ド，先行使用抗菌薬剤，炎症検査結果，主治医，転帰などを取得する．検査結果とは別に，毎日1回すべての入院患者の診療科，病棟，病室，ベッド，主治医，転帰を取得する．

SHIPLは，東邦大学 山口恵三教授を主任研究者とする厚生労働科学研究費補助金による研究の一環として，平成15～17年度に開発を行った．NUICSが病院システム，細菌検査室をもつ大病院を対象としているのに対し，SHIPLは病床200未満の中小規模病院を対象とした．ほとんどの中小規模病院が，病院システムをもたず，細菌検査を外注している現状を考慮して開発した．宮城県，茨城県，群馬県，東京都の6施設と，外注検査会社6社が開発に協力した．

検査会社は日常業務において，病院から検体と検査発注伝票を収集する．検体は，識別情報がデータ管理システムへ登録された後，検査に回される．伝票からは，検体との対応を示す識別情報，検査内容，さらに診療科，主治医，場合によって診断名，症状などが光学文字読み取り装置あるいは人手によってデータ管理システムへ登録される．検査が終了すると検査結果と伝票からの情報が結合され，結果伝票などで病院へ還元される．JCLS準拠フォーマットによる収集・報告情報の標準と，データを安全に回収する仕組みを開発した．既存の業務の流れを利用したため，データの自動取得のために病院側で新たに発生する作業はない．

NUICSと異なり，SHIPLでは検査が発注されなかった患者の情報が得られない．この違いは，病床マップなど直接ベッド情報が必要な場面以外では決定的ではない．

3）NUICS，SHIPLの機能

NUICSは病院システムから，SHIPLは検査会社からデータを自動的に取得するために，入力は完全に自動化されている．人手によるデータ入力は行われない．

いずれのシステムもおもなデータ利用機能として，異常の早期発見，異常の発生した原因の解析（対策の策定支援），対策の評価支援，基本統計の整備機能をもつ．

(1) 異常の早期発見

今日の病院感染症は，常在菌，環境菌がその原因となることが多く，起因菌となる菌種を限定できない．また，特定の菌種が，特定の施設，病棟，病室から高頻度で検出されること（異常集積）は，特定の菌種が拡散する特殊な要因の存在を意味し，不適切な対策・手技はその要因の一つである．厚生労働省の通達や病院機能評価の要件で「各種細菌の検出状況や薬剤感受性成績のパターン等が病院又は有床診療所の疫学情報として把握，活用されることを目的」とした「感染情報レポート」が週1回程度作成[6]されることや，「分離菌の種類や推移を，検体の種類や病棟別に把握している[7]」こととして不特定菌種の監視を要求しているのは，この理由によると考える．しかし，人手によって不特定菌の検出状況を継続的に観察し，異常集積を判断することは困難である．

筆者らは，偏りのない菌の分離の確率は二項分布によって計算できることを証明した[8]．これに基づいて，NUICS，SHIPLの両システムに菌の異常集積の自動検出機能を実装した（**図3**）．これらのシステムは，毎晩，検体提出患者とすべての菌の分離数を，菌ごとに，病院全体および病棟ごとに集計する．集計は，検査結果がほぼ確定する検体提出日から7日後を基準に，1日，7日，14日，30日の4つの集計期間幅で行う．図3の例は，14日の期間幅で問題が指摘された例である．実際の菌の分離を，偏りのない（sporadic；スポラディックな）分離と仮定して，菌分離のベースラインレート，被検査患者数，菌陽性患者数から確率を計算し，確率が低い場合，適合度検定によって，“偏りがあった”つまり，何らかの人的要因によって偏りが生じた可能性があると判断する．これによって異常を感度よく検出できる．さらに，ベースラインレートに全国平均のデータを用いると，自施設の菌分離状況を評価できる．

耐性菌の分離頻度を評価する機能は，耐性菌の拡散を防ぐうえで重要である．筆者らは，現在，伝達の危険性の高い多剤耐性菌を自動検出するアルゴリズムの開発を行っている．多剤耐性緑膿菌

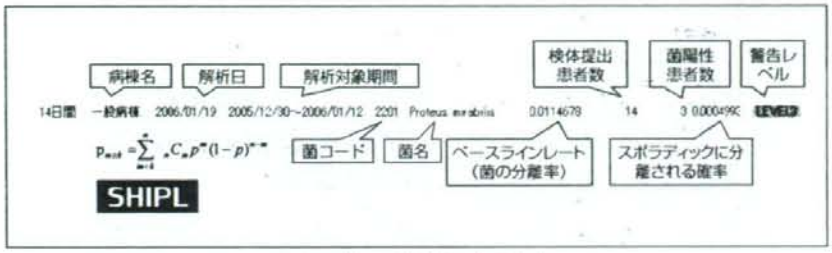

図 3　菌の異常集積の自動検出

SHIPL の例．*Proteus mirabilis* の患者当りの分離率が 0.0114678 の施設で 14 人の患者を検査して，そのうち 3 名から *P. mirabilis* が分離される確率は，分離がスポラディックであると考えると 0.0004993（1 万回に 5 回の確率）で非常にまれである．分離がスポラディックでなかった（異常集積である）可能性が高い．確率 0.001 未満の場合を警告レベル 3 に設定しているため，level 3 と判断された．左下，SHIPL の文字の上に確率の計算式を示した．

など既知の問題菌については，菌の異常集積検出技術で対応が可能である．

(2) 異常の発生した原因の解析（対策の策定支援）

施設内で菌の分離に異常が指摘された場合，①確率は低いが偶然生じたことであるのか，②実際に問題がある場合は拡散の経路などの原因を知ることが重要である．antibiogram（アンチバイオグラム，耐性パターン表）は，集積が院内で起こったかどうかを調べるのに簡便な方法である．

NUICS, SHIPL は，antibiogram を自動的に作成する．NUICS は，耐性パターンを色に変換し，3 次元の図として表示する機能をもつ（**図 4**）．比較的長期間（~1 年）の解析に有用である．NUICS, SHIPL は，病棟，病室のマップ上に特定菌の陽性患者を色分けで表示する機能（陽性患者マッピング機能）をもっている．短期間（~3 カ月）の解析に有用である．主治医も表示する．NUICS の陽性患者マップは，検査の有無にかかわらずすべての入院患者を病室ごとに 3 カ月までの期間で表示することができる．接触患者の識別に有用である（**図 5**）．

(3) 対策の評価支援

行った対策の有効性を評価する．SHIPL には，菌の分離率，陽性患者率をグラフ化する機能，対策の効果を指数化する機能がある．問題となった

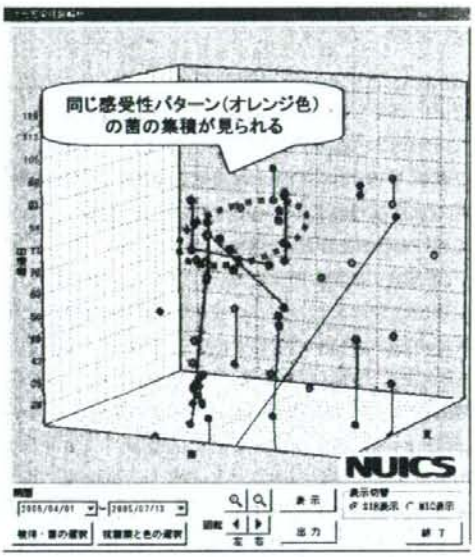

図 4　3 次元感染経路解析（NUICS）

緑膿菌分離の解析例．縦軸は時間の経過（下が過去，上が最近），平面は病院の 2 次元見取り図．1 つの丸は 1 回の菌の分離を示す．線で結ばれた丸は 1 人の患者からの分離．丸の色は耐性パターンを示す．ほぼ同時期に，病院の複数の場所で，複数の患者から同じ耐性パターンの緑膿菌が分離されたことを示している．

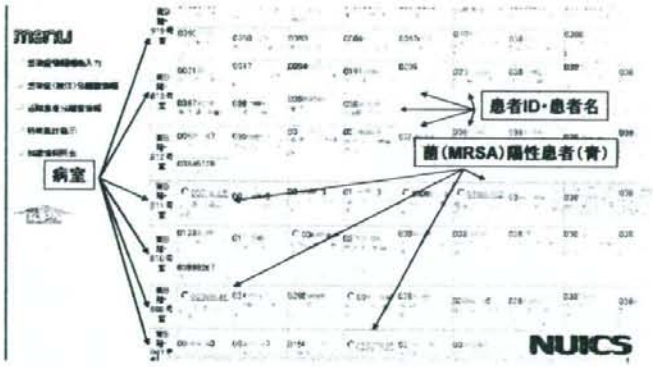

図 5　菌陽性患者マップ（NUICS）

MRSA，30 日間の表示例．検査の有無にかかわらず，病室にいたすべての患者がマップされている．陽性患者を青で示している．接触した可能性のある患者はすべてマップされる．細菌学的検査の結果もこの画面からリンクされる．

菌の分離が減少傾向にあるか否かを把握するのに有用である．異常を検出する機能は対策の評価にも利用される．菌の異常集積の自動検出で異常が検出される頻度の多寡も対策の評価になる．

(4) 基本統計の整備機能

NUICS，SHIPL いずれのシステムも，任意期間で病棟別分離菌数（すべてあるいは任意の菌種），検査材料別（すべてあるいは任意の菌種），年齢別（すべてあるいは任意の菌種），任意菌の耐性率，MIC 分布，感染情報レポート（分離背景を伴った antibiogram）を効率よく短時間（数秒）で集計し帳票化する．日常の感染対策業務，行政監査，病院機能評価などで整備すべき基本的な帳票は，すべてシステムから出力される．

電子化サーベイランスの将来と臨床検査技師の役割

電子化による自動化は，病院システム，検査システムで広く進むと考える．標準化，ネットワークの安全確保，国民の同意が進めば，感染症対策システム，サーベイランスに必要なデータは病院システムなどから低コストで自動的に得られるようになり，即時処理されるようになるであろう．処理の自動化に必要なアルゴリズムの開発は，人手による業務を置き換えるように進むと考える．

臨床検査技師の役割は，自動化の進展に伴って変化し続けるだろう．現在すでに細菌の同定，感受性検査は自動化されている．異常の自動検出，解析支援，集計・帳票の作成も自動化されている．当面，①適切な検体採取と保存・輸送，②データの解釈（異常の説明，精度管理，統計学的解釈），情報の付加と伝達，③細菌学的知識，感染対策（管理・制御）学的知識に基づいた科学的な施策の提案と評価が役割として重要になると考える．

アメリカの hospital epidemiologist[9] に対応する職域は，わが国では確立していない．今後，臨床検査技師が研鑽を積むことによって，この職域の相当部分を担うことが可能である．業務は技術の進歩によって今後も変化する．学問的な背景をもつことが重要である．学問的背景に基づいた情報の選択と伝達，学問的背景に基づいた仮説の提案と証明が重要である．

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Wadai

院内感染を防ぐ細菌院内拡散自動検出法

はじめに

本技術（「菌の異常集積の自動検出」）は，日常の細菌検査の結果のみを用い，①菌の院内拡散の早期発見，②院内感染アウトブレイクの防止を実現する．さらに，③細菌感染対策上の問題点を指摘する．

二項分布

菌の分離が偶然だけに支配され，時間的にも空間的にも偏りがない場合，1）細菌検査を受けた人の数，2）ある菌が陽性だった人の数，3）その菌の患者一人当りの分離率がわかれば，そのような（たとえば，8人中3人が陽性だったというような）菌の分離が起こる確率を簡単に求めることができる．

①菌の分離は，その菌が陽性か陰性かという二つの値のみをとり，②分離が偶然だけに支配されている場合，それぞれの分離は場所や時間，順序にかかわらず分離率にのみ支配され，毎回の分離（試行）は独立である．これらの条件を満たす場合，確率は二項分布（**図1**）によって求まる[1]．

帰無仮説

菌の分離が偶然のみに支配されたと仮定した帰無仮説のもとに確率を計算し，確率が非常に小さい場合，仮説を否定（棄却）し，何らかの集積，人為的な介在があったと判断する．

計算法

個々の確率の計算は簡単であるが，実用的に行うためには，毎日，すべての菌について，すべてのユニット（病棟など）ごとに，さらに，異なった観察幅（たとえば1日，7日，14日，30日）で集計を行うことが必要で，これを実現するためには，①自動的に細菌検査結果を収集できること，②収集したデータを自動的に集計する仕組みがあることが必要である．

$$P_{(n,m)} = {}_nC_m p^m (1-p)^{n-m} \quad \cdots\cdots ①$$

$$P_{(n,m\geq k)} = \sum_{m=k}^{n} {}_nC_m p^m (1-p)^{n-m} \quad \cdots\cdots ②$$

図1　二項分布による確率の計算

菌の分離が偶然のみに支配されている場合，ある菌の分離率（ベースラインレート）が患者一人当りpであるならば，細菌検査を行ったn人のうち，ちょうどm人からその菌が分離される確率$P(n, m)$，k人以上から分離される確率$P(n, m\geq k)$は，二項分布によってそれぞれ式①，②で与えられる．たとえば，8人を検査して3人が陽性であった場合，偶然のみに支配されて3人以上が陽性になる確率を式②で計算し，確率が小さければ，偶然だけに支配されている可能性は低いと結論できる．

警告と対応

本技術は，細菌の院内拡散を感度，特異性よく検出する．システムは，統計的に菌の分離に偏りがあったと判断できる分離に対して警告を出す．現在稼働しているシステムでは，確率の値によりレベル1（軽微），2（中程度），3（重大）の警告を出す[2,3]．警告が出た場合，利用者は，たまたま持ち込みが重なっただけなのか，院内で拡散があったのかをアンチバイオグラムや患者の動線を参考に調査する．菌の異常集積の自動検出を実装するシステムは，アンチバイオグラム作成やベッドマップ表示など，疫学解析のためのツールを提供している[3]．

警告スコア累積と対策

警告レベルを1カ月ごとに合計して指標とした．個々の警告には，たまたま起きたまれな現象も含まれるが，そのような現象が繰り返し起こる確率はきわめて低い．したがって，警告の合計（警告スコア累積）は，菌の院内拡散の指標として信頼性が高い（**図2**）．

警告スコア累積を用いてアウトブレイク事例の解析を行った．アウトブレイクの数年前から，アウトブレイクを起こした菌が潜在的な院内拡散を繰り返していたことが明らかになり，本技術に

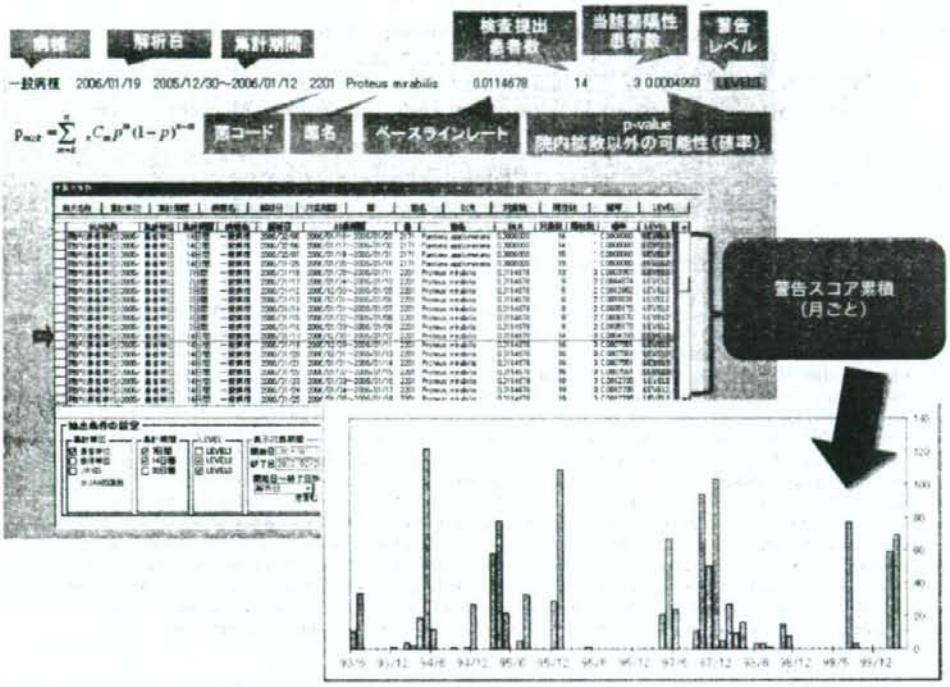

図 2　菌の異常集積の自動検出警告画面と警告スコア累積

左上：菌の異常集積自動検出の警告画面の例（SHIPL：small and medium-size hospital infection primary lookout の画面）．矢印の行を上部に拡大してある．*Proteus mirabilis* の分離率（ベースラインレート）が患者一人当り約 0.01 の施設で，12 月 30 日から翌年の 1 月 12 日までの 14 日間に，一般病棟で 14 名の患者から検体が提出され，そのうち 3 名が *P. mirabilis* 陽性であった．このような菌の分離が偶然だけに支配されたと仮定して確率を計算すると，5 10,000 程度で偶然である可能性は非常に低い．院内拡散の可能性があるとしてレベル 3 の警告が出ている．警告スコア累積は，菌の異常集積自動検出の警告のレベル値（例 1~3）を加算したもので，長期間の菌の院内拡散を評価する指標となる．

右下：グラフは 6 年間の警告スコア累積を月ごとに集計したもの．長期間の状態を俯瞰することができる．棒グラフの高さが高い期間は，対象となっている菌が異常集積を繰り返した時期に相当する．

よってこのような事故を防止できることが証明された[4]．さらに，警告累積スコアが高値を示す菌種の解析で，細菌感染対策の問題点を把握できることが明らかになっている．

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＊確率，統計の本は多数出版されている（上記は一例）．なかなかこれという一冊に巡り会えない．基礎から述べてあって，各自が読みやすいと感じる一冊を買ってほしい．
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3) 八束眞一：中小規模病院で実施可能なサーベイランス．*Medical Technology*，35 (5)：456~462，2007.
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Use of Protein Antigens for Early Serological Diagnosis of Leprosy[▽]

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Leprosy is a chronic and debilitating human disease caused by infection with the *Mycobacterium leprae* bacillus. Despite the marked reduction in the number of registered worldwide leprosy cases as a result of the widespread use of multidrug therapy, the number of new cases detected each year remains relatively stable. This indicates that *M. leprae* is still being transmitted and that, without earlier diagnosis, *M. leprae* infection will continue to pose a health problem. Current diagnostic techniques, based on the appearance of clinical symptoms or of immunoglobulin M (IgM) antibodies that recognize the bacterial phenolic glycolipid I, are unable to reliably identify early-stage leprosy. In this study we examine the ability of IgG within leprosy patient sera to bind several *M. leprae* protein antigens. As expected, multibacillary leprosy patients provided stronger responses than paucibacillary leprosy patients. We demonstrate that the geographic locations of the patients can influence the antigens they recognize but that ML0405 and ML2331 are recognized by sera from diverse regions (the Philippines, coastal and central Brazil, and Japan). A fusion construct of these two proteins (designated leprosy IDRI diagnostic 1 [LID-1]) retained the diagnostic activity of the component antigens. Upon testing against a panel of prospective sera from individuals who developed leprosy, we determined that LID-1 was capable of diagnosing leprosy 6 to 8 months before the onset of clinical symptoms. A serological diagnostic test capable of identifying and allowing treatment of early-stage leprosy could reduce transmission, prevent functional disabilities and stigmatizing deformities, and facilitate leprosy eradication.

Cases in which *Mycobacterium leprae* infection manifests to cause leprosy present as a bacteriologic, clinical, immunologic, and pathological spectrum ranging from the extremes observed in paucibacillary (PB) and multibacillary (MB) patients (21, 24). PB patients have one or a few skin lesions and a low or absent bacterial index (BI; a measure of the number of acid-fast bacilli in the dermis, expressed on a logarithmic scale) and demonstrate specific cell-mediated immunity against *M. leprae*, but they have low or absent titers of *M. leprae*-specific antibodies and a granulomatous dermatopathology. In marked contrast, MB patients have multiple symmetric skin lesions and a high BI and demonstrate high titers of anti-*M. leprae* antibodies but an absence of specific cell-mediated immunity and a dermatopathology largely devoid of functional lymphocytes (21). Despite the implementation of a WHO-directed eradication program over the last 20 years, the worldwide annual rate of new case detection for leprosy remains stable at approximately 300,000 (17, 18, 26, 27). Earlier and objective diagnosis of leprosy could interrupt transmission and, in the long term, help further reduce the number of new cases and facilitate eradication.

There is no single diagnostic laboratory test for leprosy, and diagnosis remains essentially clinical. Clinical diagnosis of leprosy is dependent upon recognition of disease symptoms and is therefore only possible once the disease has manifested. WHO experts have listed diagnostic criteria as one or more of the following: hypopigmented or reddish skin patches with definite loss of sensation; thickened peripheral nerves; acid-fast bacilli on skin smears/biopsy specimens (WHO Expert Committee on Leprosy, 1998). Pure neuritic leprosy forms, however, present with no skin lesion. Confounding WHO's implementation of a global leprosy eradication strategy is that the number of trained leprologists has diminished. This is inadvertently increasing the likelihood that a clinical diagnosis is delayed or even missed, especially in regions where leprosy has been controlled (1, 13, 16, 25).

The presence of serum immunoglobulin M (IgM) antibody to phenolic glycolipid I (PGL-I) correlates with BI in leprosy patients and has been used to support disease symptoms as a means to categorize leprosy patients. Enzyme-linked immunosorbent assay (ELISA) and rapid lateral flow test formats have been developed for the detection of anti-PGL-I antibody (3, 4, 8, 19, 22, 23, 28). In one study, a lateral flow assay correctly diagnosed 97.4% of MB patients, with a specificity of 86.2% (4). Patients toward the PB end of the leprosy spectrum have low or no BI, however, and the majority of these patients are not identified by PGL-I-based tests (4, 7, 19). In addition, false-positive results in areas of endemicity are relatively high (>10%) (4, 7, 19). Consequently, none of these PGL-I-based tests has been widely implemented in

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field situations. In addition, many studies have demonstrated that MB patients have high titers of *M. leprae*-specific antibodies but PB patients have low or absent titers. For these reasons, the potential for serological diagnosis of low-BI patients, such as PB patients or MB patients who are developing disease, has not been thoroughly pursued.

In a recent small-scale study, we demonstrated that the ML0405 and ML2331 proteins were recognized by sera from MB leprosy patients presenting with high BI (20). In the current study we demonstrate that ML0405 and ML2331 are diagnostically relevant antigens by analyzing a large panel of MB leprosy patient sera from a variety of leprosy-affected regions (the Philippines, central and coastal Brazil, and Japan). We also examine the ability of *M. leprae* protein antigens to diagnose low-BI leprosy (PB patients and early MB patients) and show here the diagnostic potential of ML0405, ML2331, and a newly discovered *M. leprae* antigen, ML1556c. Based on the results, we construct and evaluate a fusion protein comprising ML0405 and ML2331 (designated *l*eprosy *I*DRI *d*iagnostic 1 [LID-1]) and demonstrate that this construct can be used to serologically diagnose leprosy patients among presymptomatic individuals, that is, before a clinical diagnosis is possible. Moreover, ML1556c may be a valuable adduct to LID-1 for the diagnosis of PB leprosy.

MATERIALS AND METHODS

Subjects and samples. Sera were obtained from patients with leprosy (MB and PB) or tuberculosis (TB), healthy household contacts of MB leprosy patients (HHC), and endemic and nonendemic controls (EC and NEC). MB and PB leprosy patient sera used in this study were derived from recently diagnosed, previously untreated individuals who did not have signs of reversal reactions. Leprosy was classified in each case by bacterial, histological, and clinical observations carried out by qualified personnel, with the BI recorded at the time of diagnosis. HHC were defined as adults living in the same house as an MB index case for at least 6 months. TB patients were included to evaluate potential antigen cross-reactivity with other mycobacterial infection. Sera from TB patients were obtained after drawing blood from *Mycobacterium tuberculosis* sputum-positive, human immunodeficiency virus-negative individuals with clinically confirmed pulmonary TB who were undergoing treatment. Normal sera (EC and NEC) were obtained after blood draws from volunteers with no history of leprosy or TB infection. In all cases, drawing of blood was carried out with informed consent (with local institutional review board approval or local ethics committee approval in Brazil, Japan, the Philippines, Seattle, and St. Louis). The composition of each study population is summarized in Table 1.

In Cebu City, leprosy and TB patients were recruited at the Cebu skin clinic and Leonard Wood Memorial Research Center in Cebu City, Cebu (Philippines) from 2003 to 2006. Between 1985 and 1991, sera were collected prospectively from individuals who resided with MB patients (BI > 2) for at least 2 years and were free of leprosy as determined by clinical dermato-neurological examination at the inclusion point of the study. Some of these individuals developed MB leprosy as the study progressed, and these sera have previously been described (11).

In Goiânia, the state capital of Goiás State (western central Brazil), leprosy and TB patients were recruited at the main outpatient clinics of Centro de Referência em Diagnóstico e Terapêutica and Hospital Anuar Auad in 2006. PB leprosy patients were selected from a cohort of leprosy patients with a single skin lesion recruited at Brazilian sites of endemicity from 1999 to 2001, as previously described (9).

In Salvador, the state capital of Bahia State (northeast coastal Brazil), leprosy patients were recruited at Hospital Dom Rodrigo de Menezes in 2006.

In Japan, leprosy patients were recruited at the National Sanatorium Oshimaseishoen, Kagawa.

In St. Louis, sera were collected from U.S.-based individuals at a variety of times following *Mycobacterium bovis* BCG immunization.

All serum specimens were aliquoted and stored at −20°C or −80°C prior to assay.

TABLE 1. Study populations

Site	Sample categorization (total no.)	BI (mean)	Sex ratio[a]	Mean age (yr) (range)
Cebu City, Philippines	MB (17)	2.8	2.4	30 (18–55)
	PB (54)	0.5	0.4	31 (15–45)
	TB (6)		5	45 (35–53)
	EC (8)		1	26 (19–38)
	HHC (10)		0.4	38 (18–60)
Goiânia, Brazil	MB (28)	2.4	1.5	44 (19–81)
	PB (83)	0	0.4	33 (7–76)
	TB (26)		2.7	39 (17–66)
	EC (30)		0.1	20 (19–26)
	HHC (11)		0.5	28 (18–51)
Salvador, Brazil	MB (10)	NA[b]	3.5	35.1 (20–70)
	PB (6)	0	5	31.6 (12–42)
	HHC (11)		0.1	48.5 (25–57)
Kagawa, Japan	MB (30)	NA	NA	60 (48–79)
	PB (30)	0	NA	70 (55–90)
	EC (26)		NA	54 (48–62)

[a] Male/female ratio.
[b] NA, not available.

Cloning and purification of target antigens. DNA encoding selected *M. leprae* proteins was PCR amplified from *M. leprae* Thai-53 genomic DNA using *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA). PCR primers were designed to incorporate specific restriction enzyme sites 5′ and 3′ of the gene of interest and excluded in the target gene for directional cloning into the expression vector pET28a (Novagen, Madison, WI). After PCR amplification, purified PCR products were digested, ligated with vector DNA, and used to transform *Escherichia coli*, and individual clones were induced to produce recombinant proteins, as previously described (20). Recombinant proteins were quantified using the bicinchoninic acid protein assay (Pierce, Rockford, IL), and quality was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The characteristics of each *M. leprae* protein evaluated are summarized in Table 2. The ML1556c protein was included because portions of the ML1556 protein were identified in four separate clones during serological expression screening with sera from PB leprosy patients (data not shown) (20). Recognition of the clones was derived from amino acids 58 to 256 of ML1556, which are only 47% identical to the *M. tuberculosis* protein Rv2839 (compared to 82% identity across the entire amino acid sequences of ML1556 and Rv2839).

Determining patient reactivity by ELISA. ELISAs were conducted independently at IDRI, Seattle, WA (Cebu and St. Louis sera); UFG, Goiânia, and UFB, Salvador, Brazil; and NIID, Tokyo, Japan. Polysorp 96-well plates (Nunc, Rochester, NY) were coated with 1 μg/ml recombinant protein or 200 ng/ml of natural disaccharide with octyl linkage (NDO), the synthetically derived B-cell epitope of PGL-I, conjugated to bovine serum albumin (NDO-BSA; kindly supplied by John Spencer, Colorado State University, under NIH contract N01 AI-25469), in bicarbonate buffer overnight at 4°C and blocked for 1 h at room temperature with phosphate-buffered saline–Tween with 1% BSA on a plate shaker. Serum diluted appropriately in 0.1% BSA was added to each well, and plates were incubated at room temperature for 2 h with shaking. Plates were washed with buffer only, and horseradish peroxidase-conjugated IgG or IgM (Rockland Immunochemicals, Gilbertsville, PA), diluted in 0.1% BSA, was added to each well and incubated at room temperature for 1 h with shaking. After washing, plates were developed with peroxidase color substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and the reaction was quenched by the addition of 1 N H_2SO_4. The optical density (OD) of each well was read at 450 nm. Positive responses were defined as an OD of >2× the mean OD of endemic control sera or an OD of >0.1, whichever was higher.

Statistics. *P* values were determined using Student's *t* test.

RESULTS

Recognition of *M. leprae* proteins by Filipino leprosy patient sera. The majority of MB leprosy patients are readily identified

TABLE 2. Main characteristics of *M. leprae* antigens tested[a]

Gene accession no.	Functional classification[b]	Protein type	Length (bp)	Product size (kDa)	% Identity[c] with:				
					M. tuberculosis H37Rv[e]	*M. bovis* AF2122/97[e]	*M. avium* 104[d]	*M. marinum* ATCC BAA-535[e]	*M. smegmatis* MC2 155[d]
ML0091	II.C.2	28-kDa antigen precursor	711	23.7	53	53	54	54	48
ML0405	V	Conserved hypothetical	765	25.3	62	62	None	NA	None
ML1633	II.C.2	Possible secreted hydrolase	1,608	57.0	25	25	35	81	62
ML2055	IV.A	Probable cell surface protein	864	29.5	72	72	69	73	54
ML2331	II.C.2	Possible secreted protein	771	26.5	80	80	77	80	67
ML2346	VI	Hypothetical	906	33.9	None	None	None	None	None
ML1556	II.A.6	Translation initiation factor	2,775	96.6	84	82		90	

[a] Annotations for gene accession number, functional classification, and protein type are according to the Sanger database.
[b] Functional classifications: II.C.2, surface polysaccharides, lipopolysaccharides, proteins, and antigens; V, conserved hypotheticals; IV.A, virulence; VI, unknowns; II.A.6, protein translation and modification.
[c] BLAST reports were performed in September 2006; tBLASTn was used for comparisons of proteins versus translated DNA. NA, not applicable.
[d] From http://www.tigr.org.
[e] From http://www.sanger.ac.uk/Projects.

by ELISA and lateral flow tests, which assess the capacity of patient IgM to bind *M. leprae* PGL-I or its synthetic analogue (NDO) conjugated to a carrier protein (BSA). In comparison with MB leprosy patients, PB leprosy patients have low or no anti-PGL-I responses and are more difficult to diagnose serologically. We therefore sought to determine whether PB sera recognized protein antigens, expanding our previous analyses and comparing the potential of NDO-BSA, ML0405, and ML2331 to diagnose leprosy, and found that the protein antigens have a similar profile for leprosy diagnosis as that for NDO-BSA; all three test antigens were readily detected by MB patient sera, by some PB patient sera, and by few, if any, EC, HHC, or TB sera (Fig. 1). Thus, similar to NDO-BSA, ML0405 and ML2331 demonstrate good potentials for the diagnosis of leprosy.

Recognition of MB leprosy patient sera with refined ML0405 antigen constructs. To learn more regarding the seroreactivity of ML0405 and enhance recombinant ML0405 expression for purification, we expressed a variety of ML0405 polypeptide fragments and determined whether Filipino MB leprosy patient sera had similar binding capacities to these fragments and to full-length (ML0405FL) protein. All constructs were able to bind MB patient sera (Fig. 2) ($P < 0.01$ for MB versus EC). The reactivity of a truncated form (ML0405Tr) of the protein was equivalent to the reactivity of ML0405FL ($P = 0.885$ for MB patient sera), whereas the reactivity of the protein construct lacking the predicted membrane-spanning region (ML0405Tm) declined slightly (Fig. 2) ($P = 0.047$ and 0.060 for Tm versus FL and Tr forms, respectively, for MB). These data indicate that the majority, if not all, of the B-cell epitopes recognized by antibodies in patient sera are retained and accessible in the truncated form of the protein. Further testing was conducted using either ML0405FL or ML0405Tr.

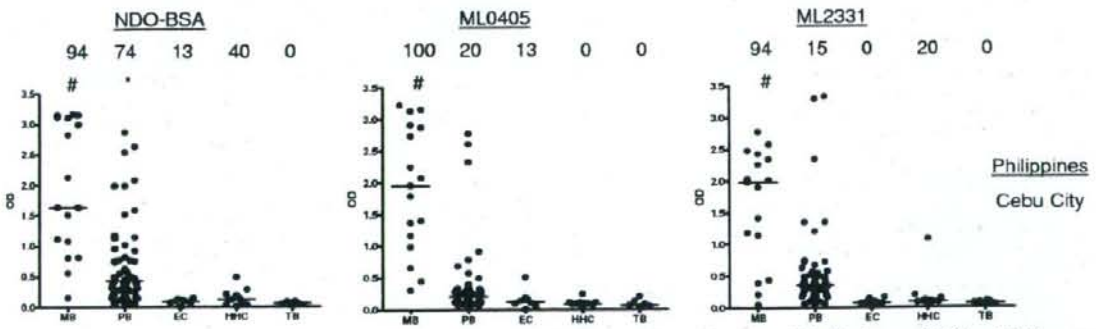

FIG. 1. Sera from Filipino leprosy patients react with recombinant *M. leprae* antigens. Sera from clinically diagnosed MB and PB leprosy patients, EC individuals, and HHC of MB leprosy patients were assessed against NDO-BSA, ML0405, and ML2331. NDO-BSA reactivity was assessed by IgM binding, and protein reactivity was assessed by IgG binding. Sera were from Cebu City, Philippines. Each point represents an individual serum sample, and the median is represented by the line. The number above each data set is the percent positive responses. *, $P < 0.05$; #, $P < 0.001$ versus EC.

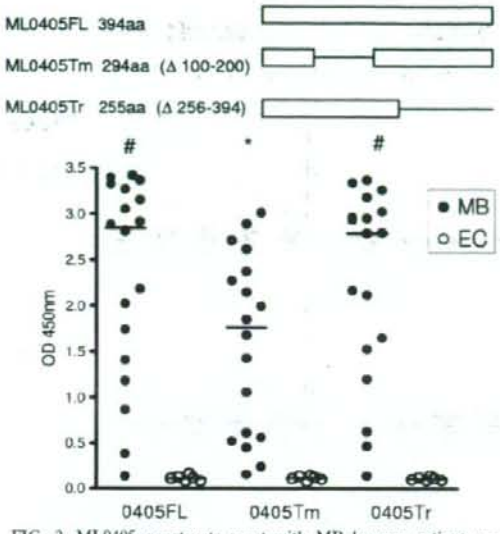

FIG. 2. ML0405 constructs react with MB leprosy patient sera. Different ML0405 constructs were created and expressed as recombinant proteins. The schematic diagram shows the sequence alignment of each of these constructs, with the deleted regions indicated by the line. Each construct was tested for IgG reactivity by ELISA with individual Filipino MB leprosy patient sera ($n = 18$) or EC sera ($n = 6$). *, $P < 0.05$; #, $P < 0.001$ versus EC.

Diagnosis of Filipino PB leprosy patients with *M. leprae* proteins. We then went on to more closely investigate the potential of *M. leprae* antigens for diagnosing PB leprosy. Sera from Filipino patients clinically diagnosed with PB leprosy and with a low BI were tested for reactivity with potential diagnostic *M. leprae* antigens (ML0405Tr, ML2331, ML1556c, and NDO-BSA). NDO-BSA was capable of identifying 57% (26 of 46) of these Filipino PB leprosy patients, but a substantial number of samples provided weak positive responses (Fig. 3). ML0405 and ML2331 also reacted with sera from some PB patients (Fig. 3A and B). Most of these Filipino sera that reacted with these proteins also demonstrated strong NDO-BSA responses, however, and so the added benefit of using these antigens for leprosy diagnosis within the Filipino population appeared minimal. In contrast, 4 of 20 sera that were weak positive/negative by NDO-BSA ELISA testing demonstrated strong reactivity to ML1556c (Fig. 3C). This result suggests that ML1556c may be useful as an adjunct to PGL-I testing, or other tests, to improve the sensitivity and clarity of leprosy diagnosis.

To test the specificity of ML1556c as a leprosy diagnostic reagent, we directly compared the reactivities of ML1556c with sera from PB leprosy patients, MB leprosy patients, TB patients, EC, and HHC of MB leprosy patients located in Cebu City, Philippines (Fig. 3D). Positive responses were observed in five of eight additional PB leprosy sera tested, with three of the sera yielding strong responses that could provide a clear diagnosis. Positive responses to ML1556c were also observed in two of seven MB leprosy sera tested in this experiment.

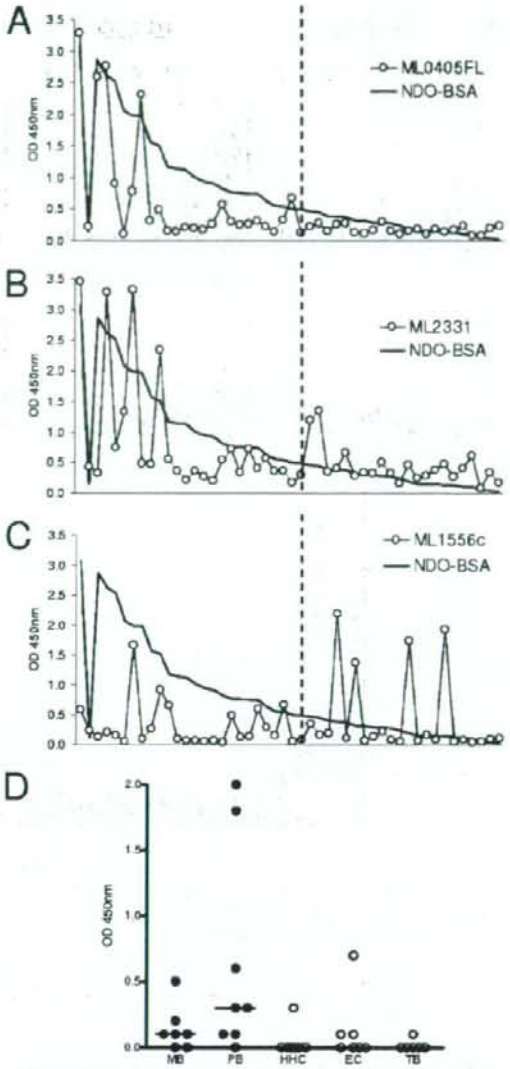

FIG. 3. *M. leprae* proteins react with PB leprosy patient sera. (A to C) Antibody reactivities of sera from a pool of clinically diagnosed MB leprosy patients, from a pool of negative control individuals, and from 46 clinically diagnosed PB leprosy patients were assessed against NDO-BSA and ML0405 (A), ML2331 (B), and ML1556c (C). NDO-BSA reactivity was assessed by IgM binding and, for reference, is shown in each plot. Recombinant protein reactivity was assessed by IgG binding. The first open circle represents the value obtained for pooled MB sera, while the next open circle represents the reactivity of pooled EC sera; individual PB sera are then arranged along the *x* axis according to their responsiveness versus NDO-BSA. The dashed line indicates the point at which diagnosis by NDO-BSA reactivity becomes unclear. ML1556c reacts with PB leprosy patient sera. (D) IgG reactivities of ML1556c with a small panel of individual sera from EC, leprosy patients (MB and PB), and TB patients were determined by ELISA using samples from Cebu City, Philippines. Each point represents an individual serum sample, and the median is represented by the line.

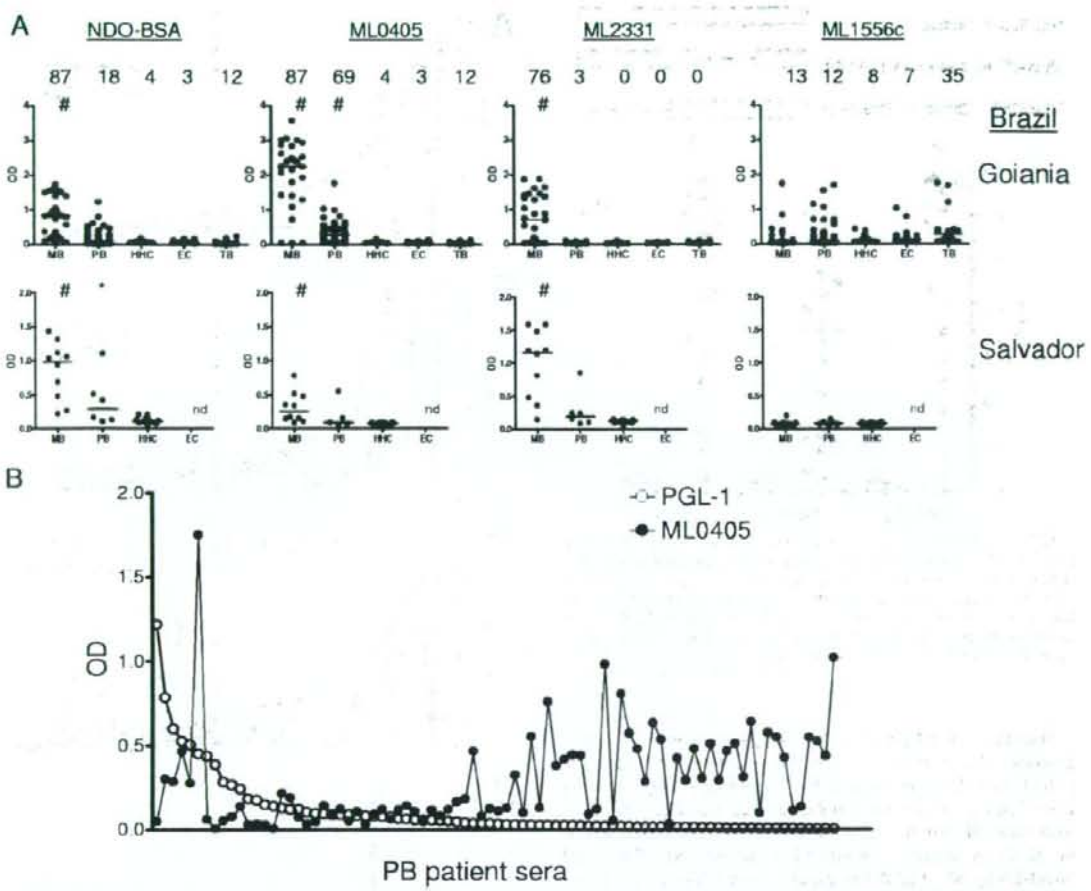

FIG. 4. Sera from Brazilian leprosy patients react with recombinant *M. leprae* antigens. Sera from clinically diagnosed MB and PB leprosy patients, EC individuals, and HHC of MB leprosy patients were assessed against NDO-BSA, ML0405, ML2331, and ML1556c. NDO-BSA reactivity was assessed by IgM binding, and protein reactivity was assessed by IgG binding. Sera were from Goiânia and Salvador (see Table 1). (A) Each point represents an individual serum sample, and the median is represented by the line. The number above each data set is the percent positive responses. *, $P < 0.05$; #, $P < 0.001$ versus EC. (B) To demonstrate complementarity, the individual PB sera from Goiânia are arranged along the *x* axis according to their responsiveness versus NDO-BSA and overlaid with the response of each serum to ML0405.

ML1556c did not react with any of the Filipino TB patient sera tested, was recognized by only one of eight HHC sera, and reacted with only one of six EC sera. Negative results were obtained upon further testing involving another 45 TB sera and 23 NEC sera (data not shown). Taken together, these results generated from sera from the Philippines suggested the utility of ML1556c to improve the diagnosis of PB leprosy.

Identification of leprosy patients in Brazil. We also examined the ability of recombinant *M. leprae* antigens to identify leprosy patients located around Goiânia, Brazil, and Salvador, Brazil. Within the clinically diagnosed leprosy population, PGL-I/NDO-BSA was capable of identifying 87% (33 of 38) of the MB patients (Fig. 4). In agreement with the results obtained by analysis of Filipino leprosy patient sera, ML0405 and ML2331 reacted with large proportions of Brazilian MB patient sera (87% [33 of 38] and 76% [29 of 38], respectively), and ML1556c reacted with only some MB patient sera (13%, 5 of 38) (Fig. 4). In Goiânia, positive responses were also observed against antigens ML0091 (71%, 20 of 28), ML1633 (32%, 9 of 28), ML2055 (75%, 21 of 28), and ML2346 (29%, 8 of 28) (data not shown). The clarity of MB leprosy diagnosis (strength of signal in positive samples versus negative samples) in Goiânia was greater when using ML0405 rather than NDO-BSA, but in Salvador it was greater when using ML2331 rather than NDO-BSA.

We also determined if these antigens were recognized by Brazilian PB patient sera. PGL-I/NDO-BSA was capable of identifying only 20% (18 of 89) of the PB patients, a level not appreciably higher than the proportion of positive responses observed with TB patients (12%, 3 of 26) (Fig. 4A). An IgG

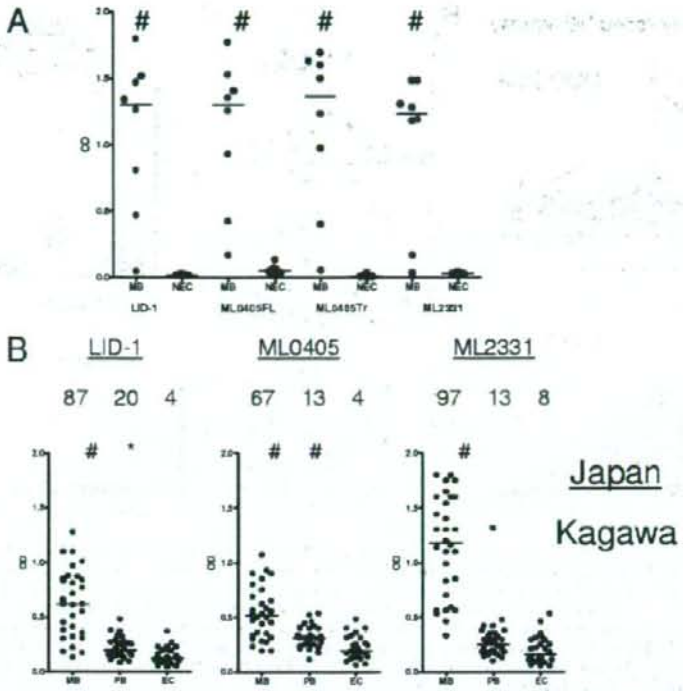

FIG. 5. LID-1 retains reactivity with leprosy patient sera. (A) LID-1 (a fusion construct of ML0405 and ML2331), ML0405FL, ML0405Tr, and ML2331 reactivities were assessed by IgG binding in an ELISA with eight MB leprosy patient serum samples from Salvador and eight NEC serum samples. (B) Sera from clinically diagnosed Japanese MB and PB leprosy patients, and Japanese EC individuals, were assessed for IgG reactivities with LID-1, ML0405, and ML2331. Each point represents an individual serum sample, and the median is represented by the line. The number above each data set is the percent positive responses. *, $P < 0.05$; #, $P < 0.001$ versus EC.

reactivity that permitted serologic diagnosis of an increased number of PB leprosy patients was observed for ML0405 (69%, 61 of 89), but responses to ML2331 were very weak, with very few positives (3%, 3 of 89) (Fig. 4A). The antigens ML0091 (6%, 5 of 83), ML1633 (17%, 14 of 83), ML2055 (13%, 11 of 83), and ML2346 (27%, 22 of 83) were recognized by some PB patient sera, but responses were generally weak (data not shown). Many of the PB patient sera that did not react with PGL-1 had a strong reactivity with ML0405 (Fig. 4B). ML1556c was recognized by only a minor subset of PB leprosy patient sera (12%, 11 of 89) and Brazilian EC individuals (6.7%, 2 of 30), but ML1556c reactivity was detected in a substantial number of Brazilian TB patients (35%, 9 of 26). These data indicate only a minor number of positive results in the Brazilian population if ML1556c is used for leprosy diagnosis, with a further complication of false-positive diagnosis in TB patients. Antigen ML0405, however, did not react with significant numbers of EC sera (3.3%, 1 of 30) or TB sera (12%, 3 of 26) (Fig. 4A). These results indicate that ML0405 can recognize some PB leprosy patients in the Brazilian population and could be used to augment leprosy diagnosis with PGL-1.

Construction of a fusion construct of ML0405-ML2331 (LID-1). Having extended our earlier observation that the single antigens ML0405 and ML2331 have the potential to diagnose leprosy (20), and given the observations that ML0405 appeared better for diagnosis in Goiânia and Cebu City but ML2331 appeared better for diagnosis in Salvador, we constructed a single fusion molecule incorporating both proteins. ML0405Tr was expressed at the C terminus of the molecule and ML2331 in the N terminus. Following recombinant expression, we validated the reactivity of the construct by assaying LID-1 versus a small panel of sera from Salvador that had bound each single component. These sera readily detected LID-1, ML0405FL, ML0405Tr, and ML2331 (Fig. 5A). Importantly, construction of the fusion protein did not introduce false-positive results with NEC sera (Fig. 5A).

We further extended our examination of sera from different geographic locations by assessing sera from Japanese leprosy patients for reactivity with ML0405, ML2331, and LID-1. Positive response were observed with MB patient sera (67% [20 of 30] for ML0405, 97% [29 of 30] for ML2331, and 87% [26 of 30] for LID-1) and PB patient sera (13% [4 of 30] for ML0405, 13% [4 of 30] for ML2331, and 20% [6 of 30] for LID-1), with few responses in EC sera (4% [1 of 26] for ML0405, 8% [2 of 26] for ML2331, and 4% [1 of 26] for LID-1) (Fig. 5B). Taken together, these data indicate that LID-1 is useful as a diagnostic antigen for leprosy.

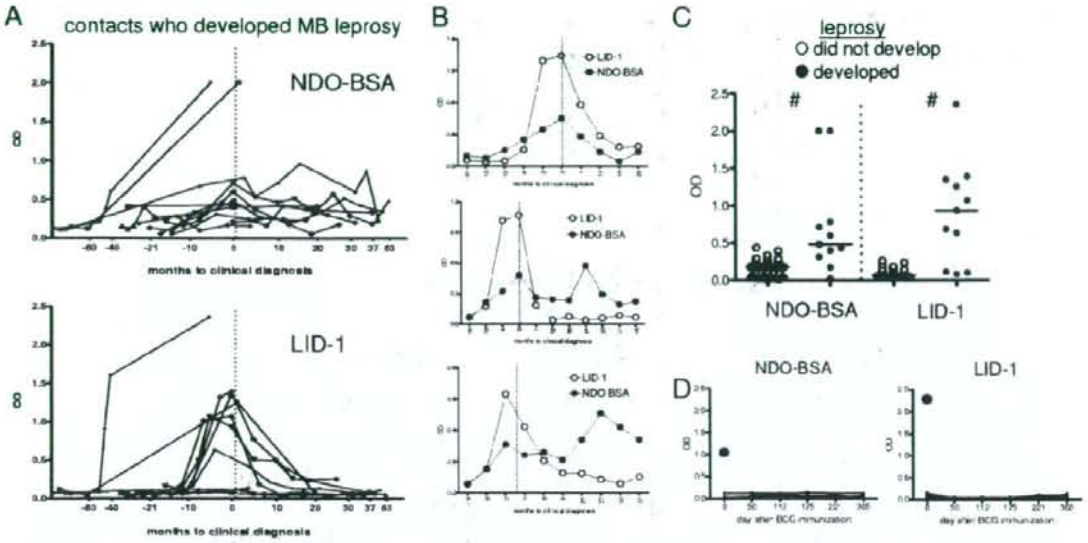

FIG. 6. LID-1 reactivity can diagnose leprosy before clinical symptoms. (A) LID-1 and NDO-BSA reactivities within sera from a prospective study conducted in Cebu City, Philippines, were assessed by either IgG or IgM binding in an ELISA. Sera were collected at a variety of times prior to the clinical diagnosis of MB leprosy in 11 patients and at a variety of times after the commencement of treatment. (B) Representative plots for individual patients are shown. (C) Sera were collected from 57 household contacts that did not develop clinical leprosy and were compared with single serum samples from each individual contact that developed leprosy (serum samples were collected within 3 months of clinical diagnosis). #, $P < 0.001$. (D) LID-1 and NDO-BSA reactivities within sera from a prospective study using 10 U.S.-based individuals who were immunized with BCG were assessed. Sera were collected at regular intervals following BCG immunization. The solid circle at day zero designates the reactivity of a leprosy patient serum sample that was included as a positive control.

LID-1 reactivity can diagnose leprosy before clinical symptoms. Having demonstrated that the LID-1 fusion molecule retained the ability to diagnose leprosy patients but lacked responses to EC sera, we obtained sera from a prospective study conducted in Cebu City, Philippines, between 1985 and 1991 (11). In that study, household contacts of leprosy patients were monitored over a prolonged period of time, and some developed clinical MB leprosy. In sera from the individuals who developed MB leprosy, as previously reported, anti-PGL-I levels increased before leprosy was diagnosed by clinical exam (Fig. 6A). Our data also indicate that anti-LID-1 antibody levels began to increase markedly as soon as 1 year prior to clinical diagnosis (Fig. 6A). For many of the patients (7 of 11, 64%) the increase in the anti-LID-1 IgG response was strikingly more obvious than the increase in the anti-PGL-I IgM response (Fig. 6B). Those patients that developed clinical leprosy had anti-PGL-1 antibody levels not dissimilar to many individuals who did not develop leprosy (Fig. 6C). The difference in anti-LID-1 antibody levels was much clearer, with a much larger differentiation between the positive responses of patients who developed leprosy compared with the extremely low levels of anti-LID-1 antibody in individuals who did not develop leprosy (Fig. 6C). Taken together, these data indicate that LID-1 is capable of providing an early serological diagnosis of leprosy.

LID-1 does not react with sera from individuals recently exposed to BCG. To examine in detail if leprosy diagnosis could be complicated by exposure to or infection with other mycobacteria, we also examined sera collected longitudinally from 10 U.S.-based individuals who were immunized with BCG. None of these BCG-immunized individuals developed positive serological responses against LID-1 or NDO-BSA (Fig. 6D). These data indicate that LID-1 can provide a clear diagnosis of *M. leprae* infection prior to the onset of signs that permit clinical leprosy diagnosis and that LID-1-based diagnostic tests could be used to expedite leprosy treatment.

DISCUSSION

Current diagnosis of leprosy is based on the appearance of clinical signs, and it is well established that the earlier a patient is identified the better their response to treatment. In addition, MB leprosy patient household contacts have a higher risk of developing clinical leprosy than contacts of PB leprosy patients (10, 12). This has been attributed to increased shedding and spreading of viable bacteria by MB leprosy patients (2). Accurate and early detection of *M. leprae*-infected individuals will open the possibility of earlier treatment that could both prevent disability and significantly reduce leprosy transmission.

We have evaluated the serological responses to a variety of *M. leprae* protein antigens in an attempt to discover antigens that can improve diagnosis of leprosy by detecting patients with a low BI (PB leprosy patients or early MB leprosy patients). We demonstrated that (i) ML0405 and ML2331 can be used to diagnose MB leprosy patients independently of geographic location; (ii) ML1556c can recognize some PB patients (al-

though it is recognized by some TB sera as well); (iii) ML0405 and ML2331 can be used for diagnosis of some PB patients; (iv) a fusion construct of ML0405 and ML2331 (LID-1) retains diagnostic capability; and (v) LID-1 can provide a clear leprosy diagnosis before the onset of clinical symptoms. These findings will improve both leprosy diagnosis and patient care.

One approach for the early detection of *M. leprae* infection is through serological diagnosis. We have conducted screening to identify *M. leprae* antigens that have not previously been described, and we then evaluated the diagnostic potential of these antigens with leprosy patient sera. In this study, the diagnostic potential of select antigens was assessed in clinically disparate leprosy patient groups, ranging from MB patients who presented with large bacterial burdens and large skin lesions to PB patients who presented with low or absent bacterial burdens and a few, small skin lesions. As expected, MB leprosy patients were easier to identify by serological assays and typically yielded higher responses than PB patients. Unexpectedly, close examination of patients with a low BI from the Philippines indicated that some patients exhibited strong responses against the ML1556c protein. The responses of Filipino PB patients to ML1556c were often greater than those of MB patients. These results suggested the utility of this protein either as an adjunct to antigens that could identify MB patients to provide a cross-spectrum leprosy diagnosis or as a stand-alone protein for PB leprosy diagnosis. An objective and differential diagnosis of MB or PB leprosy could lead to better treatment of patients by guiding the multidrug therapy regimen provided to them.

We also analyzed the diagnostic potential of each antigen within geographically disparate groups of patients, from the Philippines and two sites in Brazil. In the Brazilian (Goiânia) PB leprosy patient group, ML1556c provided only a few positive responses; this dampened the enthusiasm for ML1556c to be a widely used diagnostic or prognostic leprosy antigen. Of interest, many PB leprosy patients in Brazil (both Goiânia and Salvador) could be diagnosed by ML0405 reactivity, and several PB patients (Salvador) could be diagnosed with ML2331 reactivity. It is unclear if the differences in the responses of patients from different geographic locations are related to differences in *M. leprae* strains or to regional variations in host genetics. These possibilities might be addressed by analysis of patient sera on fragments of ML1556c or by a survey of anti-ML1556c antibody on lysates of different *M. leprae* strains. Regardless, the observed differences indicate the importance of examining antigen-specific responses in several regions when considering their ability to diagnose leprosy globally.

Given that the ML0405Tr and ML2331 proteins could provide diagnosis of leprosy, we made a fusion protein (LID-1) of these individual components. After ensuring the fusion protein retained reactivity against leprosy sera from Salvador, Brazil, we tested the antigens against sera from Japan. As with results obtained using sera from Brazil, Japanese MB leprosy patient sera reacted as strongly with the fusion LID-1 as with the ML0405 and ML2331 components. In addition, some Japanese PB leprosy patient serum antibodies recognized these antigens.

Studies have argued that the presence of anti-PGL-I antibodies is an indicator of leprosy development, but this has been debated (5, 6, 14, 15). Many contacts of leprosy patients have anti-PGL-I antibodies but do not develop disease, limiting the capacity of PGL-I-based assays to predict disease development. Indeed, PGL-I-based tests are typically marketed as a support reagent to confirm clinical diagnosis and aid leprosy classification but are not recommended for use as a stand-alone for diagnosis (19). The differential in responses of sera from contacts that developed leprosy compared with contacts that did not develop leprosy was much greater for LID-1 than PGL-1. We demonstrated that LID-1 is capable of providing an early serological diagnosis of MB leprosy. A clear and early diagnosis was achieved in 7 of 11 contacts of leprosy patients who themselves went on to develop clinical leprosy. For the small panel of sera tested, the time benefit of a LID-1-based diagnosis over a clinical-based diagnosis was 6 to 8 months. Thus, screening for LID-1-reactive antibodies, either in the general population or within more focused at-risk populations, could significantly expedite treatment of leprosy patients and, also, affect transmission rates by reducing the number of individuals who develop large bacterial burdens. As another benefit, antibody levels against LID-1 dropped following the implementation of drug treatment in these individuals, and thus the reduction and disappearance of antibodies against LID-1 may be a useful measure of multidrug therapy efficacy.

We are currently evaluating additional antigens, diagnostic formats, and different geographic sources of patient sera with the objective of early and simple identification of leprosy patients regardless of incidence locality.

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Evaluation of antimicrobial susceptibility for β-lactams using the Etest method against clinical isolates from 100 medical centers in Japan (2006)

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Abstract

This antimicrobial resistance surveillance study was performed in 100 medical centers. Susceptibility testing (Etest; AB BIODISK, Solna, Sweden) of 9152 strains including *Escherichia coli* (991 strains), *Klebsiella* spp. (1000 strains), *Enterobacter* spp. (971 strains), *Citrobacter* spp. (803 strains), indole-positive *Proteae* spp. (834 strains), *Serratia* spp. (902 strains), *Acinetobacter* spp. (874 strains), *Pseudomonas aeruginosa* (992 strains), oxacillin-susceptible *Staphylococcus aureus* (984 strains), and coagulase-negative staphylococci (CoNS; 801 strains) was performed with 7 β-lactams (cefepime, cefpirome, ceftazidime, cefoperazone/sulbactam, imipenem and piperacillin for Gram-negative bacteria, or oxacillin for Gram-positive bacteria). No strain resistance to these β-lactams (except for ceftazidime) was found in oxacillin-susceptible *S. aureus* and CoNS. Of the *E. coli* clinical isolates, 17.1% were resistant to piperacillin, whereas 2.9% or less (cefpirome = 2.9%) were resistant to other β-lactam agents. *Klebsiella* spp. strains were more susceptible to imipenem (99.9%), cefepime (99.2%), ceftazidime (98.6%), and cefpirome (98.3%). Isolates of *Enterobacter* spp., *Citrobacter* spp., indole-positive *Proteae*, and *Serratia* spp. were susceptible to imipenem, cefepime, and cefpirome as well. *Acinetobacter* spp. strains were least resistant to cefoperazone/sulbactam (0.7% resistance), imipenem (2.6%), cefepime (6.6%), and ceftazidime (7.7%) compared with other β-lactam antibiotics tested. Isolates of *P. aeruginosa* were more susceptible to ceftazidime (8.7% resistance), cefoperazone/sulbactam (9.8%), and cefepime (8.9%) than piperacillin (11.9%), cefpirome (16.2%), and imipenem (12.4%). The percentage of imipenem-resistant *P. aeruginosa* was approximately 13% in clinical isolates in Japan. The proportion of strains resistant to β-lactam antimicrobials has been decreasing compared with data from 2004, suggesting that reduced consumption of β-lactams has reflected the decreased rates of resistant bacterial isolates in Japan.

Keywords: β-Lactams; Etest; Susceptibility; Drug resistance

1. Introduction

β-Lactam antimicrobial agents have been widely used in clinical practice for more than 60 years. β-Lactamases have become the major resistance mechanism toward these agents in Gram-negative bacteria (Jacoby and Munoz-Price, 2005). Another prominent resistance mechanism in Gram-negative bacteria has been the decrease of antimicrobial concentrations to inhibit bacterial cell wall biosynthesis enzymes, for example, target enzymes (Alekshun and Levy, 2007).

Previously, extended-spectrum β-lactamase (ESBL)–producing organisms were reported in numerous countries worldwide (Canton and Coque, 2006). These ESBLs can hydrolyze penicillins and cephalosporins including oxyimino-cephalosporins. CTX-M–type ESBL enzymes prefer to hydrolyze cefotaxime as its major substrate (Ishii et al., 2007). These enzyme-producing isolates are found not only in clinical specimens (Canton and Coque, 2006; Ishii et al., 2005b) but also in animals and the environment in Japan (Ahmed et al., 2004; Kojima et al., 2005).

Plasmid-borne class B β-lactamases, metallo-β-lactamases (MBLs), are classified into 5 main molecular groups: IMP-, VIM-, SPM-, GIM-, and SIM-type enzymes (Walsh

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et al., 2005). These MBLs destroy most β-lactam antimicrobials including the carbapenems. IMP-1, the predominant MBL in Japan, has been found in clinical pathogens such as *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas stutzeri*, *Acinetobacter baumannii*, *Achromobacter xylosoxidans*, *Serratia marcescens*, *Enterobacter cloacae*, *Citrobacter youngae*, *Klebsiella pneumoniae*, or *Shigella flexneri* (Walsh et al., 2005). MBL-producing *P. aeruginosa* and *Providencia rettgeri* isolates were detected in our previously reported surveillance program in 2002 (Ishii et al., 2002, 2005a, 2006; Kimura et al., 2005a; Shiroto et al., 2005). On the other hand, nosocomial infection caused by IMP-1–producing *K. pneumoniae* has occurred in general hospital in Japan (Fukigai et al., 2007). The isolation frequencies of MBL-producing *P. aeruginosa* in 2002 and 2004 were 1.9% and 2.3%, respectively (Ishii et al., 2005a, 2006).

A surveillance program by the Japan Antimicrobial Resistance Study Group was carried out from 1997 to 2004 (Ishii et al., 2002, 2005b, 2006; Lewis et al., 1999; Yamaguchi et al., 1999). The present study was designed to provide up-to-date β-lactam antimicrobial susceptibility for clinical isolates including *Escherichia coli*, *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp., indole-positive *Proteus* spp. (*Proteus vulgaris*, *Providencia* spp., and *Morganella morganii*), *Serratia* spp., *Acinetobacter* spp., *P. aeruginosa*, oxacillin-susceptible *Staphylococcus aureus* (MSSA), and oxacillin-susceptible coagulase-negative staphylococci (CoNS) in Japan. One hundred hospitals participated in this surveillance program during 2006. Participating centers represented all geographic regions in Japan. In the present study, we compare the incidences of β-lactam–resistant bacteria and the consumption of β-lactam antimicrobials in Japan.

2. Materials and methods

2.1. Bacterial isolates

The collection and subsequent testing of clinical isolates by the 100 participant centers began in July and was concluded in September 2006. Each participant center had an average of 632 beds. Fifty-five and 32 participating centers use MicroScan WalkAway system (Dade Behring, Tokyo, Japan) and Vitek system (bioMérieux, Tokyo, Japan) to identify the organisms, respectively. Twelve centers used other systems such as the BD Phoenix system (Becton Dickinson, Tokyo, Japan), Raisus system (Nissui Pharmaceutical, Tokyo, Japan) or API sires (bioMérieux), Enterotube system (Becton Dickinson), and so on. Each laboratory was instructed to construct a collection of consecutive bacterial strains of up to 10 nonduplicate patient isolates for each designated species groups (10 total) as stated in a prevalence format. These 10 organism groups were *E. coli*, *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp., indole-positive *Proteus* spp., *Serratia* spp., *Acinetobacter* spp., *P. aeruginosa*, MSSA (oxacillin MIC, ≤2 μg/mL), and oxacillin-susceptible CoNS (MIC, ≤0.25 μg/mL). The overall collection of bacterial strains from the 100 centers totaled 9152 strains including 991 *E. coli*, 1000 *Klebsiella* spp., 971 *Enterobacter* spp., 803 *Citrobacter* spp., 834 indole-positive *Proteus* spp., 902 *Serratia* spp., 874 *Acinetobacter* spp., 992 *P. aeruginosa*, 984 MSSA, and 801 oxacillin-susceptible CoNS.

The specimens from which the strains in this study were isolated are listed on Table 1. Although compliance was complete, 1 *S. aureus* strain was omitted from the analysis because the documented oxacillin-resistant criteria was redefined by the Clinical and Laboratory Standards Institute (CLSI, 2006) during the protocol period. Also, 6 proteae isolates were omitted from analysis because these strains were identified as *Proteus mirabilis*, an indole-negative proteae by the BD Phoenix system in the coordinating laboratory (Department of Microbiology and Infectious Diseases, Toho University School of Medicine, Tokyo, Japan).

2.2. Antimicrobial susceptibility testing

Susceptibility testing of each isolate was determined by using Etest (AB BIODISK, Solna, Sweden) following the protocol described previously (Ishii et al., 2002, 2005a, 2006; Lewis et al., 1999; Yamaguchi et al., 1999). Bacteria were cultured on a 90-mm-diameter Mueller–Hinton agar (Becton Dickinson) for 16 h at 35 °C. Isolated colonies were suspended in sterile saline to obtain a turbidity of 0.5 McFarland. Each cell suspension was spread on a 135-mm-diameter Mueller–Hinton agar plate (Becton Dickinson) with a cotton swab, and the Etest strips were placed on the plates according to the manufacturer's instructions. The following strips were used: oxacillin (for Gram-positive bacteria), piperacillin (for Gram-negative bacteria), ceftazidime, cefepime, cefpirome, cefoperazone/sulbactam, and imipenem. Results were recorded after 16 to 20 h of incubation at 35 °C except for *S. aureus* and CoNS for which incubation was extended to 24 h. MIC values were interpreted as the point of intersection of the inhibition ellipse with the Etest strips edge. All clinical laboratories used the same lot of Etest strips, Mueller–Hinton agar plates, and reference strains. Clinical and Laboratory Standards Institute (2007) does not have criteria (susceptible, intermediate, or resistant) for either cefpirome or cefoperazone/sulbactam. For comparison only, the same values for cefepime (CLSI, 2007) were used as criteria for cefpirome, and the value for cefoperazone alone were used as criteria for cefoperazone/sulbactam. All 100 hospitals provided their results to the Department of Microbiology and Infectious Diseases, Toho University School of Medicine, for analysis. If uncertain data were found in the provided results, including identification and susceptibility testing, all tests were repeated. Identification

Table 1
Specimens used in this study

	S. aureus	CoNS	*E. coli*	*Klebsiella* spp.	*C. freundii*	*Enterobacter* spp.	Indole-positive *Proteae*	*Serratia* spp.	Acinetobacter spp.	*P. aeruginosa*
Urinary tract	57	108	547	252	274	157	375	148	75	220
Urine	27	65	373	156	182	95	233	92	42	116
Urinary catheter	12	10	117	74	71	40	123	51	23	88
Others	18	33	57	22	21	22	19	5	10	16
Pulmonary tract	392	96	106	411	112	418	85	487	542	449
Sputum	158	24	63	282	68	246	54	295	306	294
BALF	9	1	5	19	2	7		6	9	5
Intratracheal sputum	26	1	16	42	11	55	20	105	92	84
Pharyngeal mucus	95	24	12	47	18	67	3	44	82	28
Others	104	46	10	21	13	43	8	37	53	38
Gastrointestinal tract	24	13	98	122	246	118	139	35	28	62
Gastric or duodenal secretion	0	2	6	16	9	12	5	5	7	
Feces	20	6	59	63	179	67	112	19	13	383
Others	4	5	33	43	58	39	22	11	8	21
Blood and fluids	83	223	104	95	33	77	23	56	68	81
Blood	55	182	87	75	24	50	14	42	57	27
Spinal fluid	1	7		1	1			1	3	1
Others	27	34	17	19	8	27	9	13	8	13
Others	428	361	136	120	138	201	212	176	161	220
Drain fluid (thoracic cavity abdominal cavity)	15	10	26	24	25	37	32	19	18	29
Ophthalmic secretion	29	57	1	1	3	1	7	8	7	6
Ear secretion	95	53	3	7	4	23	10	20	23	36
Abscess	167	98	71	57	72	69	94	73	57	90
Skin or decubitus	83	55	14	9	17	17	50	26	23	27
Unspecified	39	88	21	22	17	54	19	30	33	32

BALF = bronchoalveolar lavage fluid.

and determination of MIC values was performed using the BD Phoenix system.

2.3. Quality control

For quality control (QC) of the Etest strips, the following reference strains were used: *E. coli* ATCC25922, *S. aureus* ATCC29213, and *P. aeruginosa* ATCC27853 (CLSI, 2006). CLSI does not have MIC QC ranges regarding cefpirome and cefoperazone/sulbactam. In this study, a range was determined near (±1 doubling dilution) the median MIC for cefepime and cefoperazone/sulbactam, as what was done in our previous reports (Ishii et al., 2006). The laboratories were required to test a set of all QC organisms in a replicate manner.

3. Results

3.1. Quality assurance

The validity of generated data was assured by employing appropriate QC and quality assurance measures. Values obtained for the challenge set of strains resulted in 28 of 1073 values falling out of the appropriate susceptibility category (2.6%). Of these 2.6%, 0.2% ($n = 2$ strains) were very major (false-susceptible) errors and 0.4% ($n = 4$ strains) were major (false-resistant) errors. Overall, this equates to 97.4% of MIC categoric results being accurate.

3.2. Activity against staphylococci

Because the CLSI (2006, 2007) recommends that oxacillin-resistant staphylococci be considered as resistant to all β-lactams, only oxacillin-susceptible strains were collected in this study. Of all the tested staphylococci, 984 isolates of *S. aureus* and 801 isolates of oxacillin-susceptible CoNS strains were susceptible to cefepime, cefpirome, cefoperazone/sulbactam, and imipenem (Table 2). However, 22 *S. aureus* (0.7%) and 23 oxacillin-susceptible CoNS (1.1%) were resistant to ceftazidime. The rank order of activity for all the tested agents using MIC_{90} values was imipenem (0.032 μg/mL) > oxacillin > cefpirome > cefoperazone/sulbactam > cefepime > ceftazidime (12–16 μg/mL).

3.3. Activity against E. coli and Klebsiella spp.

A total of 991 *E. coli* and 1000 *Klebsiella* spp. isolates were tested. Generally, all agents tested, except piperacillin (17.1% resistant), were highly active against *E. coli* and *Klebsiella* spp. (Table 2). No imipenem-resistant strains of *E. coli* or *Klebsiella* spp. were observed in this study.

3.4. Activity against other Enterobacteriaceae

Enterobacter spp. and *Citrobacter freundii* showed lower rates of susceptibility to piperacillin (77.6–83.4%), ceftazidime (79.5–83.0%), and cefoperazone/sulbactam

Table 2
Antimicrobial activity of 7 tested β-lactams against clinical isolates (2006)

Organism (no. tested)	Antimicrobial agent	MIC (μg/mL)		MIC (μg/mL)		Category (%)	
		50%	90%	Range		S	R
S. aureus (984)	Oxacillin	0.38	0.5	0.023	2	100.0	0.0
	Ceftazidime	12	16	0.125	48	24.7	0.7
	Cefepime	3	4	0.032	8	100.0	0.0
	Cefpirome	1	1.5	0.032	4	100.0	0.0
	CP–SB	2	3	0.032	6	100.0	0.0
	Imipenem	0.032	0.032	≤0.016	2	100.0	0.0
Coagulase-negative staphylococci (801)	Oxacillin	0.19	0.25	≤0.016	0.25	100.0	0.0
	Ceftazidime	6	12	0.125	>256	85.5	1.1
	Cefepime	1	2	0.064	>256	99.8	0.2
	Cefpirome	0.38	0.75	0.023	>256	99.6	0.2
	CP–SB	1	2	0.064	>256	99.6	0.2
	Imipenem	0.023	0.032	≤0.016	>256	99.8	0.2
E. coli (991)	Piperacillin	2	>256	0.023	>256	72.9	17.1
	Ceftazidime	0.125	0.75	0.023	>256	97.2	2.4
	Cefepime	0.032	0.125	≤0.016	>256	97.4	1.4
	Cefpirome	0.047	0.125	≤0.016	>256	95.5	2.9
	CP–SB	0.25	2	≤0.016	>256	98.7	0.4
	Imipenem	0.25	0.38	≤0.016	4	100.0	0.0
Klebsiella spp. (1000)	Piperacillin	6	48	0.125	>256	86.5	8.5
	Ceftazidime	0.125	0.5	≤0.016	>256	98.6	1.0
	Cefepime	0.047	0.125	≤0.016	96	99.2	0.4
	Cefpirome	0.047	0.125	≤0.016	>256	98.3	1.0
	CP–SB	0.25	2	≤0.016	>256	96.4	3.0
	Imipenem	0.25	0.38	0.023	6	99.9	0.0
C. freundii (803)	Piperacillin	2	>256	0.032	>256	77.6	17.9
	Ceftazidime	0.5	>256	0.047	>256	79.5	18.2
	Cefepime	0.032	1.5	≤0.016	>256	98.9	0.6
	Cefpirome	0.064	3	≤0.016	>256	96.1	2.2
	CP–SB	0.5	16	0.023	>256	90.4	2.9
	Imipenem	0.5	1	0.032	4	100.0	0.0
Enterobacter spp. (971)	Piperacillin	2	128	0.047	>256	83.4	10.5
	Ceftazidime	0.25	96	0.023	>256	83.0	13.7
	Cefepime	0.047	1	≤0.016	>256	98.4	0.6
	Cefpirome	0.064	2	≤0.016	>256	96.5	1.5
	CP–SB	0.38	16	≤0.016	>256	91.6	3.5
	Imipenem	0.5	1	≤0.064	12	99.4	0.0
Indole-positive *Proteae* (834)	Piperacillin	0.5	6	0.047	>256	92.9	4.9
	Ceftazidime	0.094	1	0.016	>256	95.4	2.6
	Cefepime	0.032	0.125	≤0.016	>256	99.4	0.2
	Cefpirome	0.064	0.38	≤0.016	>256	98.6	0.8
	CP–SB	1	3	0.032	>256	98.9	0.5
	Imipenem	1.5	3	0.047	>256	98.3	0.6
Serratia spp. (902)	Piperacillin	2	48	0.064	>256	83.3	6.9
	Ceftazidime	0.19	1	0.023	>256	96.7	2.5
	Cefepime	0.064	0.75	≤0.016	>256	97.9	0.9
	Cefpirome	0.064	0.5	≤0.016	>256	97.8	1.2
	CP–SB	1	16	0.032	>256	92.7	4.9
	Imipenem	0.5	1	≤0.016	>256	99.4	0.6
Acinetobacter spp. (874)	Piperacillin	12	64	0.032	>256	91.8	7.8
	Ceftazidime	4	16	0.032	>256	88.1	7.7
	Cefepime	2	12	0.023	>256	88.2	6.6
	Cefpirome	2	16	0.023	>256	88.9	8.5
	CP–SB	2	4	0.125	>256	97.6	0.7
	Imipenem	0.38	1	0.032	>256	95.3	2.6
P. aeruginosa (992)	Piperacillin	4	>256	0.25	>256	87.4	11.9
	Ceftazidime	2	16	0.19	>256	87.0	8.7
	Cefepime	3	24	0.19	>256	79.3	8.9
	Cefpirome	4	64	0.125	>256	70.7	16.2
	CP–SB	4	48	0.25	>256	80.5	9.8
	Imipenem	1.5	16	0.094	>256	74.8	12.4

CP–SB = cefoperazone/sulbactam (2:1); S = susceptible; R = resistance.

(90.4–91.6%) compared with the other tested β-lactams (Table 2). Susceptibility rates for cefepime (98.4–98.9%) and imipenem (99.4–100%) were superior to cefpirome (96.1–96.5%). For the indole-positive *Proteus* spp., susceptibility rates of piperacillin (92.9%) and ceftazidime (95.4%) were lower than for the other β-lactam antibiotics. *Serratia* spp. also showed lower rates of susceptibility to piperacillin (83.3%) and cefoperazone/sulbactam (92.7%) compared with the other tested β-lactams (96.7–99.4%).

3.5. Activity against nonfermentative Gram-negative bacilli

For *Acinetobacter* spp., cefoperazone/sulbactam was the most active antimicrobial (combination) (97.6% susceptible), followed by imipenem (95.3%), cefpirome (88.9%), cefepime (88.2%), and ceftazidime (88.1%). Piperacillin (91.8%) showed the lowest susceptibility rate when compared with the other tested β-lactams (Table 2).

4. Discussion

All centers participating in this surveillance were not small-sized hospitals (average number of beds = 632), so results reflect large hospital data. Imipenem maintained antimicrobial activity against Gram-positive and Gram-negative bacteria except for some indole-positive *Proteus* spp., *Acinetobacter* spp., and *P. aeruginosa* (Table 2) compared with previous studies (Ishii et al., 2002, 2005a, 2006; Lewis et al., 1999; Yamaguchi et al., 1999). Against *Acinetobacter* spp., the combination of cefoperazone and sulbactam had the most potent antimicrobial effect. Sulbactam has been recognized as one of the effective agents against carbapenem-resistant *A. baumannii* (Go et al., 1994). In 2004, the resistance rates of *Acinetobacter* spp. to cefepime or cefpirome were 7.6% and 11.6%, respectively (Ishii et al., 2006). The present surveillance data show that the resistance rates for cefepime and cefpirome were only 7.0% and 8.6%, respectively. Multidrug-resistant (MDR) *Acinetobacter* spp. isolates have become a problem in Europe (Paterson, 2006). Fortunately, these reported data suggested that expanded-spectrum cephalosporin-resistant *Acinetobacter* spp. are not increasing in Japan.

ESBL-producing Enterobacteriaceae are well known as cephalosporin-resistant strains (Ahmed et al., 2004). In this study, 6.2% (61 strains) of *E. coli* and 4.2% (42 strains) of *K. pneumoniae* showed a MIC value of 2 μg/mL or more for ceftazidime, which suggests that they are ESBL producers according to the CLSI (2006). Among *Klebsiella* spp., 26 *K. pneumoniae* and 16 *Klebsiella oxytoca* isolates were possible ESBL producers according to this screening test. These *Klebsiella* spp. isolates were collected in 33 hospitals, and the *E. coli* were isolated from 40 hospitals. ESBL producers were confirmed by the CLSI disk with clavulanate test (2007). Because some *K. oxytoca* strains produce K1 enzyme, which behaves like an ESBL and in which it was impossible to separate the K1 β-lactamase from an ESBL by a phenotypic

Table 3
Annual changing of resistant percentages of 7 β-lactams against clinical isolates (1997–2006)

Organism	Antimicrobial agents	Year (%)					
		1997	1998	2000	2002	2004	2006
S. aureus	Oxacillin	0	0	0	0	0	0
	Ceftazidime	6.4	0.5	2	2.4	2.2	0.7
	Cefepime	0	0	0	0	0	0
	Cefpirome	0	0.5	0	0	0	0
	CP–SB	0	0	0	0	0	0
	Imipenem	0	0	0	0	0	0
CoNS	Oxacillin	0	0	0	0	0	0
	Ceftazidime	15.4	1.6	2.1	3.9	2.9	1.1
	Cefepime	0	0	0	0	0.7	0.2
	Cefpirome	0	0	0	0	0.6	0.2
	CP–SB	0	0	0	0	0.5	0.2
	Imipenem	0	0	0	0	0.7	0.2
E. coli	Piperacillin	14.6	12.6	11.9	10.8	16.5	17.1
	Ceftazidime	0.5	0	1	0.5	1	2.4
	Cefepime	0.5	0	0.5	0.7	0.9	1.4
	Cefpirome	0.5	0	1	1.3	1.5	2.9
	CP–SB	0.5	0	0.5	1	0.7	0.4
	Imipenem	0.5	0	0	0	0	0
Klebsiella spp.	Piperacillin	7.2	9.6	7.2	7.4	11.2	8.5
	Ceftazidime	1.8	0.9	0.2	1	1.1	1
	Cefepime	0	0	0.2	0.2	0.8	0.4
	Cefpirome	1.4	0.5	0.7	0.3	1.4	1
	CP–SB	2.7	1.4	1.5	2.5	3.9	3
	Imipenem	0	0	0	0	0.2	0
C. freundii	Piperacillin	26.1	22.6	18.4	18.7	19.2	17.9
	Ceftazidime	25	22.1	19.5	19.7	16.7	18.2
	Cefepime	0	0	0.6	0.6	1.6	0.6
	Cefpirome	1.1	2.1	1.7	1.6	2	2.2
	CP–SB	8.3	6.3	5.8	2	5.9	2.9
	Imipenem	0	0	0	0.2	0.1	0
Enterobacter spp.	Piperacillin	18.5	25.1	18	15	14.5	10.5
	Ceftazidime	20.5	24.2	22.8	20.2	16.8	13.7
	Cefepime	1	0.5	1.3	2.1	1.7	0.6
	Cefpirome	3.9	4.3	5.3	3.7	3.4	1.5
	CP–SB	15.1	10.6	8.5	5.9	7.1	3.5
	Imipenem	0.5	0.5	0	0.5	0.1	0
Indole-positive *Proteae*	Piperacillin	8.7	8	6.3	5.5	6	4.9
	Ceftazidime	0.5	3	2.6	4.5	2	2.6
	Cefepime	0.5	0.5	0	1.4	1.2	0.2
	Cefpirome	3.1	0.5	0	0.6	1.9	0.8
	CP–SB	1.5	0	1.7	2.4	0.7	0.5
	Imipenem	1	5	0.9	3.3	0.7	0.6
Serratia spp.	Piperacillin	25	22.3	15	9.8	10.1	6.9
	Ceftazidime	9.5	6.8	8	7.1	3.7	2.5
	Cefepime	5	5.8	6.5	5.3	3.2	0.9
	Cefpirome	8.5	6.3	7.8	4.7	3.2	1.2
	CP–SB	23.5	16	14.2	10.9	6.1	4.9
	Imipenem	4.5	4.4	4.5	3.6	1.5	0.6
Acinetobacter spp.	Piperacillin	31.2	30.2	5.9	9.3	13.3	7.8
	Ceftazidime	8	4	4.5	5.8	6	7.7
	Cefepime	5	8	5.1	7.6	7	6.6
	Cefpirome	12.1	15	5.4	11.6	8.6	8.5
	CP–SB	0.5	0.5	0.3	1.5	0.8	0.7
	Imipenem	2.5	6.5	3.1	5	3.2	2.6
P. aeruginosa	Piperacillin	20.1	18.5	15.7	15	15.5	11.9
	Ceftazidime	11.4	8.7	10.8	12.3	9.9	8.7
	Cefepime	9.1	9.1	12.5	12.6	11.2	8.9
	Cefpirome	27.9	27.2	26	22.6	19.1	16.2
	CP–SB	13.7	11.5	13.2	12.5	14.9	9.8
	Imipenem	22.4	24.9	20.3	30.8	19.3	12.4

CP–SB = cefoperazone/sulbactam (2:1).