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Asian Skin and Skin Diseases

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Hansen's Disease in Asia

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Introduction

Hansen's disease (Leprosy), a chronic infectious disorder caused by the rod-shaped bacillus *Mycobacterium leprae* (*M. leprae*), primarily affects the skin and peripheral nerves[1]. Without treatment, it can be progressive and result in permanent damage to the affected areas. Patients also suffer from social stigma due to the skin lesions and deformities of the face, extremities, eyes, and other regions in advanced cases. Effective therapy using dapsone (4,4-diaminodiaphenylsulfone, DDS) was first introduced in the 1940s, followed by the introduction of multidrug therapy (MDT) in the 1980s. While the use of these drugs has significantly improved care and prognosis, the nerve damage and deformities cannot be recovered when treatment is delayed. Although the World Health Organization (WHO), community health organizations, and non-governmental organizations (NGOs) have worked to control and eliminate Hansen's disease, it is still a major public health and societal problem in many regions, including Asia. Therefore, monitoring and prevention programs must be maintained in these countries.

Bacteriology and Genomics of *M. leprae*

M. leprae is an obligate intracellular parasite that measures 0.3-0.5×4.0-7.0 μm and multiplies very slowly, with an approximate generation time of 12 to 14 days. The bacterium can remain viable for several days *ex vivo*, but cannot be cultured in artificial media. However, it multiplies extensively in the footpads of nude mice, nine-banded armadillos, and, to a limited extent, in the footpads of normal mice. It grows best around 30°C, and hence prefers the cooler areas of the human body. The cell wall is highly complex and contains proteins, phenolic glycolipid (PGL), arabinoglycan, peptideglycan, and mycolic acid, the latter possibly being responsible for its acid-fastness.

The *M. leprae* genome was completely sequenced in 2001[2]. The most striking feature of the genome is the extensive deletion and inactivation of genes, referred to as gene degradation: only 49.5% of the genome contains protein-coding genes, and 27% contains recognizable pseudogenes[2,3]. In particular, genes encoding various enzymes have been replaced by pseudogenes; which suggests limited metabolic activity in *M. leprae*. This genomic feature might correspond to its unique bacteriological characteristics such as exceptionally slow growth and failure to multiply in synthetic media.

Immunology of Hansen's Disease

Infection with *M. leprae* induces diverse clinical features corresponding to the ability of the host to mount an immune response. Hansen's disease presents as two types: polar tuberculoid and polar lepromatous, with subtypes ranging between these two polar forms. Cell-mediated immunity is the first line of defence against *M. leprae* infection. Therefore, the outcome of infection depends how the host responds to the pathogen-the magnitude of cell-mediated immunity determines the extent of the disease.

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Early pioneering works revealed an apparent relationship between the dominant cytokine profiles and the clinical presentation of Hansen's disease. Interleukin 2 (IL-2) and interferon gamma (IFN-γ) were the prevalent cytokines in tuberculoid lesions, whereas IL-4, IL-5 and IL-10 were characteristic of lepromatous lesions. The Th1-Th2 dichotomy is a central determinant of the type of host defence: the T helper type 1 (Th1) subset characterized by IL-2 and IFN-γ preferentially elicits cell-mediated immunity, whereas Th2 cells that produce IL-4, 5, and 10 augment humoral immunity. Both the classic reciprocal relationship between antibody production and cell-mediated immunity and resistance or susceptibility to *M. leprae* can be explained by different T-cell subset patterns of cytokine production.

Recognition of *M. leprae* by Toll-like receptors (TLRs) and subsequent activation of innate immune responses may also play an important role in the pathogenesis of leprosy[4]. *M. leprae* activates TLR2 and TLR1, which are found on the surface of Schwann cells, especially in tuberculoid patients. Although this cell-mediated immune defense is most active in mild forms of the disease, it might also be responsible for the activation of apoptosis genes and, consequently, the hastened onset of nerve damage found in persons with mild disease.

Disease Spectrum

According to the Ridley-Jopling classification system, which is based on the individual *M. leprae*-specific immunological status, the disease can be classified into six categories based on dermatological, neurological, and histopathological findings: indeterminate (I), tuberculoid (TT), borderline tuberculoid (BT), mid-borderline (BB), borderline lepromatous (BL), and lepromatous (LL). Most newly diagnosed patients, however, live in developing countries where sufficient medical resources are not available. Since the Ridley-Jopling classification system is not practical in these countries, the WHO established a simplified classification system that consists of just two categories-paucibacillary (PB) and multibacillary (MB), on the basis of clinical manifestations and slit skin smear results. PB leprosy

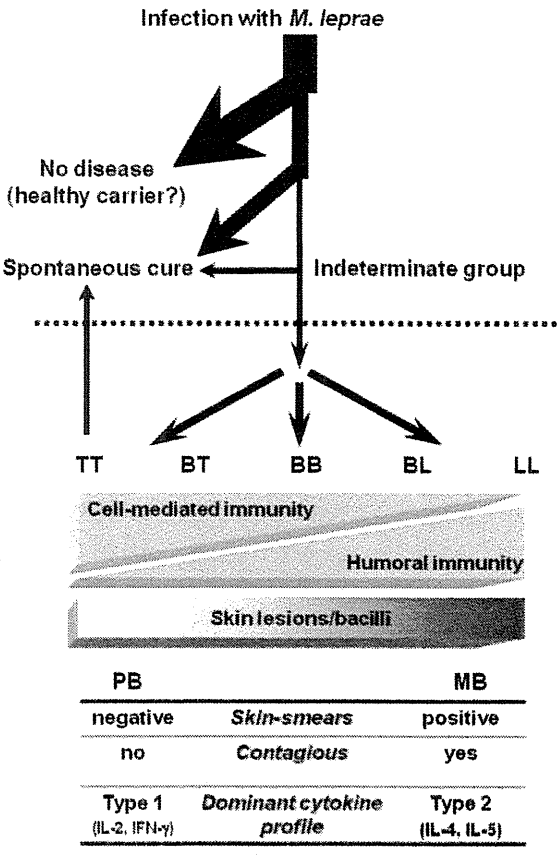

Fig. 1. Flowchart for leprosy diagnosis and classification.

is defined as five or fewer skin lesions with no bacilli in slit skin smears. MB leprosy cases have six or more lesions and may be slit skin smear positive. As for the correlation between the two classification systems, I, TT, and part of BT are generally equivalent to PB disease, while part of BT, BB, BL, and LL correspond to MB disease. Fig. 1 shows the flowchart for diagnosis and classification cited from an atlas designed for use in areas of endemicity. The procedure is simple and clear, so that patients can be diagnosed and classified without sophisticated medical facilities or staff.

Laboratory Tests and Diagnostic Procedures

Biopsies from skin or nerves of lesions are stained with hematoxylin and eosin for histological evaluation and Fite-Faraco for detection of acid-fast bacilli (Table 1). The ba-

cillus is also identified in acid-fast stains of slit skin smears taken from lesions and cooler areas of the skin, such as the ear-lobes, elbows, and knees. If acid-fast bacteria are found in slit skin smears, the patients are diagnosed with MB leprosy. Negative smears are classified as PB leprosy.

Laboratories are now encouraged to use the polymerase-chain reaction (PCR) to detect *M. leprae* DNA in infected tissues[5]. Gene variants that confer resistance to rifampicin (RFP), dapsone, and quinolone are also detectable by PCR.

Diagnosis of Hansen's disease is most commonly based

Table 1. Diagnosis and Treatment in Selected Asian Countries and Brazil

Country	Diagnosis	Classification	Treatment	Duration of treatment (MB)	Duration of treatment (PB)	Monthly supervised treatment
China	Skin lesion, Nerve, *M. leprae* (smear, pathol), Pathology	MB/PB Ridley-Jopling	WHO-MDT	2 years	6 mo	Yes
Indonesia	Skin lesion, Nerve, *M. leprae* (smear), +/-Pathology	MB/PB Ridley-Jopling	WHO-MDT	1 year	6 mo	Yes
Japan	Skin lesion, Nerve, *M. leprae* (smear, pathol, PCR), Pathology	MB/PB Ridley-Jopling	WHO-MDT	1~3 years	6 mo	No
Malaysia	Skin lesion, Nerve, *M. leprae* (smear, pathol), Pathology	MB/PB Ridley-Jopling	See below*	See below*	12 mo	Yes
Myanmar	Skin lesion, Nerve, *M. leprae* (smear), +/-Pathology	MB/PB	WHO-MDT	1 year	6 mo	Yes
Philippines	Skin lesion, Nerve, *M. leprae* (smear)	MB/PB	WHO-MDT	1 year	6 mo	Yes
Republic of Korea	Skin lesion, Nerve, *M. leprae* (smear, pathol, PCR), Pathology	MB/PB Ridley-Jopling	WHO-MDT	Until BI = 0	24 mo	
Thailand	Skin lesion, Nerve, *M. leprae* (smear, +/-pathol), +/-Pathology	MB/PB Ridley-Jopling	WHO-MDT	2 years	6 mo	Yes
Viet Nam	Skin lesion, Nerve, *M. leprae* (smear, pathol), Pathology	MB/PB Ridley-Jopling	WHO-MDT	1 year	6 mo	Yes
Brazil	Skin lesion, Nerve, *M. leprae* (smear), +/-Pathology	MB/PB Ridley-Jopling	WHO-MDT	1 year	6 mo	Yes

*Augmented Sungai Buloh Regime (Malaysia)
PB
Monthly
RFP 600 mg
CLF 300 mg
Daily
Dapsone 100 mg
CLF 100 mg
Surveillance 5 years
MB
Intensive daily till MI= 0
RFP 600 mg
CLF 100 mg
Dapsone 100 mg
then
Monthly (3 years)
RFP 600 mg
CLF 300 mg
Daily
CLF 100 mg
Dapsone 100 mg
Surveillance 10 years

on the clinical signs and symptoms (Table 1 and 2). They are easily observed and elicited by any health worker after a short period of training. In practice, the majority of persons with such complaints self-report to a health center. Only in rare instances is there a need to use laboratory and other investigative procedures to confirm a diagnosis of leprosy. Therefore, an individual should be diagnosed with leprosy if he or she shows ONE of the following cardinal signs: 1) skin lesions consistent with leprosy with definite sensory loss, with or without thickened nerves; or 2) positive slit skin smears. In developed countries, for example in Japan, both the WHO and Ridley-Jopling classifications are generally used, and in-depth examination is preferentially attempted using histopathological and molecular biological tests. We suggest a first diagnosis as PB or MB, and classification by Ridley-Jopling only if the facilities and reagents are available for histopathology and molecular testing.

Peripheral Nerve Damage and the Lepra Reaction

How does *M. leprae* invade the peripheral nerves? *M. leprae* has an extreme predilection for the Schwann cells that surround the peripheral nerve axons. A recent study demonstrated that the species-specific PGL of *M. leprae* triggers uptake into Schwann cells by creating a complex with laminin-2. The *M. leprae*/laminin-α2 complexes then bind α/β dystroglycan complexes expressed on the Schwann cell surface.

Hansen's disease results in a wide range of impairments, the most important of which is damage to the peripheral nerves. Peripheral nerve damage causes loss of sensory, motor, and autonomic nerve function to the affected region, leading in turn to deformity, secondary deformity resulting from repeated trauma to the skin, and the inability to perform daily living. The consequences of nerve damage can impact the quality of life of those affected by the disease and also generate social stigma. Prevention of nerve damage and management of impairments are important components of any Hansen's disease program. Rehabilitation should be fully integrated within existing community-based programs in developing countries on an equal basis with other disabilities.

Most of the severe nerve destruction in Hansen's disease takes place during the lepra reaction, which consists of the reversal reaction (RR; type 1 reaction) and erythema nodosum leprosum (ENL; type 2 reaction). In RR, the level of cell-mediated immunity against *M. leprae* is suddenly el-

Table 2. Who Treats New Patients of Hansen's Disease in Selected Asian Countries and Brazil

Country	Dermatologist	Hansen's disease specialist	General physician	Registered nurse	Basic health medical staff	Basic health nonmedical staff
China	○	○	×	×	×	×
Indonesia	○	×	○	○1)	○1)	×
Japan	○	×	×	×	×	×
Malaysia	○	×	×	×	×	×
Myanmar	×	○	×	×	○	×
Philippines	○	○	○		○	○
Republic of Korea	○	○	×	×	×	×
Thailand	○2)	×	○	×	×	×
Viet Nam	△3)	△3)	×	△4)	△4)	×
Brazil	○	○	○	×5)	×5)	×6)

○ : yes, × : no and/or no staff
1) : yes (○), but confirmed with district administrator for leprosy
2) : sometimes when GP in field can't diagnosis & symptom is not clear
3) : confirm diagnosis
4) : supply MDT after diagnosis
5) : yes (○) if family health program is implemented
6) : yes (○) if remote area

evated, resulting in a severe inflammatory response in the areas of the skin and nerves affected by the disease. Acute inflammation in RR can destroy nerves and result in paralysis that can be permanent if not treated promptly and adequately. Clinically detectable neural involvement occurs in approximately 10% of PB and 40% of MB patients. Prompt and adequate treatment of the lepra reaction with anti-leprosy chemotherapy is the key to preventing irreversible nerve damage and disabilities. In the unfortunate case that permanent impairment occurs, patients should be given a course of rehabilitation.

Treatment and Control Strategy

Leprosy control has three major strategic components: Early detection, adequate treatment, and care to prevent disabilities and provide rehabilitation. Since the disease is caused by an infection, treatment with antibiotics plays a pivotal role in managing newly diagnosed patients. There are several effective chemotherapeutic agents against *M. leprae*. Dapsone, RFP, clofazimine (CLF), ofloxacin (OFLX), and minocycline (MINO) are components of the MDT regimen recommended by WHO (Table 1) and are commonly used by clinicians. Levofloxacin (LVFX), sparfloxacin (SPFX), moxifloxacin (MFLX), and clarithromycin (CAM) are also effective against *M. leprae*[6]. Following classification according to the flowchart, PB patients receive 600 mg RFP monthly, supervised, and 100 mg dapsone daily, unsupervised, for 6 months. MB cases are treated a 12-month period with 600 mg RFP and 300 mg CLF monthly, supervised, and 100 mg dapsone and 50 mg CLF daily. WHO has designed blister pack medication kits for

Table 3. Global Hansen's Disease Situation by Main Asian and Endemic Countries during 2009 (WHO)

Country	No. of new cases detected, 2009	No. of new cases of MB	No. of new female cases	No. of new cases less than 15 y/o	No. of new cases with G2 disabilities
India	133,717	64,782	47,361	13,331	4,117
Indonesia	17,260	14,227	6,887	2,073	1,812
Bangradesh	5,239	2,247	2,128	366	542
Nepal	4,394	2,216	1,479	282	178
Myanmar	3,147	2,189	1,106	165	468
Philippines	1,795	1,706	387	120	87
Sri Lanka	1,875	893	816	186	119
China	1,597	1,347	511	39	364
Viet Nam	413	295	114	12	80
Thailand	300	215	108	11	41
Cambodia	351	244	105	27	35
Malaysia	187	138	54	12	8
Timor	160	119	53	13	8
Lao PDR	101	73	18	2	14
Singapore	8	2	2	0	0
Republic of Korea	5	5	4	0	3
Hong Kong	5	2	1	0	0
Japan	2	1	1	0	0
Brazil	37,610	21,414	16,865	2,669	2,436
DR Congo	5,062	3,001	2,450	594	509
Nigeria	4,219	3,733	1,772	409	494
Ethiopia	4,417	3,909	287	302	408
Tanzania	2,654	2,138	1,068	260	292
Global total	244,796	139,125	89,538	22,485	14,320

PB and MB. Each easy-to use kit contains enough medication for 28 days.

RFP is an exceptionally potent bactericidal agent against *M. leprae*. Dapsone is bacteriostatic or weakly bactericidal against *M. leprae* and was the mainstay treatment for leprosy for many years until widespread resistant strains appeared. CLF binds preferentially to mycobacterial DNA and exerts a slow bactericidal effect on *M. leprae* by inhibiting mycobacterial growth. CLF also produces anti-inflammatory properties in the control of ENL. Combination therapy has become essential in reducing or preventing the development of resistance. Direct observation or supervision of RFP treatment is very important in avoiding the development of drug resistance. Patients take dapsone (and CLF) for the other 27 days, and health workers must ensure a regular and uninterrupted supply every day.

In most patients the presence of dead bacilli in the skin and other tissues does not cause any problem, and the dead organisms are gradually cleared by the phagocytic system of the body. However, in a small proportion of patients, the antigens from dead bacilli can provoke immunological reactions, such as the lepra reaction.

In most Asian countries, MDT was introduced by the WHO in 1981 and rapidly became the standard regimen. Ensuring a free supply of drugs for MDT, and an effective distribution system, is essential in all endemic countries. However, the duration of treatment differs in each country (Table 1) and some countries extend therapy beyond the WHO guidelines. The most critical activity is patient education at the end of treatment regarding the signs and symptoms of relapse and the importance of reporting immediately to the nearest health center when such problems arise.

Epidemiology

Recent epidemiological studies indicate that transmission of the leprosy bacillus is mainly mediated by airborne droplets through the respiratory system, in which the nose plays a central role. It is believed that there is widespread subclinical transmission of *M. leprae* with transient infection of the nose in endemic areas, although most of these cases do not develop clinical disease. The incubation period is unusually long for a bacterial disease: generally five to seven years, but can be as long as 40 to 60 years, especially in developed countries. The peak age of onset is young adulthood, usually 20-30 years of age; the disease is rarely seen in children less than five years old. Elderly new patients are registered in developed countries.

WHO publishes an annual report on the worldwide incidence of leprosy, including the number of new cases, prevalence and disabilities (Table 3)[7]. Many new cases are detected in countries such as India, Indonesia, Nepal, and other Asian countries, but the number of new cases detected annually is gradually declining in many countries, including those in the Asian region. Very few new leprosy patients are registered in developed countries, and most of those cases are among immigrants from countries where the disease is still endemic. Diagnostic methods, laboratory techniques, and treatment of Hansen's disease under WHO guidance have been adopted in developing countries. Dr. Saikawa investigated the association between Hansen's disease incidence and socio-economic factors such as gross national product (GNP), personal expenditure on housing, and the number of persons per household in Okinawa, Japan and Taiwan, and concluded that the improvement in socio-economic conditions greatly contributes to the reduction of Hansen's disease in the community[8]. Therefore, Hansen's disease is referred to as a "poverty-related disease". The proportion of children under the age of 15 among newly detected cases would be a good indicator of the situation in a country/region. Similarly, the proportion of cases with grade 2 disabilities and visible disabilities among newly detected cases is a reflection of early detection and treatment.

History of Hansen's Disease

Hansen's disease was recognized and described in the old literature of India, China, and Japan. The term "leprosy" originates from the Latin word "lepros," which means defilement. Throughout history, it has been feared as an incurable disease that causes severe deformities and disabilities, and its victims have suffered both from the disease

itself and from public discrimination. Hansen's disease was considered a divine punishment in the Old Testament and as a karmic disease in Buddhism.

In many countries, patients were isolated in villages far from human dwellings[9], and in socioeconomically improved countries patients were quarantined in leprosaria. Even after dapsone was found to be effective against Hansen's disease in the 1940s, such situations did not change significantly. The memory of the history of isolation is fading, and there is a danger that history will be repeated. A lack of proper understanding and the unabated propagation of traditional myths and beliefs about the disease have led to a build-up of negative social attitudes that culminate in social discrimination and stigma against persons affected by leprosy (PAL) and their families. While discrimination refers to the unjust or prejudicial treatment of people, especially on the grounds of being affected by leprosy, stigma is an ugly "act of labeling, rejection or unexplained fear of PAL."

The history of Hansen's disease in Asia can be broadly divided into three areas: 1. The presence of leprosy prevention law and the admittance of leprosy patients into leprosaria (e.g., Japan[10]). 2. The presence of leprosy prevention law and the admittance of leprosy patients to leprosaria in the pre-sulfone era. However, the leprosy policy changed to the settlement village movement in the post-sulfone era (e.g., Korea). 3. No leprosy prevention law and the government launch of an intensive program of leprosy control in consultation with WHO (Myanmar and many other countries).

Hansen's disease has been recently recognized in societies due to the civil liberty movement of NGOs and the WHO, but the prejudice against Hansen's disease persists.

Patients in Asia

The diagnosis and treatment of leprosy is relatively easy using the WHO-MDT regimen (Tables 1 and 2). Successful treatment with WHO-MDT can completely clear patients of the *M. leprae* pathogen. However, clinically cured patients continue to suffer from the deformities caused by delayed treatment. Once the diagnosis of Hansen's disease is made, patients are often ostracized by their neighbors and societies.

Asian governments are engaged in eliminating Hansen's disease with the aid of the Sasakawa Memorial Health Foundation, the International Federation of Anti-leprosy Association (ILEP), the Japan International Cooperation Agency (JICA), the WHO, and other GOs and NGOs. The governments promote further elimination of the disease, and use the slogan "Treat Early; Prevent Disability" to emphasize that minimization of leprosy complications is vital in developing countries. Since many patients continue to experience complications following MDT, preventing the worsening of complications, self-care, occupational training, and rehabilitation are steadily conducted.

In addition to preventing complications, the governments make every possible effort to dispel prejudice against leprosy. Stigmatization and ostracization, such as occurs in Japan or Korea, are common in many developing countries. There are villages inhabited by leprosy patients alone in the mountainous districts or remote rural areas. Since the introduction of MDT, the stigma has lessened considerably; however, some people do not know about MDT and believe that leprosy is still an incurable disease.

Hansen's Disease and Dermatologists

Since leprosy is not a skin disorder, patients are sent to a leprologist or leprosaria following a diagnosis of Hansen's disease by a dermatologist. Leprologists and healthcare workers are the main caregivers for leprosy patients in developing countries. However, a few dermatologists do offer treatment in developed countries because of the low number of patients. Therefore, dermatologists should make careful preparations and be able to recognize the disease's clinical manifestations because they could encounter a patient with Hansen's disease at any time.

Acknowledgements

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3 Hansen 病

病原体名

らい菌/*Mycobacterium leprae*

微生物学，分類

抗酸菌で，人工培養に成功していない．

疾患名

Hansen 病/Hansen's disease，leprosy

らい（癩）については偏見を助長するものとして，少数の専門用語（らい菌，らい反応など）以外は使用しない．

病原体のリザーバー

ヒトの他にココノオビアルマジロ，一部のサルなど．

疫　学

日本人の新規患者は年間 0 ～ 1 人程度で，高齢者である．一方，在日外国人の新規患者は毎年 4 人程度で，約半数はブラジル人である．なお，世界ではインドやブラジルなどを中心に毎年約 25 万人の新規患者がいる[1,2]．

病原性

らい菌の増殖は遅く（世代時間：12 ～ 13 日），至適発育温度は 31℃前後で，毒力はきわめて弱い．らい菌はマクロファージ内で増殖し，皮膚症状が主で，病状は慢性に経過する．また，らい菌の膜表面にあるフェノール性糖脂質と末梢神経の Schwann 細胞表面のラミニン 2 との親和性が高いため，末梢神経の障害が起こる．

発症に大きく関与する感染の機会は，免疫能が完全でない乳幼児期に大量・頻回にらい菌を吸入すること（呼吸器感染）といわれている．発症に影響を与える因子としては，個々人のらい菌に対する特異的な細胞性免疫能の他，公衆衛生の程度，経済状態，栄養状態などの環境・社会的因子が論じられている．

感染予防策

(CDC) 標準予防策

関連法規

普通の感染症であり，現在は新規患者に関する関連法規はない．

臨床症状と経過

問診で知覚の異常（ケガをしても痛くない，頻回の火傷・熱傷，痛み・痒みのない皮疹），発展途上国出身者，高齢の日本人などの場合は鑑別の一つとして Hansen 病を頭の片隅に入れておく．

皮膚症状は環状紅斑や紅斑局面，結節など多彩で，皮疹部に一致して知覚（触覚，痛覚，温度覚）が低下していることが多い．

末梢神経も障害される．皮疹部に一致して触・痛・

表 1　らい菌検査の種類と特徴

らい菌検査の種類	サンプル	何がわかるか	長　所	短　所
皮膚スメア検査	皮膚スメア，滲出液（生検皮膚付着組織液）	菌の有無（抗酸菌染色）	準備が簡単（メスとスライドガラス），数時間で判定	菌種の同定不可能，菌少数の場合は見落としの可能性，採取の手技によって偽陰性の可能性あり
病理組織検査	生検組織（皮膚，末梢神経）	菌の有無と特異的な組織学的変化	病態の把握や治療効果判定	侵襲的検査法
PCR 法（遺伝子検査）	生検皮膚，メス刃付着組織液，滲出液	菌由来核酸成分の有無	短時間，高感度	死菌でも検出される可能性あり，検査中の汚染に注意

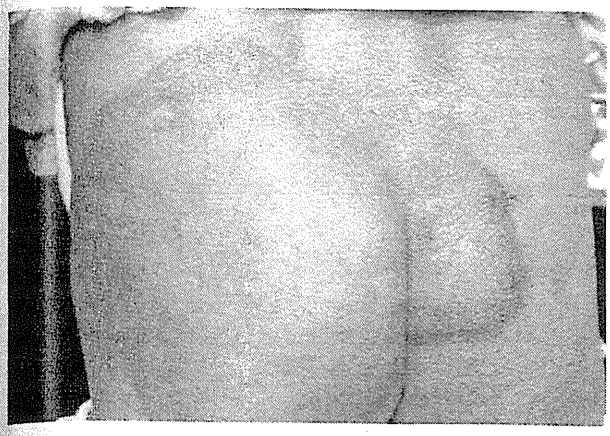

図 1　少菌型（PB，TT）Hansen 病の症例
中心治癒性の環状紅斑．中心部および皮疹部では知覚（触・痛・温度覚）低下．

温度覚の低下がみられ，外傷や火傷・熱傷などが頻発する．さらに，運動神経もおかされ，指趾などが屈曲することもある．

治療中，またその前後に急激な炎症反応（らい反応）が起こることがある．皮疹の増悪や再燃，急激な神経痛や運動障害などを起こす．

臨床検査

らい菌を検出することは診断に直結するため，以下の 3 検査の中から複数の検査を組み合せてらい菌を証明する（表 1）．①皮膚スメア検査（皮疹部からメスで組織液を採取し，抗酸菌染色し，検鏡する），②病理組織の抗酸菌染色，③PCR 法．レプロミン反応については，反応液は供給されておらず，実施されていない．

診断と病型

以下の 4 点を総合的に勘案して診断する[2]．①知覚障害のある皮疹，②神経障害（触・痛・温度覚低下，運動障害，神経肥厚），③らい菌の証明，④病理所見．

鑑別すべき疾患は，サルコイドーシス，皮膚結核，非結核性抗酸菌症，環状肉芽腫，結節性紅斑など多数である．

患者ごとに皮疹の数や形態，神経肥厚や知覚・運動障害の程度，らい菌の数，病理組織所見に差異が大きいが，これはらい菌に対する生体の免疫能を反映するものであり，これを基に病型分類を行う．菌を検出しにくい少菌型（paucibacillary：PB）（図 1）と，らい菌を検出できる多菌型（multibacillary：MB）（図 2）に分類する（表 2）．さらに Ridley-Jopling 分類を行う．

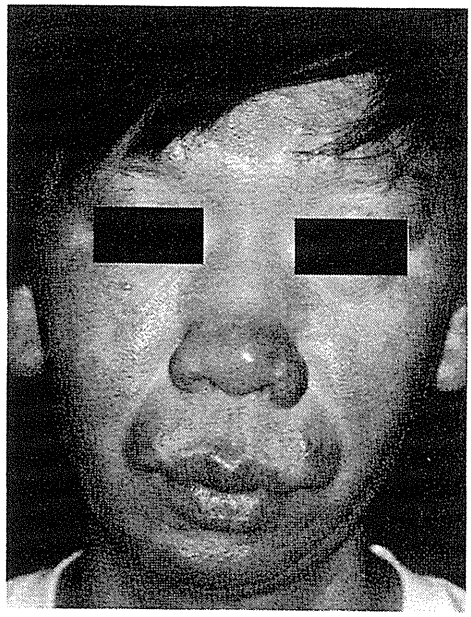

図 2　多菌型（MB，LL）Hansen 病の症例
紅色結節が多発し，表面は光沢を呈する．眉毛が薄くなっている．

治　療

治療の基本は，神経症状をコントロールしながら，らい菌を生体から排除することである．WHO の推奨する多剤併用療法（multidrug therapy：MDT）を基本に治療する．rifampicin（RFP），diaphenylsulfone（DDS），clofazimine（CLF）を，PB では前 2 剤を 6 ヵ月間，MB では 3 剤を 1 ～ 3 年間内服する．

なお，キノロン系薬，minocycline（MINO），clarithromycin（CAM）などもらい菌に対して有効である．

らい反応の治療はステロイドなどの内服を行う．

Hansen 病の歴史

Hansen 病は有効な治療薬がない時代には病状が進行し，顔面，手足に皮疹および末梢神経障害による痛覚麻痺，変形，運動障害などを形成した．そのため外見上の問題と手足の不自由による就労の困難など，さらに宗教上の問題などから，昔から世界中で偏見・差別，排除の対象となった．日本では明治時代になって救済から隔離に進む，「癩予防ニ関スル件」（1907 年），「癩予防法」（1931 年），さらに Hansen 病に有効な治療薬が開発されていた 1953 年に至っても「らい予防法」として Hansen 病に関する法律が継続した．原則的に一般保険診療の対象にならず，療養所での医療が続いてきた．医学的進歩，人権思想の高まりとともに改変されるべき法律が 1996 年まで存続してしまった．医療関係者は単に医学の進歩を追求するのみならず，病気に関連する法律や社会的状況なども考慮して，病め

表2　Hansen 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	組織球性肉芽腫
	巨細胞，神経への細胞浸潤	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	皮膚スメア検査等でのらい菌の証明
治療 multidrug therapy(MDT)	WHO/MDT：PB 6 ヵ月間 RFP，DDS	WHO/MDT：MB 1 ～ 3 年間 RFP，DDS，CLF

る人々へ，人間として最善を尽くすことが必要である．

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関連 URL

1) WHO のハンセン病欄．〈http://www.who.int/lep〉
2) 国立感染症研究所ハンセン病研究センター．〈http://www.nih.go.jp/niid/lrc/〉

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Amino Acid Substitutions at Position 95 in GyrA Can Add Fluoroquinolone Resistance to *Mycobacterium leprae*

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Amino acid substitutions at position 89 or 91 in GyrA of fluoroquinolone-resistant *Mycobacterium leprae* clinical isolates have been reported. In contrast, those at position 94 in *M. tuberculosis*, equivalent to position 95 in *M. leprae*, have been identified most frequently. To verify the possible contribution of amino acid substitutions at position 95 in *M. leprae* to fluoroquinolone resistance, we conducted an *in vitro* assay using wild-type and mutant recombinant DNA gyrases. Fluoroquinolone-mediated supercoiling activity inhibition assay and DNA cleavage assay revealed the potent contribution of an amino acid substitution of Asp to Gly or Asn at position 95 to fluoroquinolone resistance. These results suggested the possible future emergence of quinolone-resistant *M. leprae* isolates with these amino acid substitutions and the usefulness of detecting these mutations for the rapid identification of fluoroquinolone resistance in leprosy.

Leprosy is a chronic human infectious disease caused by *Mycobacterium leprae* which may cause severe disabilities due to damage to the peripheral nerves (33). The World Health Organization (WHO) reported the global number of registered new cases in 2010 to be 228,474, while during 2009 it was 244,796 (37). Although the number of new cases detected globally fell by 16,322 (6.7%) during this period, new leprosy cases are still detected every year, mainly in Asia, Latin America, and Africa (21, 37). In the 1980s, the WHO introduced multidrug therapy (MDT), composed of dapsone (DDS), rifampin (RIF), and clofazimine (36). Recently, fluoroquinolones (FQs), especially ofloxacin (OFX), have been recommended for the treatment of leprosy with a single lesion. The emergence of multidrug-resistant (MDR) leprosy, resistant to both DDS and RIF owing to therapeutic failure or low compliance, has been reported (17, 29), and FQs are thought to be important. For appropriate treatment, early assessment of drug susceptibility is essential; however, *M. leprae* cannot be cultivated on artificial media and a drug susceptibility test depending on *in vitro* growth is not available. Consequently, antibiotic susceptibility tests have relied on the mouse footpad leprosy model, requiring 8 to 12 months because of the slow growth of *M. leprae* (18). Recently, genetic analysis of drug-resistant *M. leprae* substantiated the correlation of DDS, RIF, and OFX resistance with mutations in *folP1*, encoding dihydropteroate synthetase (5, 15, 19, 23–25, 35); *rpoB* (4, 6, 12, 19, 23–25, 33), encoding the beta subunit of RNA polymerase; and *gyrA*, encoding the A subunit of DNA gyrase (4, 19, 24, 26, 40), respectively. Among these, data for *folP1* in *M. tuberculosis* are not available as DDS is not used for the treatment of tuberculosis. Mutations in *rpoB* observed in *M. leprae* showed good agreement with those obtained from RIF-resistant *M. tuberculosis*. In contrast, the distribution of mutations in *gyrA* of FQ-resistant *M. tuberculosis* was distinct from that in *gyrA* of OFX-resistant *M. leprae* (Fig. 1). Namely, amino acid substitutions at position 94 in GyrA were found in approximately half of FQ-resistant *M. tuberculosis* isolates, whereas no amino acid substitutions at position 95, equivalent to position 94 in *M. tuberculosis*, have been reported in *M. leprae*, and 11 cases with amino acid substitutions at position 91, equivalent to position 94 in *M. tuberculosis*, were reported from a total of six countries (4, 19, 24, 26, 40). Thus, elucidation of the contribution of amino acid substitutions at position 95 of GyrA in *M. leprae* to FQ resistance is important for the gene-based detection of fluoroquinolone resistance.

FQs inhibit type II DNA topoisomerases, DNA gyrase, and topoisomerase IV, which play crucial roles in DNA replication during cell division (8). As *M. leprae* has only DNA gyrase, this is the sole target of FQs. DNA gyrase, consisting of two GyrA and two GyrB subunits, catalyzes the negative supercoiling of the circular bacterial chromosome by cleaving double strands and passing the enwrapped DNA, followed by resealing the double strands (8, 13). To reveal the significance of amino acid substitution at position 95 to FQ resistance, we conducted the FQ-mediated supercoiling activity inhibition assay and DNA cleavage assay using recombinant DNA gyrases having an amino acid substitution in GyrA at position 95, Asp to Gly (GyrA-Asp95Gly) or Asp to Asn (GyrA-Asp95Asn). These mutations are frequently found in FQ-resistant *M. tuberculosis* strains (1, 7, 9, 10, 32, 34, 39) but not in FQ-resistant *M. leprae* strains.

MATERIALS AND METHODS

Materials. The Thai-53 strain of *M. leprae* (22), maintained at the Leprosy Research Center, National Institute of Infectious Diseases (Tokyo, Japan), was used to prepare *M. leprae* DNA. *Escherichia coli* strains TOP-10 (Life Technologies Corp., Carlsbad, CA), Rosetta-gami 2, and BL21(DE3)(pLysS) (Merck KGaA, Darmstadt, Germany) were used for cloning and protein expression. GyrA and GyrB expression plasmids were constructed on the basis of pET-20b (+) (Merck KGaA). OFX and gatifloxacin (GAT) were purchased from LKT Laboratories, Inc. (St. Paul, MN); moxifloxacin (MXF) was from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Sitafloxacin (SIT) was a gift from Daiichisankyo Pharmaceutical, Co., Ltd. (Tokyo, Japan). Ampicillin was purchased from Meiji Seika Pharma, Ltd. (Tokyo, Ja-

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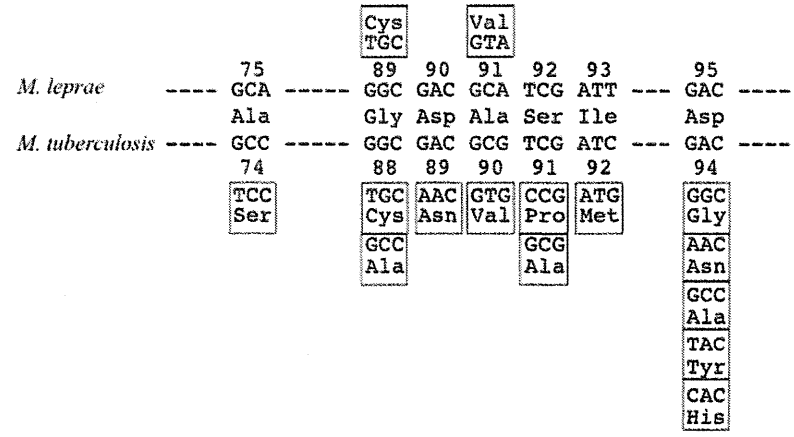

FIG 1 Nucleotide substitutions encoding the quinolone resistance-determining region in *gyrA* of WT and FQ-resistant *M. leprae* and *M. tuberculosis*. Nucleotide sequences encoding the quinolone resistance-determining region of WT *M. leprae* and *M. tuberculosis* GyrA were aligned with the amino acid sequence at the corresponding positions indicated by the numbers. Altered amino acids and the corresponding nucleotide substitutions of *M. leprae* and *M. tuberculosis* are placed above and below WT sequences, respectively.

pan). Oligonucleotide primers were synthesized by Life Technologies Corp. Restriction enzymes were obtained from New England BioLabs, Inc. (Ipswich, MA). The supercoiling assay kit and supercoiled and relaxed pBR322 DNA were purchased from John Innes Enterprises Ltd. (Norwich, United Kingdom).

Construction of recombinant wild-type (WT) and mutant DNA gyrase expression plasmids. DNA gyrase expression vectors were constructed basically as previously described (16), and Fig. 2 presents an overview of the procedure. The sequences of the primers used in the study

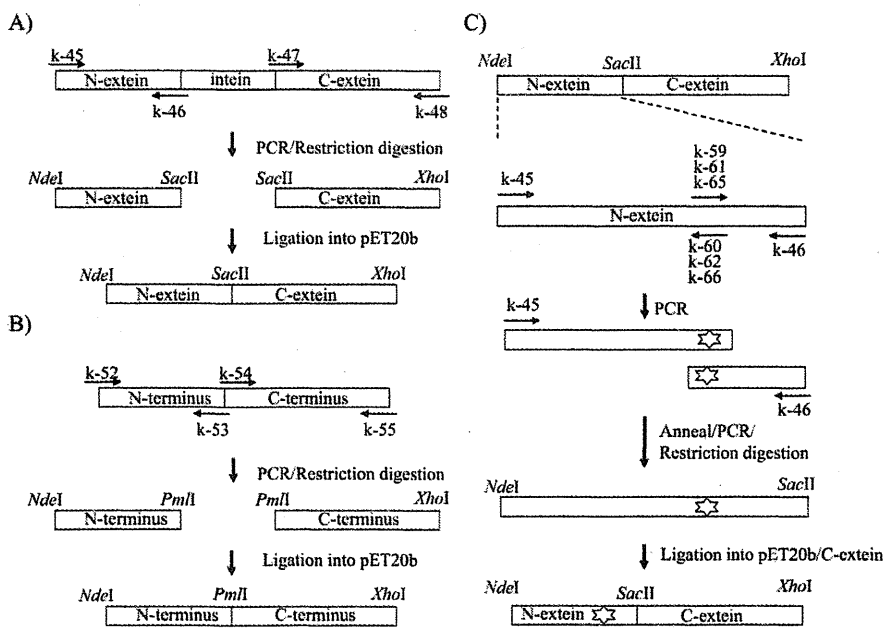

FIG 2 Construction of WT and mutant DNA gyrase expression plasmid. (A) DNA fragments encoding N-extein (amino acids 1 to 130) and C-extein of GyrA (amino acids 125 to 830) were amplified by PCR with primer pairs k-45/k-46 and k-47/k-48 (Table 1), respectively. Similarly, those encoding the N-terminal (amino acids 1 to 428) and C-terminal (amino acids 424 to 679) regions of GyrB were amplified with primer pairs k-52/k-53 and k-54/k-55 (Table 1), respectively. PCR products encoding N-extein and C-extein of GyrA were digested by NdeI-SacII and SacII-XhoI, respectively, and introduced simultaneously into NdeI-XhoI-digested plasmid pET-20b (+). (B) DNA fragments encoding the N-terminal and C-terminal regions of GyrB were digested by NdeI-PmaCI and PmaCI-XhoI, respectively, and introduced into pET20b as described above. (C) Primer pairs consisting of primer k-45 and primer k-60, k-62, or k-66 (Table 1) were used for amplifying the DNA fragment encoding the N-terminal portion (amino acids 1 to 94) of N-extein carrying Ala91Val, Asp95Gly, and Asp95Asn, respectively. Primer pairs consisting of primer k-46 and primer k-59, k-61, or k-65 (Table 1) were used for amplifying the DNA fragment encoding the C-terminal portion (amino acids 88 to 130) of N-extein carrying Ala91Val, Asp95Gly, and Asp95Asn, respectively. To complete the N-extein-encoding cassette, DNA fragments encoding the N-terminal and C-terminal regions of N-extein of GyrA were annealed and reamplified by PCR using the primer pair k-45/k-46. The mutated *gyrA*-N cassettes were digested with NdeI and SacII restriction endonucleases and ligated into the expression plasmid containing WT *gyrA* C-extein DNA fragment digested by the same enzymes.

TABLE 1 Nucleotide sequences of primers used in PCR

Primer name	Primer sequence (nucleotide positions)[a]
k-45	5′-GGCATATGACTGATATCACGCTGCCACCAG-3′ (1–25)
k-46	5′-ATAACGCATCGCCGCGGGTGGGTCATTACC-3′ (361–390)
k-47	5′-CACCCGCGGCGATGCGTTATACCGAGGCTCGGCTTACTCC-3′ (371–410)
k-48	5′-GGCTCGAGTTAATGATGATGATGATGATGACCGACACCGCCGTCGG-3′ (2471–2490)
k-52	5′-GGCATATGGCTGCCCAGAGGAAG-3′ (1–18)
k-53	5′-CTAACTCACGTGCTTTACGTGCAGCTATTC-3′ (1259–1288)
k-54	5′-CGTAAAGCACGTGAGTTAGTGCGTCGAAAAAGTGCC-3′ (1270–1305)
k-55	5′-GGCTCGAGCTAATGATGATGATGATGATGGACATCCAGGAAACGAACATCC-3′ (2013–2037)
k-59	5′-GCACGGCGAC**GTG**TCGATTTATG-3′ (261–283)
k-60	5′-CATAAATCG**ACAC**GTCGCCGTGC-3′ (261–283)
k-61	5′-CATCGATTTAT**GGC**ACGTTAGTGC-3′ (272–295)
k-62	5′-GCACTAACGT**GCC**ATAAATCGATG-3′ (272–295)
k-65	5′-CATCGATTTAT**AAC**ACGTTAGTGC-3′ (272–295)
k-66	5′-GCACTAACG**TGTT**ATAAATCGATG-3′ (272–295)

[a] Six-histidine tag codons are underlined, and mutated codons are shown in bold type.

are shown in Table 1. All PCRs were carried out in a thermal cycler (Applied Biosystems) under the following conditions: predenaturation at 98°C for 2 min; 35 cycles of denaturation at 98°C for 10 s, annealing at 50 to 60°C for 15 s, and extension at 68°C for 1 to 2.5 min; and then a final extension at 68°C for 2 min. The nucleotide sequences of the DNA gyrase genes in the plasmids were confirmed using a BigDye Terminator (version 3.1) cycle sequencing kit (Life Technologies Corp.) and an ABI Prism 3130*xl* genetic analyzer (Life Technologies Corp.) according to the manufacturer's protocol.

Expression and purification of recombinant DNA gyrase. DNA gyrase subunits were purified as previously described (2, 3, 16, 20, 21, 31). Expression plasmids carrying the *gyrA* (WT and mutants) and WT *gyrB* genes of *M. leprae* were transformed into *E. coli* Rosetta-gami 2 and BL21(DE3)(pLysS), respectively. Expression of GyrA and GyrB was induced with the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (Wako Pure Chemical Industries Ltd., Tokyo, Japan), followed by further incubation at 14°C for 16 h. The recombinant DNA gyrase subunit in the supernatant of the sonication lysate (by Sonifier 250; Branson, Danbury, CT) was purified by nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Life Technologies Corp.) column chromatography. The protein fractions were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

DNA supercoiling activities and inhibition by FQs. ATP-dependent and quinolone-inhibited DNA supercoiling assays were carried out as previously described (2, 3, 16, 20, 21, 31) with the following modifications. DNA supercoiling activity was examined with a reaction mixture (total volume, 30 μl) consisting of DNA gyrase reaction buffer, relaxed pBR322 DNA (300 ng), and purified GyrA and GyrB (50 ng each) subunits. Reactions were performed at 30°C for 1.5 h and stopped by adding an equal volume of chloroform-isoamyl alcohol (24:1 mixture) and 3 μl of 10× DNA loading dye. The total reaction mixtures were subjected to electrophoresis in a 1% agarose gel in 1× Tris-borate-EDTA (TBE) buffer, followed by ethidium bromide (0.7 μg/ml) staining. Supercoiling activity was evaluated by tracing the brightness of the bands with the software ImageJ (http://rsbweb.nih.gov/ij). Gyrase bearing an Ala91Val amino acid substitution in GyrA was used as a positive control for all assays (20). The inhibitory effect of FQs on DNA gyrases was assessed by determining the drug concentrations required to inhibit the supercoiling activity of the enzyme by 50% (IC_{50}s). All enzyme assays were performed at least three times to confirm reproducibility.

Quinolone-mediated DNA cleavage assay. DNA cleavage assays were carried out as previously described (16, 20, 21, 31). The reaction mixture (total volume, 30 μl) contained DNA gyrase reaction buffer, recombinant DNA gyrase subunits (50 ng), supercoiled pBR322 DNA (300 ng), and 2-fold serially increasing concentrations of FQs. After incubation for 2 h at 30°C, 3 μl of 2% SDS and 3 μl proteinase K (1 mg/ml) were added to the reaction mixture. After subsequent incubation for 30 min at 30°C, reactions were stopped by the addition of 3 μl of 0.5 mM EDTA, 30 μl chloroform-isoamyl alcohol (24:1 mixture), and 3 μl of 10× DNA loading dye. The total reaction mixtures were subjected to electrophoresis in 0.8% agarose gels in 1× TBE buffer, followed by ethidium bromide staining. The extent of DNA cleavage was quantified with ImageJ, and the quinolone concentrations required to induce 25% of the maximum DNA cleavage (CC_{25}s) were determined.

Temperature sensitivity of *M. leprae* DNA gyrase. The reactions with mixtures (total volume, 30 μl) consisting of DNA gyrase reaction buffer, relaxed pBR322 DNA (300 ng), and recombinant DNA gyrase subunits

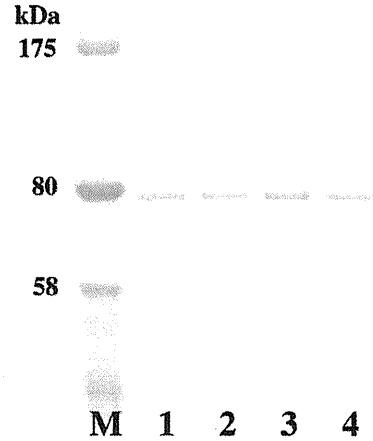

FIG 3 SDS-PAGE analysis of purified *M. leprae* DNA gyrases. The His-tagged recombinant DNA gyrases were overexpressed in *E. coli* and purified by Ni-NTA affinity resin chromatography. Lanes: M, protein marker (NEB); 1, WT GyrA; 2, GyrA-Ala91Val; 3, GyrA-Asp95Gly; 4, GyrA-Asp95Asn; 5, WT GyrB. Three hundred nanograms of each protein was loaded onto a 5 to 20% gradient polyacrylamide gel.

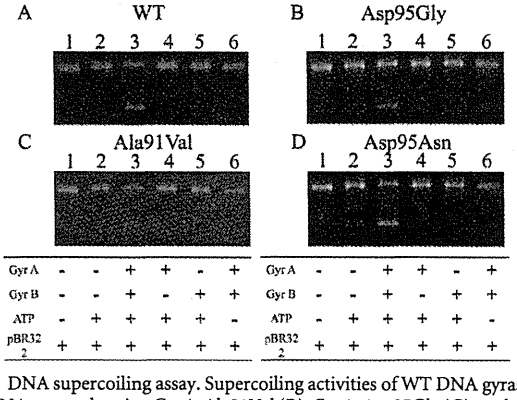

FIG 4 DNA supercoiling assay. Supercoiling activities of WT DNA gyrase (A) and DNA gyrases bearing GyrA-Ala91Val (B), GyrA-Asp95Gly (C), and GyrA-Asp95Asn (D) were analyzed. Relaxed pBR322 (0.3 μg) was incubated with GyrA (50 ng) or GyrB (50 ng), or both. Lanes: 1, relaxed pBR322 alone; 2, relaxed pBR322 and ATP; 3, relaxed pBR322, ATP, GyrA, and GyrB; 4, relaxed pBR322, ATP, and GyrA; 5, relaxed pBR322, ATP, and GyrB; 6, relaxed pBR322, GyrA, and GyrB.

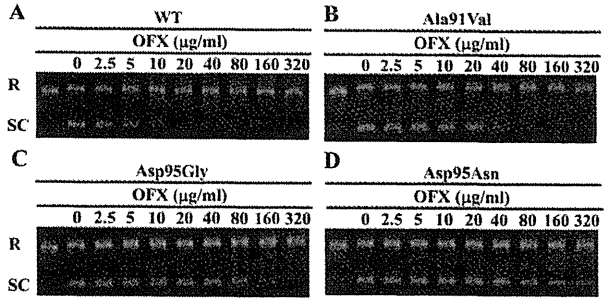

FIG 5 OFX-inhibited DNA supercoiling assay. Relaxed pBR322 (0.3 μg) was incubated with GyrA (50 ng) and GyrB (50 ng) in the presence of the indicated concentration of OFX. Quinolone-inhibited supercoiling activity assay was performed with combinations consisting of WT GyrB-WT GyrA (A), GyrA-Ala91Val (B), GyrA-Asp95Gly (C), and GyrA-Asp95Asn (D). R and SC, relaxed and supercoiled pBR322 DNA, respectively.

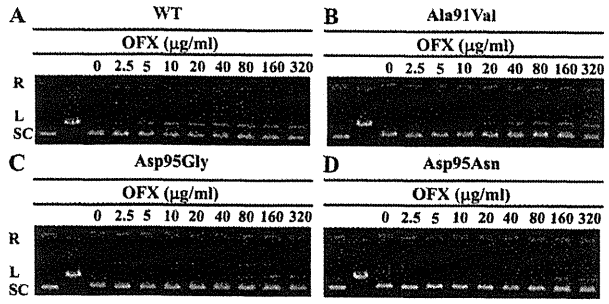

FIG 6 OFX-mediated DNA cleavage assay. Supercoiled pBR322 (0.3 μg) was incubated with GyrA (50 ng) and GyrB (50 ng) in the presence of the indicated concentration of OFX. DNA cleavage assay was performed with combinations consisting of WT GyrB-WT GyrA (A), GyrA-Ala91Val (B), GyrA-Asp95Gly (C), and GyrA-Asp95Asn (D). R, L, and SC, relaxed, linear, and supercoiled pBR322 DNA, respectively.

(50 ng) were run at 25, 30, 33, 37, and 42°C for 1.5 h. Supercoiling activities of recombinant DNA gyrases were evaluated at each reaction temperature as described above.

RESULTS

Construction and purification of recombinant His-tagged GyrA and GyrB proteins. DNA fragments, including the *gyrA* and *gyrB* genes, were successfully amplified from *M. leprae* Thai-53 strain DNA and inserted in frame downstream of a T7 promoter in pET-20b (+). GyrA and GyrB were expressed as C-terminal hexahistidine-tagged proteins for ease of purification, as the His tag has been shown not to interfere with the catalytic functions of GyrA and GyrB (2, 3, 16, 20, 21, 31). Expressed recombinant WT and mutant DNA gyrase subunits were purified as 0.3 to 1.5 mg soluble His-tagged 80-kDa protein of GyrA and 75-kDa protein of GyrB from 500-ml cultures. The purity of the recombinant proteins was confirmed by SDS-PAGE (Fig. 3). All of the recombinant proteins were obtained with high purity (>95%).

DNA supercoiling activities. Combinations of GyrA WT, Ala91Val, Asp95Gly, or Asp95Asn and WT GyrB subunits were examined for DNA supercoiling activities using relaxed pBR322 DNA as a substrate in the presence or absence of ATP (Fig. 4). DNA supercoiling activities were observed in the presence of ATP and recombinant DNA gyrase subunits (Fig. 4A to D, lane 3), while neither subunit alone exhibited DNA supercoiling activity (Fig. 4A to D, lanes 4 and 5). In addition, no supercoiling activity was observed when ATP was omitted from the reaction mixture (Fig. 4A to D, lane 6).

Inhibition of DNA gyrase activities by FQs. The IC_{50}s of FQs were determined using the quinolone-inhibited DNA supercoiling assay (Fig. 5). Representative data showing the inhibitory effects of OFX against DNA gyrase are shown in Fig. 5, and data for other FQs are presented in Fig. S1 in the supplemental material. IC_{50}s of each FQ against WT and mutant DNA gyrases are summarized in Table 2. Each FQ showed dose-dependent inhibition, with IC_{50}s ranging from 0.4 to 262.3 μg/ml. DNA gyrases bearing GyrA-Asp95Gly and -Asp95Asn showed significantly higher IC_{50}s to quinolones (Table 2; Fig. 5; see Fig. S1 in the supplemental material) than WT gyrase (Table 2). These DNA gyrases also showed higher resistance than DNA gyrase bearing GyrA-Ala91Val, which was simultaneously analyzed as a positive control for resistance to FQs. Inhibitory effects of FQs were ranked SIT > GAT > MXF > OFX in all DNA gyrases.

FQ-mediated DNA-cleavable complex formation. The CC_{25}s of FQs were determined. Figure 6 shows the result of a DNA cleavage assay using OFX, and Fig. S2 in the supplemental material presents the results using GAT, MXF, and SIT. Table 2 summarizes the CC_{25}s of each DNA gyrase. DNA gyrases bearing GyrA-Asp95Gly and -Asp95Asn showed significantly higher CC_{25}s to quinolones than WT gyrase (Table 2). These DNA gyrases also showed higher CC_{25}s than gyrase bearing GyrA-Ala91Val (Table 2). Effects on cleavable complex formation were ranked SIT > GAT > MXF > OFX in all DNA gyrases.

Temperature sensitivity of *M. leprae* DNA gyrase. Figure 7 shows the effects of temperature on DNA gyrase activities. The highest DNA supercoiling activities were observed at 33°C in all DNA gyrases. WT and GyrA-A91V DNA gyrases showed reduced DNA supercoiling activities at 37°C, whereas Gyr-Asp95Gly and Asp95Asn DNA gyrases maintained activities comparable to those at 33°C. No supercoiling activities were observed in any of the DNA gyrases at 42°C.

TABLE 2 IC_{50}s and CC_{25}s of FQs against WT and mutant DNA gyrases[a]

Drug	IC_{50}				CC_{25}			
	WT	Ala91Val	Asp95Gly	Asp95Asn	WT	Ala91Val	Asp95Gly	Asp95Asn
OFX	6.8 ± 0.8	39.4 ± 15.5 (5.8)	161.2 ± 44.2 (23.7)	262.3 ± 105.8 (38.6)	7.3 ± 0.5	75.5 ± 16.8 (10.1)	240.5 ± 30.7 (32.1)	269.5 ± 76.5 (35.9)
GAT	1.0 ± 0.1	3.1 ± 0.7 (3.1)	7.5 ± 1.6 (7.5)	13.8 ± 1.6 (13.8)	1.1 ± 0.2	4.3 ± 0.2 (3.9)	15.6 ± 3.6 (14.2)	13.5 ± 3.1 (12.3)
MXF	1.5 ± 0.3	5.2 ± 1.0 (3.5)	21.5 ± 4.7 (14.3)	34.7 ± 3.1 (23.1)	1.0 ± 0.1	4.5 ± 1.0 (4.5)	25.5 ± 3.7 (25.5)	20.8 ± 5.0 (20.8)
SIT	0.4 ± 0.0	1.0 ± 0.2 (2.5)	2.2 ± 0.5 (5.5)	3.9 ± 0.6 (9.8)	0.3 ± 0.0	0.9 ± 0.0 (3.0)	2.2 ± 0.6 (7.3)	2.3 ± 0.4 (7.7)

[a] IC_{50}s and CC_{25}s are in μg/ml, and data in parentheses represent the fold increase compared to WT.