2 構造評価

構造に関するものは組織，人員，財源に関するものである。現在ではほとんどの病院に感染対策委員会が設置されているが，今後は病院のリスクマネジメント，医療安全，医療の質管理の面から，専従組織として機能する必要がある。院内感染も医療事故も1度起こすと病院の経営基盤を大きく揺るがすため，病院経営の危機管理としての重要性が高まっている。また，院内感染対策に関する指揮命令系統の確立や院内感染対策委員会の権限も重要である。規模が大きな病院になるほど診療科の縦割り管理が強くなり，院内感染のように横断的にしかも全組織をあげて取り組まなければならない活動は十分に機能することができない。したがって，院内感染に関するすべての権限は院内感染対策委員会に委譲する必要がある。特に重要なのは，隔離やCohorting，病棟や手術室の閉鎖に加えて，抗菌薬や消毒薬の採用，適正使用に関して，診療科の権限を超えた強力な権限が与えられる必要がある。また，院内感染対策に関する十分な教育や訓練を積んだ専門職が配置されているかが評価基準となる。院内感染に関する専門職教育は現在では日本看護協会による認定看護師として提供されているが，将来は病院のマネジメントや危機管理の一環として院内感染が専門職大学院の中に組み込まれることが必要となる。これらの構造評価に関するものは数値化して評価することは難しいため，通常は有無によって評価することになり，総合得点化して全体評価することも可能である。

3 プロセス指標

1）リスク調整感染率

プロセス管理として院内感染の発生率を比較するには，院内感染に関連すると証明されている交絡因子でリスク調整を行った後にその発生率を比較することが必要となる。リスク調整された院内感染の発生率はその施設の院内感染防止機能を反映し，施設間比較や施設間のばらつきの評価に使用される。したがって，一般病棟などケースミックスの場合にはリスク調整を行わないで単純な感染率を比較すると，時系列での比較研究や施設間比較も正確に評価することはできない。特に，ケースミックスでは従来から行われてきたデバイス装着日当たりの感染患者数を用いる方法ではリスク調整としては不十分である。

入院患者が院内感染を獲得するリスク因子としては患者自身に内在するもの（内部リスク因子）と医療環境に関するもの（外部リスク因子）が指摘されており，その内訳を表-2に示した。わが国の患者を対象として，これらのリスク因子の院内感染発生に対する寄与率を総合的に調査した研究はICU入室患者を対象としたもの以外にはない。

表-2 院内感染のリスク因子

リスクの種類	項　　目
内部リスク因子	年齢 性 原疾患 重症度 併存合併症
外部リスク因子	装着医療器具（CVカテーテル，人工呼吸，尿道カテーテル，ドレーン） 薬剤（抗菌薬，免疫抑制薬） 手術/処置 施設治療/看護能力 院内感染対策（衛生管理，マニュアル，サーベイランス，教育）

2) CDC/NNISのリスク調整

アメリカではCDC/NNISが院内感染サーベイランス初期に全入院患者を対象としたサーベイランスを実施した。その結果，院内感染のタイプと頻度から，CR-BSI（カテーテル関連血流感染），人工呼吸器関連肺炎，尿道カテーテル関連尿路感染，術後創感染の頻度が高いことが判明した。また，ICU，NICU，手術（術後創感染）が患者転帰（医療コストを含む）に与える影響が最も大きな領域であることが確定されたため，この領域に限定した監視サーベイランスを継続的に行うこととした。このCDC/NNISサーベイランスでは，内部リスク因子はデバイス利用率によって一部反映されているが，原疾患や重症度などの内部リスク因子は考慮されていない。このため，外部リスク因子としての延べデバイス日によってリスク調整されて，施設間比較が行われている。しかし，アメリカのICUは，冠動脈疾患ICU，脳外科ICU，心臓外科ICU，小児ICU，呼吸器疾患ICUなどと疾患ごとに分かれており，それぞれのICUでの患者の内部リスク因子は似ている。つまり，ICUを対象としたCDC/NNISの感染率は，ICUが機能分化していることで内部リスク因子がある程度調整されており，そのうえで外部リスク因子（延べ器具装着日）が考慮されて比較されていることになる。

3) わが国のリスク調整感染率のあり方

わが国では一般病棟で感染率を測定する場合はCDC/NNISと同様の方法でデバイス装着日数のみでリスク調整がされていることが多いが，このリスク調整のみで十分か否かの検討はわが国の入院患者を対象としては行われていない。唯一，ICUにおいてはAPACHEスコアを用いて重症度分類が行われ，ICUでの院内感染獲得に関する種々のリスク因子の検討が行われてきている[3]。厚生労働省院内感染対策サーベイランス事業

ICU部門で収集したデータに基づいてリスク因子を解析した結果は表-3のごとくである。

表-3に示すように，ICU内での感染症獲得に関するリスク因子は術後以外の理由での入室，APACHEスコア，緊急手術，人工呼吸器の使用であった。デバイス使用期間がリスク因子かどうかの検討はされていないが，人工呼吸器の使用以外にも前記のリスク因子が存在することが証明されている。

また，一般的には患者が重症であれば院内感染を獲得する確率が高いといわれているが，それを統計学的に証明したデータは少なくてもわが国には存在しない。ICUにおいては入室時の重症度に応じて経日的な院内感染の獲得率は異なることが証明されている（図-1）。したがって，重症度によるリスク調整はより詳細な調整が必要となる。

図-1に示すように，重症例ではICU在室日数に応じて感染率が低下するのに対して，軽症例では在室17日まで感染率が上昇する。入室後0～2日の感染症は厳密には院内感

表-3 ICUで獲得した感染症に対する調整ハザード比と95%信頼区間（ダミー変数を使用しない場合）

	ハザード比（95%信頼区間）
ICU	
術後患者49%以下	0.78（0.62-0.97）
術後患者50-79%	1.00（reference）
術後患者80%以上	1.46（1.22-1.75）
性（対男性）	0.77（0.65-0.91）
年齢	
16-44歳	1.00（reference）
45-54歳	0.78（0.58-1.07）
55-64歳	0.92（0.70-1.20）
65-74歳	0.93（0.72-1.19）
75歳以上	0.84（0.64-1.10）
APACHEスコア	
0-10	1.00（reference）
11-20	1.67（1.34-2.09）
21以上	2.52（1.99-3.19）
手術	
なし	1.00（reference）
待機手術	0.95（0.76-1.18）
緊急手術	1.24（1.03-1.50）
デバイス	
人工呼吸器	1.70（1.28-2.28）
中心静脈カテーテル	1.16（0.87-1.54）
尿路カテーテル	1.20（0.77-1.88）

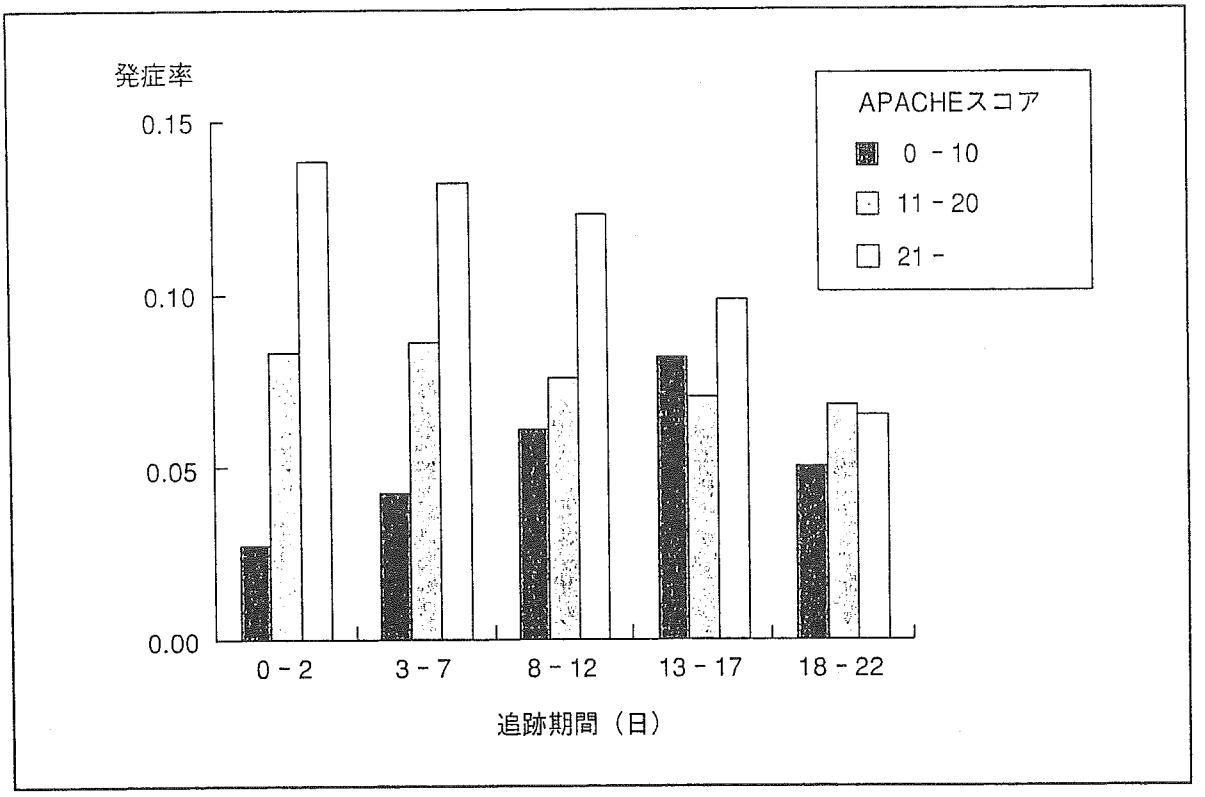

図-1 重症度によるICUでの経日的院内感染獲得率

染ではないが，この間に感染症を発生した患者も検討対象に加えてある。したがって，デバイス装着日でリスク調整しても，入室時の重症度によって院内感染獲得率が違うとすれば，重症度を考慮に入れたリスク調整が必要となる。これが一般病棟となれば，原疾患，並存症，重症度などのリスク因子がデバイス装着日に加わるため，感染率の解析·評価は困難を極める。したがって，ICUも含めて入院患者での院内感染の獲得に関するリスク因子は十分に検討されているとは言いがたく，そのため，デバイス装着日だけでリスク調整された院内感染発生率を用いた施設間比較や経時的変化によるインターベンションの効果の評価には大きな限界がある。

4 成果指標

院内感染の成果（アウトカム）指標としての検討は，わが国においては厚生労働省院内感染対策サーベイランス事業ICU部門において重点的に行われてきた[4]。ICU退室患者の病院死亡に関するリスク因子は表-4に示した。退院時死亡に影響を与えるリスク因子としては年齢75歳以上，APACHEスコアによる重症度，人工呼吸器の使用，中心静脈ラインの使用，多剤耐性菌による感染であり，これらの因子が退院時死亡を有意に上昇させていた。また，待機か緊急かにかかわらず，術後にICUに入室した場合は逆に退院時転帰を改善させていた。つまり，術後以外の理由でICU入室した患者で

表-4 ICUで獲得した感染症が退院時転帰に及ぼす影響

性（対男性）	1.06（0.95－1.19）
年齢[1]	
45－54	1.19（0.94－1.49）
55－64	1.06（0.85－1.31）
65－74	1.11（0.91－1.35）
75－	1.33（1.09－1.62）
APACHEスコア[2]	
11－15	1.68（1.37－2.06）
16－20	2.66（2.18－3.25）
21－25	4.28（3.48－5.27）
26－30	5.92（4.76－7.37）
31－	7.88（6.23－9.97）
待機手術	0.29（0.24－0.34）
緊急手術	0.68（0.59－0.77）
人工呼吸器	1.78（1.49－2.12）
中心静脈カテーテル	1.23（1.04－1.47）
尿道カテーテル	0.70（0.54－0.90）
ICU内獲得感性菌感染症	1.11（0.94－1.31）
ICU内獲得耐性菌感染症	1.42（1.15－1.77）

1）45歳未満を基準にした
2）0－10を基準にした

退院時転帰が悪化していた。

また，APACHE Ⅱを用いた重症度分類でリスク調整し，ICU入室患者で感染症なしを基準とした場合の退院時死亡のオッズ比を表したのが表-5である。感性菌による感染も多剤耐性菌による感染も退院時転帰とICU退室時転帰を有意に悪化させていることがわかる。多剤耐性菌による感染はその影響がさらに強く現れることが判明している。

さらにICUで獲得した院内感染の在院日数とICU在室日数を比較したのが表-6である。ICUで院内感染を獲得すると在院日数もICU在室時間も有意に延長するが，多剤耐性菌によって感染を獲得するとその影響はさらに強くなることが判明している。

ICU以外の一般病棟においても同じような傾向があると推察できるが，一般病院のケースミックスを対象とした退院時転帰に関する検討はなされていない。したがって現状では院内感染に関する成果指標はわが国ではICU以外には存在しない。院内感染に関する成果指標が必要であるとしても，それをどのように指標化するかに関しては今後さらなる検討が必要となる。以上のような，厚生労働省院内感染対策サーベイランス事業ICU部門に参加すると，上記のような全国平均値と比較した参加施設の機能評価を行うことができる。

表-5 ICUで獲得した感染の退院時死亡とICU死亡に及ぼす影響

	オッズ比	95%CI
退院時死亡		
感性菌感染	1.4	1.2 - 1.6
耐性菌感染	1.9	1.5 - 2.3
ICU内死亡		
感性菌感染	1.4	1.1 - 1.7
耐性菌感染	2.0	1.6 - 2.9

表-6 ICUで獲得した感染の在院日数とICU在室日数に及ぼす影響

在院日数	日 数	95%CI
感染症なし	49.1	47.1 - 51.0
感性菌感染	61.1	56.4 - 65.8
耐性菌感染	76.7	66.5 - 86.9
ICU在室時間	時 間	95%CI
感染症なし	169.8	164.4 - 175.3
感性菌感染	335.5	321.9 - 349.2
耐性菌感染	394.8	370.9 - 418.7

5 当面の成果指標算出のあり方

すでに述べてきたように，わが国に限らず，院内感染に関してはリスク調整されたプロセス指標も成果指標も十分には確定されてはいない。したがって，リスク調整された臨床指標を必要とするのであれば，今まで判明したリスク因子に加えて，可能性のあるリスク因子を網羅した患者·病院情報を集積し，院内感染の獲得および患者転帰に関する総合的リスク調整が必要となる。現実的には包括評価の枠組みを利用して，年齢，性，原疾患，並存症，合併症，重症度，デバイス，抗菌薬などを加味したリスク因子関連情報の収集と院内感染の獲得，および退院時患者転帰への影響を検討し，予後予測式を確定する必要がある。また，その予測式の適合性を新たな患者集団を用いて検証する必要がある。わが国では2003年度から特定機能病院を対象として包括評価が導入されている。包括評価は基本的には診療報酬支払いシステムであるが，その中には臨床指標に利用することができる患者情報が含まれている。2004年度からは中央社会保険医療協議会DPC評価分科会でDPC収集データの中に医療の質と安全に関する臨床指標が加えられて収集されることになる。院内感染に関する臨床指標もそのデータを利用して確立されることが望まれる。DPCで収集される院内感染に関する情報とDPCに直接には関連しないために，DPCと別枠で収集することが必要なデータを表-7に示した。

今後は，臨床指標は手入力なしに，電子化された病院間患者情報システムから直接に収集，集計，解析され，その評価が医療機関や国民に開示されることになると思われる。

表-7　院内感染の臨床指標を確定するためのデータ収集システム

指　標	DPC関連情報	DPC非関連情報
構造評価指標	感染認定看護師数	Closed/Open ICU
プロセス評価指標	年齢，性，病名（ICD-10），手技・手術（Kコード），並存症，合併症，重症度（外保連A-E分類）入院ー手術期間	抗菌薬投与歴（DDD），デバイス装着日，起炎菌（耐性・感性）
成果評価指標	在院日数，退院時転帰，退院先，医療費	患者満足度

注）2004年から収集されることが予定されているDPCの様式3のデータを含む。

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Extended-Spectrum-β-Lactamase-Producing *Escherichia coli* Strains Isolated from Farm Animals from 1999 to 2002: Report from the Japanese Veterinary Antimicrobial Resistance Monitoring Program

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A nationwide surveillance for antimicrobial susceptibility in *Escherichia coli* strains isolated from food-producing animals in Japan was conducted from 1999 to 2002. Eighteen cefazolin-resistant *E. coli* strains were isolated from broilers. Six were CTX-M-type producing, and eight were CMY-2 producing, while eight had mutations at the *ampC* promoter region.

Recently, the relationship between the use of antimicrobials in food-producing animals and the emergence of resistant bacteria in the food chain has become of great concern and has been the subject of numerous international meetings (6, 11, 12). However, until recently there was a lack of nationwide information available on antimicrobial resistance of bacteria isolated from animal origins. Consequently, we established the Japanese Veterinary Antimicrobial Resistance Monitoring program in 1999 (9).

In Japan, CTX-M-type extended-spectrum-β-lactamase (ESBL)-producing *Enterobacteriaceae* are important in nosocomial infections. Yagi et al. reported that Toho-1-like ESBLs were the most prevalent type of ESBL in clinical isolates of *Escherichia coli* (13, 14). The aim of this study was to characterize cephalosporin-resistant *E. coli* strains recovered from healthy animals and especially to investigate isolates resistant to ceftiofur, an expanded-spectrum cephalosporin used in animals.

Fresh fecal samples were collected from healthy farm animals. In principle, one fecal sample per farm was collected and two *E. coli* isolates from each sample were kept using desoxycholate-hydrogen sulfate-lactose agar. Overall, a total of 2,747 isolates (872 isolates from 453 cattle farms, 793 isolates from 417 pig farms, 406 isolates from 219 layer farms, and 676 isolates from 354 broiler farms) were collected during 4 years (1999 to 2002).

MICs were determined by the agar dilution method (4, 5). The cefazolin MIC for 18 isolates from 12 broiler farms was ≥32 μg/ml, and these isolates were further investigated in this study. The MICs of 19 antibiotics for the 18 cefazolin-resistant isolates are shown in Table 1. The resistance profiles of isolates collected from the same farm were always identical to each other, suggesting that those isolates were likely replicates. Six isolates from four farms were also resistant to ceftiofur, cefpodoxime, cefotaxime, and cefepime while retaining susceptibility to cefoxitin. A double-disk synergy test for detection of ESBLs, carried out as described previously (3), revealed synergy between clavulanate and cefotaxime, ceftadizime, cefpodoxime, or aztreonam disks (Nissui Pharmaceutical, Co., Ltd, Tokyo, Japan) with these six isolates, suggesting production of an ESBL (Table 2). The remaining 12 isolates exhibited increased cefoxitin MICs while retaining very low cefepime MICs, suggesting the production of a class C β-lactamase. Double-disk synergy testing yielded negative results with these isolates (Table 2).

Detection of several β-lactamase genes, including bla_{TEM}, bla_{SHV}, bla_{PSE-1}, $bla_{CTX-M-2}$, $bla_{CTX-M-9}$, bla_{CMY-1}, bla_{CMY-2}, and bla_{FOX}, and amplification of the promoter region of the *ampC* gene were carried out by PCR (94°C for 3 min; 30 cycles of amplification at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and 72°C for 7 min) using primers listed in Table 3. Nucleotide sequences were determined on both strands, directly on PCR products. The DNA alignments and deduced amino acid sequences were examined using the BLAST program (1). Mutations in the *ampC* promoter region were defined in comparison with *E. coli* K-12 strain LA5 (7).

The results of β-lactamase gene detection and analysis of the *ampC* promoter region in the cefazolin-resistant isolates are reported in Table 2. Acquired β-lactamase genes were detected in most isolates. CTX-M-2 or CTX-M-18 β-lactamase genes were detected in the six ceftiofur-resistant isolates, in agreement with the resistance phenotype. The four CTX-M-2-producing *E. coli* strains were isolated from three different farms (farm F is 500 km away from farm G and 400 km away from farm K; farm G is 100 km away from farm K) in different years. A CMY-2 β-lactamase gene, alone or in combination with bla_{TEM-1} or bla_{PSE-1}, was detected in eight of the other

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TABLE 1. MICs of various antimicrobial agents for 18 cefazolin-resistant field strains isolated from 1999 to 2002

Antimicrobial	MIC (μg/ml) for isolate(s):											
	11-C-217 and 11-C-218 (Form A, 1999)	12-C-015 and 12-C-016 (Form B, 2000)	12-C-129 (Form C, 2000)	12-C-139 (Form D, 2000)	12-C-140 and 12-C-141 (Form E, 2000)	13-C-005 and 13-C-006 (Form F, 2001)	13-C-033 (Form G, 2001)	13-C-034 (Form H, 2001)	13-C-073 (Form I, 2001)	13-C-099 and 13-C-100 (Form J, 2001)	14-C-020 (Form K, 2002)	14-C-079 and 14-C-080 (Form L, 2002)
Amoxicillin	>512	>512	256	>512	>512	>512	>512	>512	256	>512	>512	>512
Cefazolin	>512	>512	>512	256	>512	>512	>512	>512	128	128	>512	>512
Ceftiofur	16	8	8	8	16	>512	>512	16	1	1	>512	>512
Ceftazidime	32	16	16	8	16	2–4	4	16	4	2	4	1
Cefotaxime	4–8	2–4	8	16	8	>512	>512	8	8	1	>512	>512
Cefpodoxime	128	128	128	64	128	>512	>512	256	32	16	>512	>512
Cefepime	≤0.25	≤0.125	≤0.125	≤0.125	≤0.125	32–64	64	≤0.125	≤0.125	≤0.125	>512	>512
Cefoxitin	64	64	64	128	128	4	4	128	32	16	4	4
Moxalactam	0.5	0.25	0.25	0.5	1	≤0.125	≤0.125	0.5	0.5	≤0.125	≤0.125	≤0.125
Aztreonam	8	4	4	8	8	16	16	4	4	2	16	8–16
Imipenem	0.25	0.25	0.25	0.25	≤0.125	≤0.125	≤0.125	0.25	0.25	≤0.125	≤0.125	0.25
Amoxicillin-clavulanic acid[b]	32/16	32/16	32/16	32/16	32/16	8/4	8/4	32/16	32/16	32/16	8/4	8/4
Kanamycin	2	256	4	16	2	128	2	256	2	256	1	512
Dihydrostreptomycin	>512	>512	2	2	>512	256	128	>512	2	256	64	128
Oxytetracycline	256	256	1	256	>512	256	1	256	256	256	256	256
Nalidixic acid	4	4	4	512	4	512	4	2	32	128	2	4
Enrofloxacin	≤0.125	≤0.125	≤0.125	16	0.25	0.25	≤0.125	≤0.125	≤0.125	0.25	≤0.125	≤0.125
Chloramphenicol	512	128	8	512	512	512	256	256	4	4	8	8
Sulfadimethoxine	512	512	512	512	512	≥512	512	512	512	512	512	≥512

[a] Isolates from the same form always exhibited the same resistance pattern.
[b] Combination of amoxicillin and clavulanic acid in a ratio of 2 to 1.

TABLE 2. Phenotypes and β-lactamase genotypes of 18 cefazolin-resistant *E. coli* field isolates

Isolate(s)	Farm	Double-disk synergy test result	*bla* gene(s) detected	Mutation(s) at *ampC* promoter region	Frequency of cefazolin resistance transfer
11-C-217 and 11-C-218	A	−	bla_{CMY-2}	−18, −1, and +58	1.0×10^{-8}
12-C-015 and 12-C-016	B	−	bla_{IEM-1} and bla_{CMY-2}	No mutation	1.5×10^{-4}–2.6×10^{-4}
12-C-129	C	−	bla_{CMY-2}	No mutation	3.5×10^{-3}
12-C-139	D	−	bla_{TEM-1}	−42, −18, −1, and +58	Not transferred
12-C-140 and 12-C-141	E	−	bla_{CMY-2}	−18, −1, and +58	3.2×10^{-4}–4.3×10^{-4}
13-C-005 and 13-C-006	F	+	$bla_{CTX-M-2}$	No mutation	1.5×10^{-3}–4.7×10^{-3}
13-C-033	G	+	$bla_{CTX-M-2}$	No mutation	2.2×10^{-6}
13-C-034	H	−	bla_{PSE-1} and bla_{CMY-2}	No mutation	8.9×10^{-5}
13-C-073	I	−	Not detected	−42, −18, −1, and +58	Not transferred
13-C-099 and 13-C-100	J	−	bla_{IEM-1}	−42, −18, −1, and +58	Not transferred
14-C-020	K	+	$bla_{CTX-M-2}$	No mutation	2.6×10^{-6}
14-C-079 and 14-C-080	L	+	$bla_{CTX-M-18}$	No mutation	3.7×10^{-4}–4.3×10^{-6}

isolates, in agreement with the resistance phenotype. In the remaining four isolates, either a bla_{TEM-1} gene or none of the acquired β-lactamase genes searched in this work was detected. In these isolates, however, mutations at positions −42 (C→T), −18 (G→A), −1 (C→T), and +58 (C→T) were detected. Though we did not perform enzyme expression experiments, mutations at these points could be associated with AmpC hyperproduction (2) and thus explain the resistance phenotype.

Conjugation experiments were carried out as described previously (15) using a rifampin-resistant mutant of *E. coli* INVαF′ (Invitrogen Corp. Carlsbad, CA) generated in our laboratory. Transconjugants were selected on LB agar (Difco Laboratories, Detroit, MI) containing rifampin (50 μg/ml) and cefazolin (50 μg/ml). Cefazolin-resistant transconjugants were obtained from 14 isolates, including those producing CTX-M-type and CMY-2 enzymes. Resistance profiles of the transconjugants were consistent with transfer of a CTX-M-type or CMY-2 β-lactamase gene, respectively (Table 4). The presence of the respective β-lactamase genes was confirmed in all transconjugants by PCR analysis with primers encoding CTX-M types or CMY-2. The transfer of resistance traits to non-β-lactam agents was also observed in most cases (Table 4), suggesting that additional resistance genes were cotransferable with the β-lactamase genes.

Plasmid restriction profiles of the six CTX-M-producing transconjugants are shown in Fig. 1. Restriction profiles of plasmids carrying the same type of bla_{CTX-M} gene were identical or similar to each other, suggesting a common origin. On the other hand, restriction profiles of plasmids carrying different types of CTX-M determinants were remarkably different from each other.

TABLE 3. Primers used for PCR and DNA sequencing

Target	Nucleotide sequence (5′ → 3′)	Accession no.	Positions
bla_{IEM}	ATGAGTATTCAACATTTTCG TTACCAATGCTTAATCAGTG	AB194682	215–234 1075–1066
bla_{SHV}	ATGCGTTATATTCGCCTGTG TTAGCGTTGCCAGTGCTCGA	AF148850	6–25 866–847
bla_{PSE-1}	ATGCTTTTATATAAAATGTG TCAGCGCGACTGTGATGTAT	AB126603	150–169 1064–1045
$bla_{CTX-M-2}$	ATGATGACTCAGAGCATTCG TCAGAAACCGTGGGTTACGA	AY750915	1–20 876–857
$bla_{CTX-M-9}$	ATGGTGACAAAGAGAGTGCAACGG TCACAGCCCTTCGGCGATGATTCT	AJ416345	132–155 1007–984
bla_{CMY-1}	ATGCAACAACGACAATCCATCCTG TCAACCGGCCAACTGCGCCAGGAT	X92508	333–356 1481–1458
bla_{CMY-2}	ATGATGAAAAAATCGTTATGCT TTATTGCAGCTTTTCAAGAATGCG	X91840	1924–1945 3069–3046
bla_{FOX}	ATGCAACAACGACGTGCGTTCGCG TCACTCGGCCAACTGACTCAGGAT	X77455	701–724 1849–1826
$bla_{frdD-ampC}$	ATGATTAATCCAAATCCAAAGCGT CAAATGTGGAGCAAGAGGCGGTAA	U14003	70194–70171 69718–69741

TABLE 4. MICs of various antimicrobial agents for transconjugants and recipient strain

Antimicrobial	MIC (μg/ml) for transconjugant(s)[a]									
	217-1 and 218-3[b]	15-7 and 16-2[b]	129-2[b]	140-1 and 141-3[b]	5-4 and 6-8[c]	33-13[c]	34-5[b]	20-5[c]	79-6 and 80-12[d]	*E. coli* INVαF′ -Rif[r]
Amoxicillin	128	128–>512	128	128	>512	>512	256	>512	>512	2
Cefazolin	128	128	128	128	>512	>512	256	>512	>512	0.5
Ceftiofur	8	8	8	8	256	256	8	128	128	≤0.125
Ceftazidime	8	8	8	8	2–4	1	8	4	1	≤0.125
Cefotaxime	4	4	4	4	128	32	4	32	32	≤0.125
Cefpodoxime	128	128	128	128	512	256	128	256	256	0.25
Cefepime	≤0.125	≤0.125	≤0.125	≤0.125	16–32	4	≤0.125	8	4	≤0.125
Cefoxitin	32	16–32	32	32	2	2	32	8	2	2
Moxalactam	0.25	0.25	0.25	0.25	≤0.25	≤0.125	0.25	≤0.125	≤0.125	≤0.125
Aztreonam	1	1	1	1	16–32	8	1	8	4	≤0.125
Imipenem	0.25	0.25	0.25	0.25	≤0.25	0.25	0.25	≤0.125	0.25	≤0.125
Amoxicillin-clavulanic acid	32/16–64/32	64/32	64/32	64/32	16/8	16/8	64/32	16/8	16/8	2/1
Kanamycin	0.5	0.5	0.5	0.5	0.25–0.5	0.25	512	0.25	0.25–0.5	0.25
Dihydrostreptomycin	128	128–256	0.5	128	64	64	128	64	0.5	0.5
Oxytetracycline	64	64–128	0.5	64	128	0.5	64	256	0.5–2	0.5
Nalidixic acid	32	32	32	32	32	32	32	64	32	32
Chloramphenicol	128	128	4	128	2	2	128	2	2–4	2
Sulfadimethoxine	>512	>512	32	>512	>512	>512	>512	>512	16	16
Rifampicin	>512	>512	>512	>512	>512	>512	>512	>512	>512	>512

[a] Transconjugants were derived from following field isolates: 217-1 and 218-3 were 11-C-217 and 11-C-218; 15-7 and 16-2 were 12-C-015 and 12-C-016; 129-2 was 12-C-129; 140-1 and 141-3 were 12-C-140 and 12-C-141; 5-4 and 6-8 were 13-C-005 and 13-C-006; 33-13 was 13-C-033; 34-5 was 13-C-034; 20-5 was 14-C-020; and 79-6 and 80-12 were 14-C-079 and 14-C-080, respectively.
[b] CMY-2 producer.
[c] CTX-M-2 producer.
[d] CTX-M-18 producer.

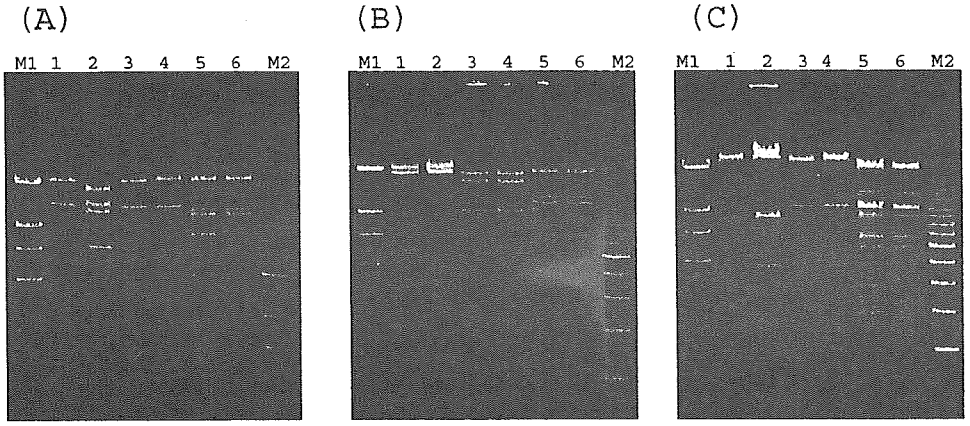

FIG. 1. Restriction profiles of plasmids from CTX-M-producing transconjugants digested with ClaI (A), EcoRI (B), and SphI (C). The plasmids shown in lanes 1 (plasmid pC5-4; 65.1 kbp), 2 (pC6-8; 68.1 kbp), 3 (pC33-13; 66.8 kbp), 4 (pC20-5; 67.4 kbp), and 5 and 6 (pC79-6 and pC80-12; 97.3 kbp) were derived from field isolates 13-C-005, 13-C-006, 13-C-033, 14-C-020, 14-C-079, and 14-C-080, respectively. M1 and M2, lambda DNA digested with HindIII marker and 1-kb DNA ladder marker, respectively (Takara Bio Inc., Shiga, Japan).

In our survey, cefazolin-resistant *E. coli* strains were isolated only from broilers. In Japan, six cephalosporins are approved for parenteral use, but in cattle and pigs only. Some reports discuss the relationship between the use of ceftiofur and the appearance of resistant strains in cattle and/or pigs (8, 10). However, our results suggest that the use of the expanded-spectrum cephalosporins in healthy animals at the farm level does not directly influence the appearance of resistant strains. For some reason, ESBL-producing *E. coli* strains were only isolated from broilers and not layers, which suggests there might be some other factor, possibly in their specific environment, that introduces the plasmids encoding CTX-M-type ESBLs into *E. coli* during the husbandry of broilers.

In conclusion, we report on the emergence of extended-spectrum class A and class C β-lactamases in *E. coli* strains from healthy broilers. Even if at present there is a low level of isolation in food-producing animals, it is necessary to monitor the spread of expanded-spectrum cephalosporin-resistant bacteria and further research including animals and humans and their environments should be carried out.

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Metallo-β-lactamase IMP-1 in *Providencia rettgeri* from two different hospitals in Japan

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In 2002, 495 indole-positive proteae strains were isolated from patients at 60 hospitals in Japan. Nine indole-positive proteae strains had reduced susceptibility to imipenem (MIC ≥ 8 μg ml^{-1}) and were identified as *Providencia rettgeri* by BD Phoenix. Eight of the nine *Prov. rettgeri* isolates were confirmed as metallo-β-lactamase producers by the double-disc synergy test. All the metallo-β-lactamases were classified as IMP-1 by PCR and DNA sequence analysis. These bla_{IMP-1} genes were encoded in the integron structure on conjugative plasmids. These plasmids could transfer from *Prov. rettgeri* clinical isolates to *Escherichia coli* ML4903 at a frequency between $1{\cdot}5 \times 10^{-5}$ and $5{\cdot}5 \times 10^{-7}$. The eight bla_{IMP}-positive strains were isolated from two hospitals, and showed two different PFGE patterns, two different integron structures and two different incompatibility groups, which corresponded to the two hospitals. These results strongly suggest the possibility of nosocomial infections by bla_{IMP-1}-producing *Prov. rettgeri* isolates.

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INTRODUCTION

Proteae are normal inhabitants of the gut of animals including humans and are also found in the environment. Proteae were ranked as the fourth and fifth leading cause of urinary-tract infections in Europe and North America, respectively, in 1997 (Jones *et al.*, 1999; Fluit *et al.*, 2000). Normally, *Proteus mirabilis*, as the only indole-negative proteus, has a high susceptibility to antibiotics except for nitrofurantoin. Indole-positive proteae, such as *Proteus vulgaris*, *Proteus penneri*, *Providencia alcalifaciens*, *Providencia rettgeri*, *Providencia stuartii* and *Morganella morganii*, are susceptible to expanded-spectrum cephalosporins, cefoxitin, cefepime, aztreonam, imipenem and aminoglycosides (Murray *et al.*, 2003).

Nosocomial infections caused by extended-spectrum β-lactamase (ESBL)-producing indole-positive proteae have been reported (Ma *et al.*, 2002; Pagani *et al.*, 2003; Tumbarello *et al.*, 2004) in which the strains were resistant to most β-lactams including expanded-spectrum cephalosporins. ESBL-producing *Providencia* species and *Prot. vulgaris* that produced CTX-M-type or SHV-type enzymes remained susceptible to carbapenems including imipenem. Tumbarello *et al.* (2004) described ESBL-producing multidrug-resistant *Prov. stuartii*. The strains were resistant to penicillins, cephalosporins, aminoglycosides and fluoroquinolones, but were susceptible to imipenem.

Shibata *et al.* (2003) reported metallo-β-lactamase (MβL)-producing *Prov. rettgeri* and *M. morganii* that produced the IMP-1 enzyme. In the case of IMP-1-producing *Pseudomonas aeruginosa* and *Serratia marcescens*, the strains showed resistance to carbapenems such as imipenem, meropenem, panipenem, biapenem and doripenem; however, the results of drug susceptibility testing were not described. IMP-1 producers have no susceptibility to any β-lactams except for aztreonam. The genes that encode these MβLs are located in an integron structure on a plasmid (Arakawa *et al.*, 1995). An integron is one of the genetic elements capable of integrating gene cassettes by a site-specific recombination mechanism (Fluit & Schmitz, 2004). Therefore, horizontal spread of these resistance determinants can be anticipated.

In 2002, we conducted a surveillance programme involving 60 hospitals that were widely distributed geographically throughout Japan. The aim of the study was to gain a detailed understanding of β-lactam antibiotic susceptibility data (Ishii *et al.*, 2005). Of 495 indole-positive proteae isolates, nine, from two hospitals, showed reduced susceptibility to imipenem (MIC ≥ 8 μg ml^{-1}). The aim of this study was to characterize the imipenem-resistance mechanism(s) and to investigate the possible clonal origins of the isolates.

Abbreviations: DDST, double-disc synergy test; ESBL, extended-spectrum β-lactamase; MβL, metallo-β-lactamase.

METHODS

Bacterial strains. In 2002 a total of 495 single strains of indole-positive proteae were isolated from patients at 60 hospitals in Japan that were widely distributed geographically (Ishii *et al.*, 2005). Each participating laboratory performed its own identification tests. The species and number of isolated strains are listed in Table 1. Sources of the specimens isolated are described in Table 2.

Antimicrobial susceptibility testing. Susceptibility testing of each isolate was performed by Etest strip (AB Biodisk) following the manufacturer's instruction manual. All clinical laboratories used Etest strips with the same lot number. The non-susceptible break point of indole-positive proteae against imipenem (MIC $\geqslant$ 8 μg ml^{-1}) was based on that defined by the Clinical and Laboratory Standards Institute, formerly known as the National Committee for Clinical Laboratory Standards (NCCLS). Quality control of Etest strips was performed using the following reference strains: *Staphylococcus aureus* ATCC 21293, *Escherichia coli* ATCC 25922 and *Ps. aeruginosa* ATCC 27853. In addition, identification and susceptibility of all isolates collected were re-evaluated at the Department of Microbiology and Infectious Diseases, Toho University School of Medicine using the BD Phoenix system (Becton Dickinson).

Screening of MβL producers. Strains selected by the criteria described above were subjected to a screening test for MβL production by using the double-disc synergy test (DDST) reported by Arakawa *et al.* (2000). The test was performed by placing a SMA (sodium mercaprotoacetic acid) disc containing 3 mg sodium mercaptoacetic acid (Eiken) and two commercially supplied Kirby–Bauer (KB) discs, each containing 30 μg ceftazidime or 10 μg imipenem (Eiken), on Mueller–Hinton agar plates. The distance between the two ceftazidime and imipenem discs was kept at about 3 cm, and the SMA disc was placed near one of the discs with a centre-to-centre distance of 1·5–2·0 cm. The plates were then incubated at 35 °C for 16–18 h. If the inhibition zone around the disc nearer to the SMA disc was bigger by more than 5 mm than that of ceftazidime or imipenem alone, the strain was considered to be an MβL producer.

Table 1. Species of indole-positive proteae collected

Species*	No. (%) of isolates
Morganella morganii	272 (54·9)
Proteus vulgaris	127 (25·7)
Providencia rettgeri	62 (12·5)
Providencia stuartii	7 (1·4)
Unspeciated†	27 (5·5)
Total	495 (100)

*These results were reported by each hospital.

†Reported only as *Proteus* spp. by each hospital.

Table 2. Details of clinical specimens of indole-positive proteae

Source	No. (%) of isolates
Urinary tract	213 (43·0)
Gastrointestinal tract	76 (15·4)
Respiratory tract	
Upper	14 (2·8)
Lower	40 (8·1)
Skin	23 (4·7)
Blood	17 (3·4)
Other	112 (22·6)
Total	495 (100)

PCR and sequencing of MβL genes. The MβL gene cassettes and integrons were detected by a PCR method using previously described specific primer sets for bla_{IMP}, bla_{VIM}, bla_{SPM}, *intI1*, *intI2* and *intI3* (Table 3). Template DNA from original strains and their transconjugants was used. PCR was performed in a GeneAmp PCR system 2400 thermal cycler (Applied Biosystem). The thermocycle protocol used was: an initial denaturation step at 94 °C for 2 min, followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 90 s, plus a final extension step at 72 °C for 7 min. The resulting PCR product was purified with QIAquick PCR purification kit (Qiagen), prepared with ABI Prism Big Dye Terminator version 3.1 cycle sequencing ready reaction kit (Applied Biosystems) and sequenced with the automatic sequencer ABI Prism 310 genetic analyser (Applied Biosystems) using sequence specific primers for bla_{IMP} and *aacA4*, one of the aminoglycoside acetyltransferases. A similarity search for the deduced amino acid sequences against sequence databases was done using the BLAST program at the DNA Database of Japan (Shizuoka, Japan).

Conjugation experiments. Conjugation experiments were performed by the broth method using rifampicin-resistant *E. coli* ML4903 as a recipient (Ishii *et al.*, 1995). A 0·9 ml : 0·1 ml mixture of exponentially growing donor and recipient isolates was incubated in 1 ml Mueller–Hinton broth at 35 °C for 1 h. *E. coli* transconjugants were selected on Drigalsky agar medium (BTB Agar, Eiken) containing ceftazidime (5 μg ml^{-1}) and rifampicin (25 μg ml^{-1}). Frequency of transfer was expressed as the number of transconjugants per number of donors. Susceptibility testing for donor, recipient and transconjugants was performed by the microdilution method according to the Clinical and Laboratory Standards Institute, formerly known as the NCCLS, document M7-A6 (NCCLS, 2003). Incompatibility tests were carried out as described in a previous report (Chabbert *et al.*, 1972; Ishii *et al.*, 1995). Briefly, the transconjugant was used as a donor and mixed with a recipient *E. coli* C600 that harboured plasmids of a known incompatibility group (Ishii *et al.*, 1995). The conditions used were the same as for the conjugation experiments described above. Transconjugants were selected for resistance to ceftazidime and the resistance marker drugs of each plasmid.

Plasmid elimination. Plasmid elimination was performed to prove that the resistance gene was on a transferable plasmid. The transconjugants of TUM1933 and TUM1936 were used as a recipient, and mixed with a donor *E. coli* C600 that harboured a plasmid with the same incompatibility group as the recipient. Conjugation conditions were the same as those mentioned above. Transconjugants were selected for resistance to rifampicin and the resistance marker drugs of each plasmid.

PFGE. PFGE analysis was performed with a modified version of the instruction manual from Bio-Rad Laboratories. Agarose plugs containing genomic DNA were digested with *SfiI* (Bio-Rad Laboratories). Fragments were separated using SeaKem Gold Agarose gel (FMC Bioproducts) in 0·5 TBE buffer (0·089 M Tris base, 0·089 M boric acid and 0·002 M EDTA) at 14 °C for 20 h on a CHEF Mapper apparatus (Bio-Rad Laboratories). The banding patterns were evaluated by using Finger printing II DST software (Bio-Rad Laboratories) with Dice and UPGMA coefficients (Mariani-Kurkdjian *et al.*, 2004). Isolates with a genetic similarity of > 80 % according to dendrogram results were considered to be from the same origin.

RESULTS

Nine of 495 indole-positive proteae clinical isolates were not susceptible to imipenem (MIC $\geqslant$ 8 μg ml^{-1}) by Etest strip

Table 3. PCR primers

Gene	Primer sequence (5′–3′)	Tm (°C)	Expected size of amplicon (bp)	Reference
MβL genes				
bla_{IMP}	F1 CTACCGCAGCAGAGTCTTTG	62·7	587	Kimura *et al.* (2005)
	R1 AACCAGTTTTGCCTTACCAT	59·9		
bla_{IMP-2}	F2 GTTTTATGTGTATGCTTCC	51·8	678	Shibata *et al.* (2003)
	R2 AGCCTGTTCCCATGTAC	55·6		
bla_{VIM-1}	F3 AGTGGTGAGTATCCGACAG	57·5	261	Shibata *et al.* (2003)
	R3 ATGAAAGTGCGTGGAGAC	58·7		
bla_{VIM-2}	F4 ATGTTCAAACTTTTGAGTAAG	52·7	801	Shibata *et al.* (2003)
	R4 CTACTCAACGACTGAGCG	57		
bla_{SPM-1}	F5 GCGTTTTGTTTGTTGCTC	59·4	786	Shibata *et al.* (2003)
	R5 TTGGGGATGTGAGACTAC	55·6		
Integrase genes				
*intI1**	F6 ATCATCGTCGTAGAGACGTCGG	67·4	-	Kimura *et al.* (2005)
	R6 CTCTCTAGATTTTAATGCGGATG	60·6		
intI2	F7 CACGGATATGCGACAAAAAGGT	66·7	789	Shibata *et al.* (2003)
	R7 GTAGCAAACGAGTGACGAAATG	63·4		
intI3	F8 ATCTGCCAAACCTGACTG	58·7	922	Shibata *et al.* (2003)
	R8 CGAATGCCCCAACAACTC	64·2		

**intI1* detects the area between 5′-CS and 3′-CS, which is a variable region.

during evaluation at each medical centre, and confirmed at the Department of Microbiology and Infectious Diseases, Toho University School of Medicine. All nine isolates were identified as *Prov. rettgeri* by the BD Phoenix system. Eight of the nine isolates were positive on screening for MβL production by DDST. These isolates were from two hospitals, in Nagasaki prefecture and Mie prefecture. Six strains from one hospital were urine isolates from different patients. In the other hospital, the two IMP-1-producing *Prov. rettgeri* strains were isolated from sputum and blood from different patients (Table 4).

The types of MβL were assessed by PCR. Eight of the nine DDST-positive isolates were confirmed as carriers of IMP-1 type MβL, which was encoded by a gene located in a class 1 integron structure. The types of MβL were confirmed by sequencing analysis. PCR amplification of the variable region (located between the 5′- and 3′-conserved sequences) of the class 1 integron using the conjugative plasmids as the template yielded a 2·5 kb or 3·5 kb product. Sequence analysis of both the 2·5 kb and the 3·5 kb integrons revealed a structure with at least two gene cassettes containing bla_{IMP-1} and *aacA4*, which encodes resistance to tobramycin and amikacin. In the 2·5 kb product, the bla_{IMP-1} gene cassette was located immediately downstream of the 5′-CS and was followed by the *aacA4* gene cassette. On the other hand, in the 3·5 kb product, the *aacA4* gene cassette was located immediately downstream of the 5′-CS, and was followed by the bla_{IMP-1} gene cassette and another cassette, which did not code any protein.

Table 4. Characteristics of bla_{IMP-1}-positive *Prov. rettgeri* clinical isolates

Strain	Region	Source	Antimicrobial susceptibility (μg ml^{-1})					
			Piperacillin	Ceftazidime	Cefotaxime	Aztreonam	Imipenem	Meropenem
TUM1965	Nagasaki	Urine	32	>16	>32	2	>8	>8
TUM1966	Nagasaki	Urine	32	>16	>32	2	>8	>8
TUM1933	Nagasaki	Urine	>64	>16	>32	2	8	>8
TUM1934	Nagasaki	Urine	>64	>16	>32	>16	>8	>8
TUM1935	Nagasaki	Urine	>64	>16	32	2	>8	>8
TUM1967	Nagasaki	Urine	>64	>16	>32	2	>8	>8
TUM1936	Mie	Blood	8	>16	>32	2	>8	>8
TUM1937	Mie	Sputum	>64	>16	>32	2	>8	>8

The MβL genes of the eight isolates from two different hospitals were transferable to *E. coli* ML4903 at a frequency between $1{\cdot}5 \times 10^{-5}$ and $5{\cdot}5 \times 10^{-7}$. When production of MβL was checked by DDST for the *E. coli* transconjugants, all showed MβL production. Moreover, most β-lactam MICs of the transconjugants were similar to those observed for *Prov. rettgeri* isolates, but the imipenem MICs (MIC 4 μg ml^{-1}) tended to be lower than those of the donors (MIC 128 μg ml^{-1}).

Incompatibility testing showed two groups. Group H1-harbouring strains were found in Nagasaki, and group T-harbouring strains were found in Mie. The transconjugants of TUM1933 and TUM1936 lost their plasmid when conjugated with *E. coli* C600. MICs of β-lactam antibiotics for the products of this second conjugation were lower than those of the transconjugants of TUM1933 and TUM1936, which proved that the resistance gene was on a transferable plasmid. In addition, the MICs of the resistance marker drugs were the same as for *E. coli* C600 (Table 5). This result reflected the phenomenon of incompatibility. Two plasmids could not stably coexist in the same host when these plasmids had the same incompatibility groups.

The genetic similarity of the eight bla_{IMP-1}-positive isolates was evaluated by using PFGE. Two types of PFGE patterns were observed (Fig. 1). The strains TUM1965, TUM1966, TUM1933, TUM1934, TUM1935 and TUM1967, isolated from the hospital of Nagasaki prefecture, and strains TUM1936 and TUM1937, from the hospital of Mie prefecture showed the same chromosomal DNA banding pattern.

DISCUSSION

The spread of nosocomial strains producing MβL has been reported around the world and is regarded as a serious clinical problem (Nordmann & Poirel, 2002; Walsh *et al.*, 2005). MβL-encoding genes are located in an integron structure on a conjugative plasmid (Arakawa *et al.*, 1995). These structures are one of the most important factors for multidrug-resistant bacteria because they can easily transfer from one strain to another, even to other species. Most MβL-producing strains show resistance not only to β-lactams (except monobactams) but also to other antibacterial agents such as aminoglycosides, quaternary ammonium compounds, trimethoprim and sulfonamides because these resistance genes are located on the same integron structure (Laraki *et al.*, 1999).

Recently, it has been reported that isolates of MβL-producing *Ps. aeruginosa* and *Serratia marcescens* probably have a high incidence (Kurokawa *et al.*, 1999). The reported isolates were mainly isolated from immunocompromised hosts who had pre-existing conditions such as malignant diseases, and were caused by nosocomial spread (Hirakata *et al.*, 1998). Among other Gram-negative bacteria, the isolation of MβL producers is also increasing. Shibata *et al.* (2003) reported that bla_{IMP-1}, bla_{IMP-2} and bla_{VIM-2} as MβL genes were

Table 5. Results of the plasmid elimination experiments

Strain	Incompatibility group	Resistance marker	Antimicrobial susceptibility (μg ml^{-1})							
			Piperacillin	Ceftazidime	Cefotaxime	Aztreonam	Imipenem	Meropenem	Rifampicin	Streptomycin	Tetracycline
Donor (*E. coli* C600)	H1	Tetracycline	2	≤0·25	≤0·25	≤0·25	≤0·25	≤0·25	8	4	256
Transconjugant			2	≤0·25	≤0·25	≤0·25	≤0·25	≤0·25	>256	4	256
Recipient (TUM1933 transconjugant)	H1	Rifampicin	>256	>256	64	≤0·25	4	4	>256	4	8
Donor (*E. coli* C600)	T	Streptomycin	16	≤0·25	≤0·25	≤0·25	≤0·25	≤0·25	8	256	4
Transconjugant			16	≤0·25	≤0·25	≤0·25	≤0·25	≤0·25	>256	256	4
Recipient (TUM1936 transconjugant)	T	Rifampicin	>256	>256	64	≤0·25	4	2	>256	4	4

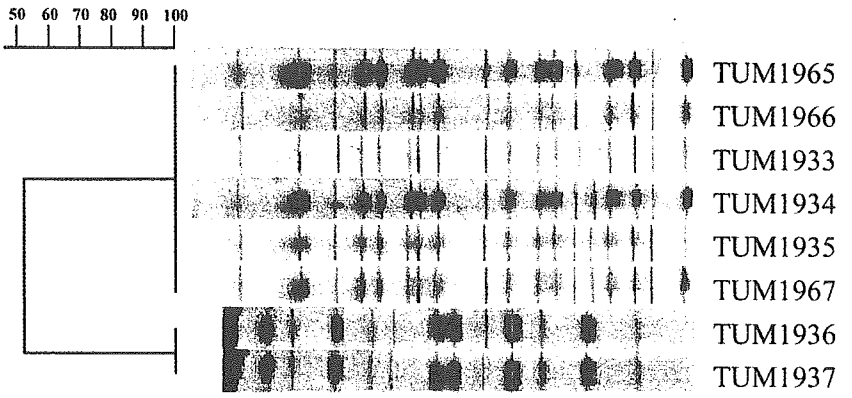

Fig. 1. PFGE profiles obtained with *SfiI* chromosomal digestion of *Prov. rettgeri* carrying the IMP-1 metallo-β-lactamase.

detected in *Klebsiella oxytoca*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *M. morganii* and *Prov. rettgeri* by PCR analysis. However, their study analysed only ceftazidime-resistant strains for β-lactam resistance factors and integron structure.

In this study 495 indole-positive proteae strains were isolated. Among these, 54·9 % were *M. morganii*. It appears that this species is quite common in this part of the world, having a high incidence in Korea as well (Kim *et al.*, 2003), even though it has been reported as rare in other places (Murray *et al.*, 2003). *Prot. vulgaris* was second in frequency of isolation (25·7 %) in the current study and followed by *Prov. rettgeri* (12·5 %) (Table 1). Of the proteae, 43 % (213/495) were urinary-tract isolates (Table 2). Of the *Prov. rettgeri* isolates, 69·3 % (43/62) were isolates from the urinary tract. Proteae have been recognized as pathogens in urinary-tract infections, and the majority of these urinary-tract infections are a consequence of urinary-tract catheterization and instrumentation (Warren, 2001). Stickler *et al.* (1998) reported that *Prov. rettgeri* can form crystalline biofilms that rapidly encrust and block catheters. This study did not distinguish catheter specimens from other urine specimens. For future surveillance, it will be necessary to specify the origin and to evaluate biofilm formation as one of the pathogenic factors in this species.

The isolation frequency of MβL-producing *Prov. rettgeri* strains was 1·6 % (8/495). Kimura *et al.* (2005) reported an isolation frequency of MβL-producing *Ps. aeruginosa* of 1·9 % (11/594) in 2002 using strains from the same surveillance programme as the current study. It is of interest that no MβL-producing *Prov. rettgeri* were isolated from hospitals where MβL-producing *Ps. aeruginosa* were isolated. Eight MβL-producing *Prov. rettgeri* strains were isolated from only two hospitals, Mie and Nagasaki, which are separated by over 600 km. The genetic relatedness was evaluated by pulsed-field gel electrophoresis, integron structure and plasmid incompatibility group. These data show that the resistant *Prov. rettgeri* strains had two different origins, which coincided with the two different hospitals where they were isolated. The strains isolated in each hospital shared the same integron structure and also the same incompatibility group. These results strongly suggest that nosocomial infection by *Prov. rettgeri* occurred in the two different hospitals. Moreover, these bla_{IMP-1}-encoding plasmids could transfer from *Prov. rettgeri* isolates to other species and their incompatibility groups could expand to other *Enterobacteriaceae*. This result suggests that the spread of this imipenem-resistance factor to other *Enterobacteriaceae* is not very difficult.

In conclusion, we report the finding of *Prov. rettgeri* isolates that harbour a conjugative plasmid containing an integron on which bla_{IMP-1} is encoded. Our results very strongly suggest that nosocomial infections by IMP-1-producing *Prov. rettgeri* occurred at two hospitals. IMP-1-producing *Enterobacteriaceae* could become a serious problem in the future. Thus, it is important to continue surveillance and monitoring of carbapenem resistance and reduced susceptibility *Enterobacteriaceae* including indole-positive proteae.

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ORIGINAL ARTICLE

Semi-quantitative analysis of Streptococcus pneumoniae urinary antigen: Kinetics of antigen titers and severity of diseases

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Abstract

Detection of urinary antigen by a rapid immunochromatographic membrane test (Binax NOW) was widely accepted as a powerful tool for diagnosis of Streptococcus pneumoniae pneumonia. This is a qualitative kit, so the value of quantitative analysis of urinary antigen, especially correlation of antigen titers and severity of diseases, remained to be determined. We examined semi-quantitative antigen titer in urines collected from urinary antigen-proven S. pneumoniae pneumonia on admission, and analyzed the kinetics of antigen titer and its relation to severity of diseases. After serial 2-fold dilution of urine, the highest dilution for positive results was determined, and this was designated as maximum dilution factor (MDF). MDFs varied from 1 to 4096 in 29 patients examined (mean MDF, 317.8). Importantly, severe cases of S. pneumoniae pneumonia were higher values of MDFs (mean MDF: 760.5) than those of non-severe cases (mean MDF: 5.4). The patients with high MDFs ($\geq$64) demonstrated higher values of LDH, CRP and lower values of WBC and PaO_2 compared to those of low MDFs group ($\leq$32). There was no clear correlation between CRP values and antigen titers, and conversely the majority of severe cases showed relatively weak CRP responses, despite high levels of bacterial antigen. Kinetic analysis of urinary and serum antigen titers in 4 cases of S. pneumoniae pneumonia exhibited consistently higher values of antigen titers in urine than those in serum. The half lives of urinary and serum antigen titers were calculated to be 1.0–3.4 and 1.1–2.3 weeks, respectively. These data suggest that quantitative analysis of urinary antigen may be a useful indicator for severity of disease and course of S. pneumoniae pneumonia. Our results demonstrate an application for S. pneumoniae antigen titer determination in urine and serum, which may be crucial not only for diagnostic measures, but also may provide a better understanding of the pathogenesis of S. pneumoniae infection.

Introduction

Streptococcus pneumoniae has been consistently shown to be the most common cause of community-acquired pneumonia (CAP) in both adults and children. This organism accounts for about two-thirds of cases where an etiologic diagnosis is made [1]. In particular, systemic pneumococcal infection is a major cause of morbidity and mortality, especially for young children, people with underlying diseases, and the elderly. Rapid diagnosis and proper antibiotic chemotherapy, in addition to correct evaluation of severity of disease, may be crucial for determining course and outcome of S. pneumoniae infection [2,3].

Recently, a new, rapid immunochromatographic membrane test, the NOW S. pneumoniae urinary antigen test (Binax, Inc., Portland, Maine), has been developed for the detection of antigens of S. pneumoniae in urine samples [4]. This test is simple to perform, detects the C-polysaccharide cell wall antigen common to the majority of S. pneumoniae strains, and provides results within 15 min. The utility of this assay has been repeatedly demonstrated, in which sensitivity and specificity of this kit was shown to be 65.9–82.0% and 89.7–100%, respectively [5–9]. It is likely that recovery of bacterial antigen from urine may be associated with invasion of bacteria or dispersion of bacterial components and products into the blood stream.

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Thus, it is reasonable to query whether intensity of urinary antigen reflects intravascular loading of bacteria, and more importantly, severity of disease.

To answer this question, we have examined intensity of urinary antigen by diluting urine samples in a serial 2-fold fashion. Our data suggest a correlation between urinary antigen titers and severity of pneumonia. Furthermore, the kinetics and the half lives of urinary and serum antigens were demonstrated in patients with S. pneumoniae pneumonia.

Materials and methods

Collection of clinical data and samples in S. pneumoniae pneumonia cases

Urine samples from CAP patients admitted to Toho University Hospital were tested for pneumococcal antigen using the NOW S. pneumoniae urinary antigen test. Urinary antigen positive cases (29 cases; age range 18–86 y; 66% male) were recruited as part of the present study. Clinical and laboratory data were recorded, and urine samples were stored at −80°C until used. In cases presenting with dyspnea, arterial blood-gas analysis was performed before and after oxygen supplementation. All enrolled patients had an acute illness with clinical features of pneumonia and radiographic pulmonary shadowing that was at least segmental or present in 1 lobe and was neither pre-existing nor due to some other known causes. Patients were excluded when pneumonia was not the principal reason for admission. Also, patients with history of pneumonia within past 3 months were not included in this study.

In 4 cases of urinary-antigen proven S. pneumoniae pneumonia, urine and serum samples were consecutively collected over 17 weeks after admission, and these samples were also stored at −80°C until used.

Evaluation of severity of diseases

There are no universally accepted criteria for severe or non-severe CAP [2,3,10–12]. According to previous reports, we have used 1 set of variables that has been proposed as a reliable predictor defining severe CAP: the presence of 2 out of 3 possible minor criteria (systolic blood pressure <90 mmHg, multilobar disease, $PaO_2/FiO_2 < 250$), or 1 of 2 major criteria (need for mechanical ventilation or septic shock).

Qualitative and semi-quantitative analysis of pneumococcal antigen

The urine samples were tested using the immunochromatographic assay Binax NOW S. pneumoniae antigen (Binax). This test detects the C-polysaccharide antigen from the cell wall of S. pneumoniae that is believed to be specific for a majority of pneumococcal serotypes. The test was performed in accordance with the manufacture's instructions. A swab was dipped into the urine sample and then inserted into the test device. A buffer solution was added, and the device was closed, bringing the sample into contact with the test strip. The test was read at 15 min and was interpreted by noting the presence or absence of visually detectable pink lines. A positive test result was indicated by the detection of both sample and control lines, and a negative result was indicated by the detection of a control line only. The results were read by 2 observers and consensus data were used for following analysis.

For semi-quantitative analysis in urine and serum, serial 2-fold dilution was performed with phosphate-buffered saline, and then presence of pneumococcal antigen was examined by the NOW S. pneumoniae urinary antigen test, as described above. The dilution at which urinary antigen is positive, but negative at the next 2-fold dilution, was designated to be maximum dilution factor (MDF) in the present study.

Statistical analysis

We used Student's t-test to compare quantitative variables. A 2-tailed p-value of 0.05 was considered to be statistically significant.

Results

Urinary antigen titers on admission

MDFs of 29 cases of S. pneumoniae pneumonia were examined in urine samples, which were obtained and stocked on admission (Figure 1). MDFs varied from 1 to 4096, with the median value calculated to be 317.8. Next, we examined the correlation of urinary antigen titers and severity of disease, as defined in Materials and methods. MDFs of 17 cases with non-severe diseases ranged from 1 to 32, with a median MDF value of 5.4. In contrast, MDFs of 12 cases with severe pneumonia were widely distributed from 1 to 4096, with the median MDF value calculated to be 760.5 ($p < 0.05$). In the present study, 2 lethal cases were included, and MDFs of urines in these cases were 4 and 4096. These data demonstrated that severe cases of S. pneumoniae pneumonia appear to have higher MDFs in urine than those of non-severe cases.