detection of potentially vaiable *Mycobacterium leprae* in human biopsies. *FEMS Microbiol lett* 65: 305-310.

Zhang L, Budiawan T & Matsuoka M (2004). Diversity of potential short tandem repeats in *Mycobacterium leprae* and application for Molecular typing. *J Clin Microbiol* 43: 5221-5229.

Chapter 9

Serology

Masanori Kai

The key to controlling Hansen's disease lies in curbing the number of new cases that has shown little decline over the years, requiring the stemming of disease through early detection and treatment. Although the advanced methods of late using molecular techniques to diagnose the disease are useful, the less costly and more convenient conventional serodiagnostic methods continue to be of value. Serological methods to detect specific antibodies against *Mycobacterium leprae* can be used for both early diagnosis, and for monitoring treatment effect in patients under treatment with antibiotics. Their use in the detection of relapse and for identifying patients at risk of developing type 1 or type 2 reactions are also under consideration. Furthermore, the seropositive rate in a given group or geographical area is believed to correspond to the infection rate by *M. leprae* in that group or region. Monitoring using serological methods can also determine the efficacy of the various countermeasures being taken for leprosy.

In this section, the serology of Hansen's disease is reviewed through description of the various antigens of *M. leprae*, and the methods used for their serodiagnosis.

9.1 INTRODUCTION

The current treatment for leprosy consists primarily of the multi-drug therapy (MDT) regimens recommended by the World Health Organization (WHO). The MDT has been effective in reducing the number of registered leprosy cases in the world to date. However, we have yet to see comparable reduction in the number of new cases of leprosy, and at present, the number of both registered cases and new cases has plateaued at 210,000 and 250,000 cases, respectively (WHO, 2009). As such, emphasis in the next stage of leprosy control is the identification and eradication of sources of infection, and the control of relapses and intractable cases through early detection and treatment.

Clinically, diagnosis of leprosy is based on histopathological detection of the acid-fast *M. leprae* bacilli in skin smears or biopsies, in patients suspected of Hansen's disease given clinical evidence of skin lesions with peripheral nerve damage or enlargement. Recent advances in diagnostic methods now allow for detection of *M. leprae* DNA sequences from biopsy samples (Donoghue *et al*, 2001; Phetsuksiri *et al*, 2006). However, it is difficult to incorporate such methods in developing countries that still have leprosy hot spots, because such methods require expensive machinery and materials, as well as skilled technicians. In the interest of leprosy control, early detection of patients in the stage before onset of clinical manifestations is desired. However, it is difficult to obtain consent to biopsies from asymptomatic persons, even when they are household contacts (HHC) of leprosy patients. In contrast, serological tests employing blood samples sharply reduce subject load increasing compliance to testing. Serological tests that detect antibodies against components of *M. leprae* can be utilized for both monitoring the effectiveness of drug treatment as well as early diagnosis of infection (Roche *et al*, 1993). In addition, the serological tests are applicable for the early diagnosis of relapse, and determination of patients at risk of developing type 1 or type 2 reactions during the course of therapy. However, despite these benefits, serological methods are still regarded as being no more than complementary, as they are unable to detect all types of leprosy under all conditions. Detection of specific antibodies is difficult in many paucibacillary type (PB) patients whose response to Hansen's disease is believed to be the result of mainly cell-mediated immunity. Various antigens have been reported as target candidates for serodiagnosis, including the glycolipids lipoarabinomannan (LAM) (Gelber *et al*, 1989) and phe-

nolic glycolipid-I (PGL-I) (Patil *et al*, 1990), and proteins with relative molecular masses of 10-kDa (Rojas *et al*, 1997), 15-kDa (Britton *et al*, 1988), 18-kDa (Mohanty *et al*, 2004), 25-kDa (Schorey *et al*, 1995), 27-kDa (Young *et al*, 1985), 28-kDa (Mohanty *et al*, 2004), 30-kDa (Filley *et al*, 1994), 35-kDa (Roshe *et al*, 1999), 36-kDa (Klatser *et al*, 1985), 45-kDa (Rinke-de-Wit *et al*, 1992), 48-kDa (Britton *et al*, 1988), 65-kDa (Meeker *et al*, 1989), and 70-kDa (Britton *et al*, 1988). However, further study on their specificity and reproducibility have reduced these possibilities to a few, such as PGL-I, and the 35-kDa and 45-kDa proteins that have been popularly used. As it now stands, serodiagnosis using these antigens allow for detection of 90-100% of patients in the active phase of the disease, but only 40-60% of those in the early phase (Sengupta, 1990).

This chapter provides an overview of leprosy serology with reference to Buchanan's review (Buchanan, 1994), incorporating some of the more recent applications and methods in serodiagnosis.

9.2 LIPID ANTIGENS

9.2.1 LAM

The *M. leprae* bacillus is surrounded by a coating of lipoarabinomannan (LAM), and many serological tests have been developed to detect antibodies against the LAM antigen (Gelber *et al*, 1989; Mwatha *et al*, 1988; Jayapal *et al*, 2001). Mwatha et al used a competitive inhibition assay employing an RI-labeled monoclonal antibody, ML34, which responds to an epitope on the LAM antigen. Gelber et al and Jayapal et al have measured antibodies to LAM directly in a microtiter enzyme-linked immunosorbent assay (ELISA). Serum antibodies in leprosy patients, especially multibacillary type (MB) patients can be detected well by either method. However, as LAM is not structurally or antigenically unique to *M. leprae*, the detection of antibodies to LAM can not rule out the possibility of infection by other LAM-carrying bacteria.

9.2.2 PGL-I

Apart from the LAM antigen, the cell surface of *M. leprae* is studded with characteristic phenolic glycolipids. The main component of these phenolic glycolipids is phenolic glycolipid-I (PGL-I) (Brennan & Barrow, 1980), which is characterized by a terminal trisaccharide unique to *M. leprae* consisting of three immunodominant saccharides—methylglucose, methylramnose, and methylramnose. Many serological studies have been conducted following discovery of this molecule, and various methods have been developed to measure specific antibodies against PGL-I, employing the whole molecule either alone (Cho *et al*, 1983), or within liposomes (Schwerer *et al*, 1989), in deacylated form (Young & Buchanan, 1983), or as synthetic forms of the terminal monosaccharide (Douglas *et al*, 1988), disaccharide (Cho *et al*, 1983; Petchlai *et al*, 1988), or trisaccharide (Izumi *et al*, 1990). Detection of antibody to PGL-I has for most part been by direct ELISA, although Izumi *et al* have developed a unique gelatin particle agglutination test (Izumi *et al*, 1990) (Fig.9.1).

Figure 9.1 "Serodia-Leprae" is commercially available from Fujirebio Inc.,Japan, which was used MLPA (*M. leprae* particle agglutination) method originally developed by Izumi *et al.*

The PGL-I structure with its unique trisaccharide was analyzed in detail, and mixed synthetic saccharides (mono-, di-, or tri-saccharides) were engineered for laboratory use. As patients with Hansen's disease exhibit high antibody titers to PGL-I, this has been the most intensely studied of *M. leprae* antigens to date. The study of responses to PGL-I by antibody class has demonstrated that while IgG, IgA, and IgM antibodies can recognize PGL-I antigen, detection of IgM antibodies has become standard in analyses for PGL-I, given predominance of the IgM response (Praputpittaya *et al*, 1990). Comparative analyses between PGL-I and LAM (Jayapal *et al*, 2001; Sekar *et al*, 1993) have demonstrated that both PGL-I and LAM are capable of detecting high antibody responses in MB patients, but not in PB patients, and that sensitivity of PGL-I was slightly higher than that of LAM, supporting the popular use of PGL-I for serodiagnosis.

9.2.3 Other glycolipids

Cord factor—identified as one of the virulence factors in *M. tuberculosis*—is chemically a type of glycolipid, trehalose dimycolate (TDM). TDM is present in many mycobacteria, but the structure of one of its components—mycolic acid—is slightly different between species. Using this slight difference, TDM has been utilized as a serodiagnostic antigen (Wang *et al*, 1999), although until the recent extraction of TDM from *M. leprae*, the TDM used in ELISA tests for leprosy had been derived from *M. bovis* BCG. Recent progress in technology has enabled the detection, analysis, and extraction of *M. leprae* TDM, which is now regarded as a candidate antigen for the serodiagnosis of leprosy (Kai *et al*, 2007).

9.3 PROTEIN ANTIGENS

M. leprae has few surface-exposed proteins in contrast to other bacteria, but some proteins are known to be processed and presented on the surface of antigen-presenting cells (macrophage or dendritic cells), and specific antibodies have been detected to such processed proteins or secretory proteins. Leprosy patients infected with *M. leprae* produce antibodies against such protein antigens, although not at levels noted in response to PGL-I and LAM. The major protein antigens with possible serodiagnostic value reported to date are described below.

9.3.1 30-kDa protein

Three types of 30-kDa protein—85A, 85B, and 85C—are secretory or membrane-binding proteins known as the Antigen 85 complex. The homology rates between each of the three proteins exceed 80%. The 85A and 85B proteins have a fibronectin-binding domain similar to the fibronectin-binding protein (Thole *et al*, 1992). The 85B protein is involved in mycolic acid biosynthesis (Anderson et al, 2001), and the Antigen 85 complex comprised of these antigens reacts well with leprosy patient serum but not with TB patient serum (Filley *et al*, 1994).

9.3.2 35-kDa protein (MMP-I)

The 35-kDa protein is a membrane-binding protein known as MMP-I (major membrane protein-I), and is one of the most well-analyzed and reported antigens (Sinha *et al*, 1983) recognized as having serodiagnostic value approaching that of PGL-I (Roshe *et al*, 1999; Jayapal *et al*, 2001; Parkash *et al*, 2002).

9.3.3 MMP-II (22-kDa)

MMP-II (major membrane protein-II) was identified as one of the major proteins in *M. leprae* by Hunter *et al* (Hunter *et al*, 1990), also shown to be a mycobacterial bacterioferritin (Pessolani *et al*, 1994). MMP-II, with a molecular weight of 22-kDa, has been shown to induce both humoral and cellular immune response in leprosy patients (Ohyama *et al*, 2001), indicating possible serodiagnostic value. Maeda *et al* have identified, extracted, and cloned MMP-II protein as a cellular membrane protein that reacts with serum of PB patients (Maeda *et al*, 2007). Using purified MMP-II protein fused with maltose binding protein (MBP) (64-kDa), they demonstrated high reactivity with serum from Japanese leprosy patients, in terms of both sensitivity and specificity. In particular, MMP-II was found to react more strongly with PB patient serum compared to other *M. leprae* antigens. Good results were also obtained in a recent survey using MMP-II ELISA conducted in other countries where leprosy continues to be endemic (Kai *et al*, 2008; Hatta *et al*, 2009). Moreover, while comparison with PGL-I ELISA showed comparable sensitivity and specificity between MMP-II and PGL-I, slightly higher positive rates have been obtained with MMP-II ELISA in PB patients.

9.3.4 45-kDa protein (ML0411: serine-rich protein)

The 45-kDa serine-rich protein derived from *M. leprae* was reported as a *M. leprae*-specific protein by Rinke de Wit *et al* (Rinke de Wit *et al*, 1993), in spite of homology with that of *M. tuberculosis* (Rinke-de-Wit *et al*, 1992). Although sensitivity does not equal PGL-I, there have been reports of positive results being obtained in many samples negative by PGL-I ELISA (Thole *et al*, 1995), indicating possible utility as a serodiagnostic antigen.

9.3.5 CFP-10 and ESAT-6

Parkash *et al* analyzed the usefulness of *M. leprae* CFP-10 and ESAT-6 in independent experiments (Parkash *et al*, 2006a; Parkash *et al*, 2006b). The possible utility of these secretory proteins was confirmed, with 82-83% of MB patients and 18% of PB patients being seropositive for these antigens.

9.3.6 10-kDa protein

The 10-kDa protein is known as heat shock protein GroES, that binds to form a chaperone with heat shock protein GroEL. The two heat shock proteins found in *M. leprae* and *M. tuberculosis* are almost identical, accounting for the cross-reactivity with TB patients (Young & Buchanan, 1983).

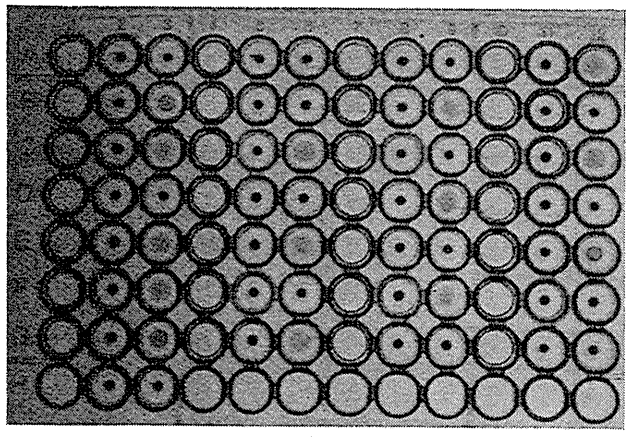
9.3.7 Pepetide antigens

An attempt to identify T cell epitopes on the 45-kDa serine-rich protein of *M. leprae* was conducted using 17 overlapped peptides synthesized to cover the entire protein, and analyzing its response in leprosy patients in Pakistan and healthy (non-exposed) controls in England (Brahmbhatt *et al*, 2002). T-cell recognition of some peptides in PB patients suggested possible diagnostic potential of such T-cell epitopes, although unfortunately, T cells from many TB patients also responded to the peptides. Many of these initially studied antigens are proteins found in abundance in the *M. leprae* bacillus, but utility was often limited by the cross-reactivity with other mycobacteria.

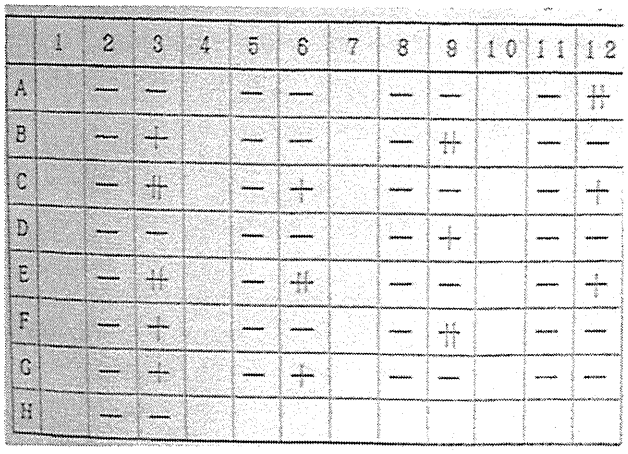
9.4 SEROLOGICAL METHODS

9.4.1 Advanced rapid serodiagnosis (ML-flow test)

Although methods such as radio-immunoassay and monoclonal antibody inhibition tests are available, the most common method in laboratory serodiagnosis is probably the ELISA. The ELISA is used to detect specific antibodies in subject serum, plasma, or whole blood, in microtiter plates coated with various antigens. As mentioned above, high sensitivity and specificity have been reported for various antigens specific to *M. leprae* as tested by the ELISA. However, ELISA testing requires skilled technicians and costly specialized equipment and facilities. Additionally, the test routinely requires a full day to obtain results. Such being the case, development of simpler methods with higher cost performance was desired for field use in endemic areas. Multiplex ligation-dependent probe amplification (MLPA) was applied to develop the first such simple agglutination test using PGL-I antigen (Izumi *et al*, 1990) (Fig. 9.1, 9.2). It was followed by development of a simple card test using 35-kDa antigen (Roche *et al*, 1999). These simple tests are no longer in wide circulation, being replaced by the dipstick test developed in 1998 by Buhrer *et al* as a simple method capable of producing results in 3 hours (Buhrer-Sekula *et al*, 1998), while showing more than 97% agreement with the ELISA test. More recently, the same group has developed the ML-flow test (lateral flow test for *M. leprae*) using the same principle, capable of detecting antibody with comparable reliability from a single drop of whole blood in just 10 minutes (Buhrer-Sekula *et al*, 2003) (Fig. 9.3). The plastic strip system used in the ML-flow test is the same as the used in diagnostic kits for TB, filariasis, and other such diseases, which is becoming a standard tool in the diagnosis of infectious diseases.



A



B

Figure 9.2 An example of MLPA qualitative assay (Serodia-Leprae kit).
A: Two-fold serum dilutions from 1:4, 1:8, 1:16 were made in 3 wells (for example: from A-1, -2, -3 to G-1, -2, -3). Buffer is added to H-1,-2,-3 as negative control. The PGL-I sensitized particles are added to lane 3, 6, 9, 12 and unsensitized particles are added to lane 2, 5, 8,11. The result is interpreted according to the instructions after incubation at room temperature for 2 hrs.
B: The agglutination pattern is determined as follows: +: positive, ++: strong positive, –: negative

9.4.2 Other methods and modifications

(1) Low temperature ELISA.

Parkash et al have reported that both sensitivity and specificity of PGL-I ELISA could be en-

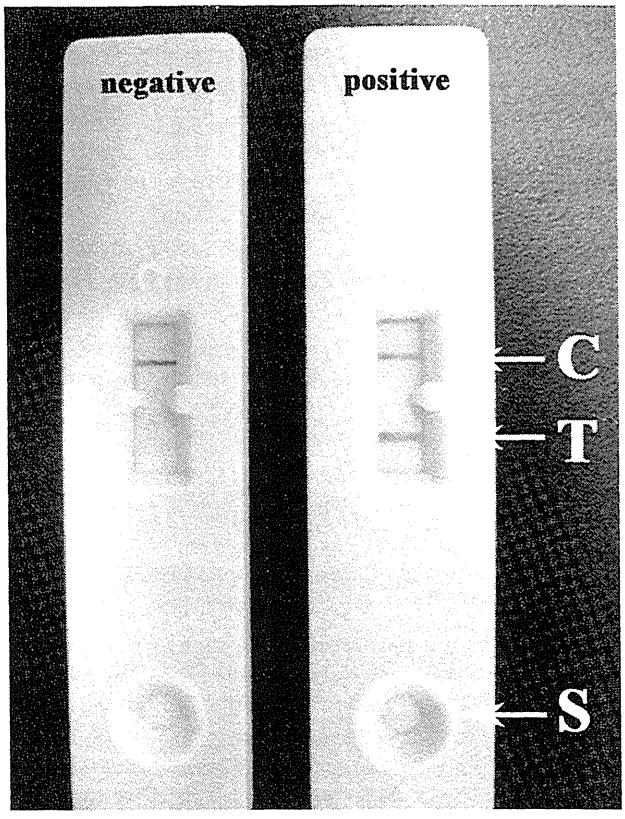


Figure 9.3 The latest serodiagnostic kit, ML flow test. Sample (serum or blood) is put into the lower circle (S) and the reaction buffer is add to the same circle. After 10 min the result is visualized as lines in the upper windows. Single band at position C indicates negative for anti-PGL-I antibodies. Double bands at position C and T indicate positive.
*The kit was purchased from KIT Biomedical Research in Netherland.

hanced by carrying out the test at low temperatures (Parkash *et al*, 2007a). Positive results could be obtained in 50-70% of PB patients using this modification. However, two drawbacks have been noted. First, this effect was not evident in ELISA using another protein antigen, 45-kDa, indicating this is not a general improvement applicable to all ELISAs. Secondly, the low temperature treatment requires an additional 24 hours to produce results, making it an even more time-consuming procedure.

(2) Double usage.

Parkash *et al* also tested the use of two antigens—45kDa protein and PGL-I—in low temperature ELISA (Parkash *et al*, 2007b). A positive rate of 100% in MB patients and 76% in PB patients was achieved with this double usage, more than making up for the aforementioned disadvantages, awaiting further study and evaluation by other researchers to confirm these findings.

(3) CMI (cell mediated immunity) method

The CMI method was adapted from its original use in diagnosing tuberculosis, in a new attempt for the early diagnosis of patients in early stage PB disease, in whom cell mediated immunity is the primary response (Ferrara *et al*, 2006)). The CMI method measures γ-IFN produced by T cells stimulated by specific bacterial antigens to determine the presence of infection. Geluk *et al* first demonstrated utility of the CMI method in leprosy diagnosis using various *M. leprae* antigens (Geluk *et al*, 2005). In their report, 90% of PB patients who were PGL-I ELISA negative, and 70% of household contacts (HHC) of leprosy patients were positive by CMI, while positive readings in healthy controls amounted to only about 7%. Effective use of this method is visualized in combination with other conventional forms of serodiagnosis such as PGL-I ELISA.

Recently, several other antigens of *M. leprae* were analysed and reported by members of the IDEAL Consortium (Geluk *et al* 2009).

9.5 SERODIAGNOSIS AND THE CLINICAL STATE

Reports of serodiagnosis using PGL-I have indicated that while it is possible to identify 75-100% of MB patients, positive results could be obtained in only 15-40% of patients with PB disease (Oskam *et al*, 2003). This means that it is not possible to diagnose all types of leprosy with a single serodiagnostic test using any of the antigens studied to date. However, the ability to detect most all cases of MB disease is of definite value. Use of the serological test in combination with clinical information such as the number of skin lesions has also been proposed as a useful method for proper determination of the type of disease, for accurate diagnosis and selection of optimum treatment. In terms of treatment, Buhrer *et al* have come up with concrete suggestions regarding interpretation of serodiagnostic results, recommending application of MB treatment for all seropositive patients even when less than six lesions are noted to reduce the number of patients who exhibit insufficient response to therapy (Buhrer *et al*, 2000).

With regard to the correlation between treatment course and serodiagnostic findings, antibody titers generally decrease with progress of treatment, although this is not a constant feature, being largely dependent upon the individual. Patients sometimes remain seropositive for many years after treatment (Gelber *et al*, 1989), a possible cause of such persistency being the continuing presence of dead or dormant bacteria inside the body (Meeker *et al*,

1990).

Fine has expressed negative views regarding utility of serological tests for either early detection of disease or monitoring of drug effect (Fine, 1989). Reporting that many healthy seropositive individuals become seronegative in subsequent follow-up without developing leprosy, Fine questioned the utility of serodiagnosis for large-scale screening in low incidence areas.

As such, given the correlation between decrease in antibody titers and bacillary index noted through serial follow-up in individual patients, serological testing is believed to have greater utility as a complementary method for monitoring progress in individual patients rather than as a tool for mass screening.

9.6 SERODIAGNOSIS IN THE FIELD

In addition to the utility of serodiagnosis for early detection and treatment of Hansen's disease, its validity in identifying subclinical patients who may be possible sources of infection among the house-hold contacts of leprosy patients has been studied in the interest of decreasing the incidence of new cases (Kai *et al*, 2004). However, correct interpretation of results from such surveys is difficult. Comparison of serological test results between healthy individuals and HHC in a given area shows a general tendency of higher seropositivity of the HHC group. However, this tendency does not necessarily hold true for all regions, for example, there have been reports of no difference in positive rates between the general population and HHC in prevalent areas, where as much as 1.7-3.1% of the general population has been reported seropositive for *M. leprae*.

On the other hand, Douglas *et al* have demonstrated that seropositive HHC in a region of the Philippines are at increased risk of developing leprosy (Douglas *et al*, 1988). This suggests the possibility that serological testing may have practical utility in identifying high-risk individuals among the contacts of leprosy patients, and furthermore, allow for prophylactic treatment of seropositive HHCs, heralding a new dimension in leprosy control.

REFERENCES

Anderson D. H, Harth G, Horwitz M. A & Eisenberg D (2001). An interfacial mechanism and a class of inhibitors inferred from two crystal structures of the *Mycobacterium tuberculosis* 30 kDa major secretory protein (Antigen 85B), a mycolyl transferase. *J Mol Biol* 307: 671-681.

Brahmbhatt S, Hussian R, Zafar S, Dawood G, Ottenhoff T. H, Drijfhout J. W, Bothamley G, Smith S, Lopez F. V & Dockrell H. M (2002). Human T-cell responses to peptides of the *Mycobacterium leprae* 45kDa serine-rich antigen. *Clin Exp Immunol.* 128: 140-148.

Brennan P. J & Barrow W. W (1980). Evidence for species-specific lipid antigens in *Mycobacterium leprae. Int J Lepr Other Mycobact Dis* 48: 382-387.

Britton W. J, Hellqvist L, Garsia R. J & Basten A (1988). Antigens of *Mycobacterium leprae* identified by immunoprecipitation with sera from leprosy and tuberculosis patients. *Clin exp Immunol* 71: 394-398.

Buchanan T. M (1994). Serology of leprosy. In: Hastings R. C & Opromolla D. V. A (eds) Leprosy, Churchill Livingstone, Edinburgh, pp. 169-176.

Buhrer-Sekula S, Cunha M. G, Ferreira W. A & Klatser P. R (1998). The use of whole blood in a dipstick assay for detection of antibodies to *Mycobacterium leprae*: a field evaluation. *FEMS Immunol Med Microbiol* 21: 197-201.

Buhrer-Sekula S, Sarno E. N, Oskam L, Koop S, Wichers I, Nery J. A, Vieira L. M, de Matos H. J, Faber W. R & Klatser P. R (2000). Use of ML dipstick as a tool to classify leprosy patients. *Int J Lepr Other Mycobact Dis* 68: 456-463.

Buhrer-Sekula S, Smits H. L, Gussenhoven C, van Leeuwen J, Amador S, Fujiwara T, Klatser P. R & Oskam L (2003). Simple and fast lateral flow test for classification of leprosy patients and identification of contacts with high risk of developing leprosy. *J Clin Microbiol* 41: 1991-1995.

Cho S. N, Yanagihara D. L, Hunter S. W, Gelber R. H & Brennan P. J (1983). Serological specificity of phenolic glycolipid 1 from *Mycobacterium leprae* and use in serodiagnosis of leprosy. *Infect Immun* 41: 1077-1083.

Donoghue H. D, Holton J & Spigelman M (2001). PCR primers that can detect low levels of *Mycobacterium leprae* DNA. *J Med Microbiol* 50: 177-182.

Douglas J. T, Steven L. M, Fajardo T, Cellona R. V, Madarang M. G, Abalos R. M & Steenberger G. J (1988). The effects of chemotherapy on antibody levels in lepromatous patients. *Lepr Rev* 59: 127-135.

Ferrara G, Losi M, D'Amico R, Roversi P, Piro R, Meacci M, Meccugni B, Dori I. M, Andreani A, Bergamini B. M, Mussini C, Rumpianesi F, Fabbri L. M & Richeldi L (2006). Use in routine clinical practice of two commercial blood tests for diagnosis of infection with *Mycobacterium tuberculosis*: a prospective study. *Lancet* 367: 1328- 1334.

Filley E, Thole J. E, Rook G. A, Nagai S, Waters M, Drijfhout J. W, Rinke de Wit T. F, De Vries R. R & Abou-Zeid C (1994). Identification of an antigenic domain on *Mycobacterium leprae* protein antigen 85B, which is specifically recognized by antibodies from patients with leprosy. *infect Dis* 169: 162-169.

Fine P. E (1989). Immunological tools in leprosy control. *Int J Lepr Other Mycobact Dis* 57: 671-686.