Table II. Zinc(II)-ligand distances (Å) and angles (°) for IND-7 and CcrA metallo-β-lactamases.

		IND-7	CcrA[a]	
Zn(II)-ligand[b]		Distances		
Zn1	His116NE2	2.1	2.1/2.2	
	His118ND1	2.0	2.0/2.1	
	His196NE2	2.0	2.0/2.0	
	O(Wat1)	1.9	1.9/2.0	
	O(Wat2)	2.6		
Zn2	Asp120OD2	2.1	2.3/2.1	
	Cys221ASG	2.2	2.3/2.4	
	His263ANE2	2.1	2.1/2.2	
	O(Wat1)	2.1	2.1/2.2	
	O(Wat3)		2.3/2.2	
Zn1	Zn2	3.6	3.5/3.5	
Ligand-Zn(II)-Ligand[b]			Angles	
His116(96)NE2	Zn1	His118ND1	99	105/100
	Zn1	His196NE2	102	100/106
	Zn1	O(Wat1)	108	119/111
His118(98)ND1	Zn1	His196NE2	116	107/108
	Zn1	O(Wat1)	116	112/109
His196(159)NE2	Zn1	O(Wat1)	113	113/121
O(Wat2)	Zn1	His116NE2	169	
	Zn1	His118ND1	71	
	Zn1	His196NE2	88	
	Zn1	O(Wat1)	73	
Asp120(100)OD2	Zn2	Cys221ASG	113	97/97
	Zn2	His263ANE2	91	86/82
	Zn2	O(Wat1)	94	86/81
Cys221A(179)SG	Zn2	His263ANE2	110	107/110
	Zn2	O(Wat1)	118	115/117
His263A(220)NE2	Zn2	O(Wat1)	125	138/132
O(Wat3)	Zn2	Asp120OD2		161/164
	Zn2	Cys221ASG		101/110
	Zn2	His263ANE2		83/89
	Zn2	O(Wat1)		92/95

[a] The distances and angles for CcrA metallo-β-lactamase are quoted for molecules A and B in the asymmetric unit. [b] Ligand numbers are indicated by the BBL numbering scheme and numbers of amino acid resides from the N-terminus for each mature protein are omitted for simplification.

|structural parameter $\tau = (\beta - \alpha)/60)$ (56)], where $\alpha = 116°$ for His118(98)ND1−Zn1 − His196(159)NE2 and $\beta = 169°$ for O(Wat2) − Zn1 − His116(96)NE2 were the two basal angles ($\beta \geq \alpha$) representing the change in trigonal distortion from a square pyramidal geometry: $\tau = 0$ for an ideal square pyramid and 1 for an ideal trigonal bipyramid. Two His residues, His118(98) and His196(159), and either one oxygen from a water molecule or a hydroxide ion (labeled Wat1) made a trigonal plane (the average angle of the corresponding angles was 115°). Wat1, which is thought to act as the attacking nucleophile on the CO group of the β-lactam ring (32, 57, 58), bridged to both Zn1 and Zn2, and also formed a hydrogen bond with one oxygen atom of Asp120(100) with a distance of 2.7 Å. The apical positions were occupied by His116(96) and Wat2, where Wat2 weakly interacted with Zn1 at a distance of 2.6 Å [the average Zn − Wat bond distance in zinc(II) enzymes is reportedly 2.2 Å (59)]. In the crystal structure of VIM-2 metallo-β-lactamase, Zn1 is also coordinated to five ligands in a distorted trigonal bipyramidal geometry (37), as seen in the IND-7 structure. However, the Zn1 − Wat bond distance (2.8 Å) at the apical position was longer by 0.2 Å than that of IND-7 (Zn1 − Wat2). These observations suggest that Wat2 can more easily

accommodate replacement by the Zn1 coordination sphere upon uptake of substrates and inhibitors. We expected the coordination structure of the dinuclear zinc(II) active site in IND-7, especially pentacoordination at the Zn1 site, to be of major benefit to a favourable Michaelis complex, because Wat2 was thought to be the carbonyl oxygen atom of the β-lactam ring that interacted with Zn1 upon formation of the Michaelis complex (Fig. 4).

The Zn1−His and Zn1−Wat1 bond distances were 2.0 − 2.1 Å and 1.9 Å, respectively, which were almost identical to those found in CcrA (2.0 − 2.2 Å for Zn1 − His and 1.9 − 2.0 Å for Zn1 − O, respectively). Zn2 was tetrahedrally coordinated with Asp120(100), Cys221A(178), His263A(220), and Wat1, with bond distances of 2.1 Å (Fig. 3B), and 'the apical water Wat3', observed in CcrA, was missing from the IND-7 structure. The Zn2 − Wat1 bond distance of 2.1 Å was somewhat longer than that of Zn1−Wat1 and this tendency was also found in CcrA. The ligand−Zn2−ligand bond angles of 91−125° (the average angle; 109°) were close to the optimal tetrahedral angles. The Zn1 − Zn2 distance was 3.6 Å and this bond distance was close to that of CcrA (3.5 Å). Thus, the zinc(II) coordination geometry was notably

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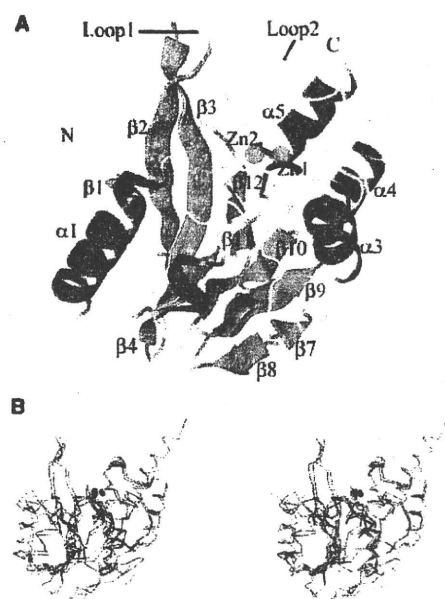
Fig. 2 Overall structure of IND-7 from *C. indologenes*. (A) A ribbon diagram of IND-7 is shown with the secondary structures labeled. α-Helices, β-strands and the loops are shown in red, green and yellow, respectively. Zinc(II) ions are represented as orange spheres. The figure was prepared with MolFeat software (FiatLux Corporation). (B) Superposition of the C_α tracing of IND-7 (red) with CcrA from *B. fragilis* [blue. PDB code: 1ZNB, (*32*)]. In the CcrA structure, only molecule A is depicted.

different from those of other crystal structures of subclass B1 metallo-β-lactamases, which includes CcrA.

Role of Arg121 and hydrogen-bonding networks around the dinuclear zinc(II) active site

Arg121 is well conserved in IND variants (*39, 40*), VIM variants (*20*), BlaB (*60*) and BcII (*7, 32*), and is thought to be responsible for the reduced binding affinity for Zn2 (*29, 33, 35*). Indeed, BcII metallo-β-lactamase has a binding affinity that differs from that of the two zinc(II) binding sites (*61–63*). By contrast, residues at position 121 in CcrA and IMP variants are occupied by Cys121(104) and Ser121(82), respectively, and these metallo-β-lactamases display high binding affinity at both the Zn1 and Zn2 sites (*64, 65*).

As seen in Fig. 3C, a positively charged guanidinyl group of Arg121(101) was situated below the floor of the Zn2 site. A $2|F_o| - |F_c|$ electron density map clearly indicated Arg121(101) alternated in the crystal, and the conformations Arg121A(101) and Arg121B(101) fit the map well. The former corresponded to a Zn2-coordinated form of Cys221(178) and His263(220), whereas the latter corresponded to a Zn2-uncoordinated form with no bound Zn2. Therefore, the corresponding atoms in the two conformations were refined with occupancies of 0.7 and 0.3, respectively. In Arg121A(101), the electrostatic repulsion between a positively charged bulky guanidinyl group of Arg121 and Zn2 cause movement of the guanidinyl group to move away from Zn2

[4.7 Å from Arg121A(101)NE and 5.7 Å from Arg121A(101)NH2], where the guanidinyl group is maintained by four hydrogen bonds between Asn70(52), Ser71(*53*), Asp84(66) and Asp120(100): Arg121A(101)NE Asp120(100)O = 2.8 Å, Arg121A (101)NH1·Asp84(66)OD2 = 3.2 Å, Arg121A(101) NH2·Asn70(52) = 2.7 Å and Arg121A(101)NH2· Ser71(*53*)OG = 3.0 Å. Ser71(53) is conserved in IND-1, IND-3, and IND-5—IND-7 but is Gly in IND-2, IND-2a, and IND-4 (Fig. 1). The substitution of residues at position 71(*53*) in IND variants is expected to have an effect on the changing of the binding affinity of Zn2 and on the hydrolysis of β-lactams.

In Arg121B(101), with a rotation of the CG—CD bond of the side chain in Arg121B(101), the plane formed by the atoms CD, NE, NH1 and NH2 of Arg121B(101) was nearly perpendicular to the corresponding plane of Arg121A(101) and the guanidinyl group of Arg121B(101) lay close to the dinuclear zinc(II) active site [3.5 Å from Arg121B(101)NE and 3.6 Å from Arg121B(101)NH2]. The guanidinyl group made four hydrogen bonds with Asn70(*52*), Asp84(66), Asp120(100) and Gly262(219): Arg121B (101)NE Asp120(100)OD2 = 2.9 Å, Arg121B(101) NH1 Asn70(52)O = 3.0 Å, Arg121B(101)NH1 Asp84 (66)OD2 = 3.2 Å and Arg121B(101)NH2 Gly262 (219)O = 2.7 Å, of which two hydrogen bonds of Arg121B(101) with Asp120(100) and Gly262(219) pulled the guanidinyl group close to the dinuclear zinc(II) active site. These observations highlight the importance of Arg121(101) for the binding affinity of Zn2 by cooperation between the electrostatic effect and the change in the hydrogen-bonding network. In addition, Arg121(101) also appeared to partially contribute to the preservation of the moderate orientation of Asp120(100) relative to Zn2.

We compared the hydrogen-bonding networks around the dinuclear zinc(II) active site to estimate the primary cause of the differences in coordination structure between IND-7 and CcrA metallo-β-lactamases.

In CcrA, the side chain NZ of Lys224(184) produced hydrogen-bonding networks with Wat3 coordinated to Zn2, the side chain ND1 of His196(162), and the main chain oxygen atoms of Cys221(181), Ile231(191) and Asn233(193) through well-ordered water molecules (Fig. 5A). Lys224 located on loop 2 was assumed to interact with the carboxylate of the β-lactams (*32, 35*). Moreover, the side chain of ND2 of Asn233(193) formed a hydrogen-bonding network with bridging water between Zn1 and Zn2, Wat1, via a water molecule. Conversely, the hydrogen-bonding networks in IND-7 were somewhat different from those of CcrA (Fig. 5B). Lys224(181), which was ~7 Å away from the zinc(II) active site, did not participate in the hydrogen-bonding networks between the bulk water molecules. In the case of IND-7, Asn233(193) in CcrA was replaced with Tyr233(190), which was located on loop 2. Tyr233(190) rotated ~74° around the CB—CG bond and the hydroxy group of the phenyl ring was ~8 Å away from Zn1. The main chain oxygen atoms of Leu231(188) and Tyr233(190) were hydrogen bonded to a water molecule, which is

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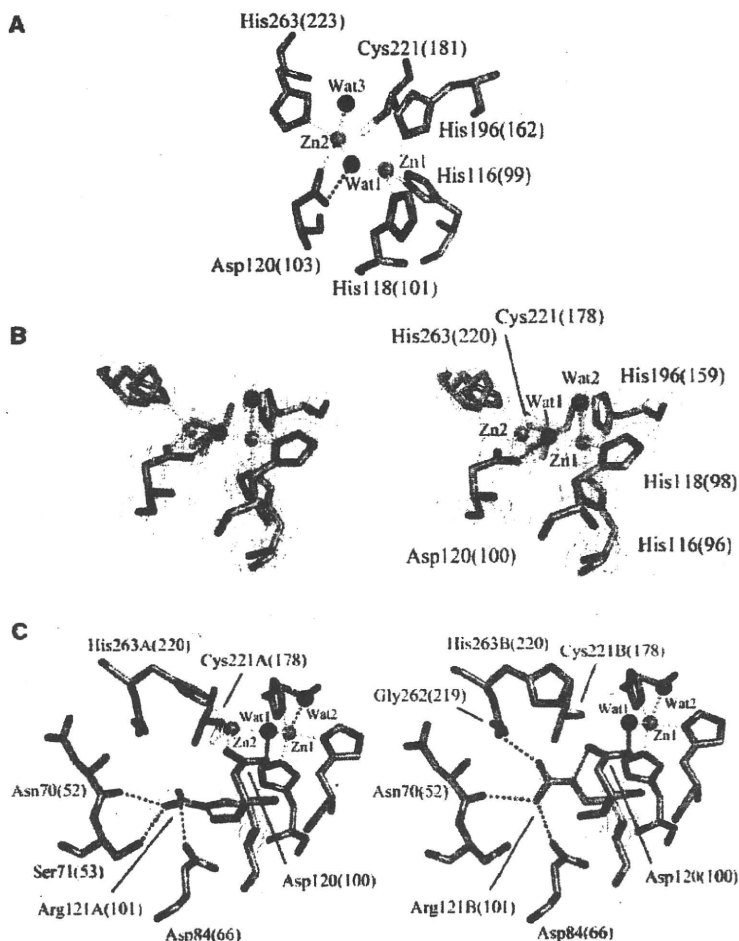
Fig. 3 Comparison of the dinuclear zinc(II) active site structures in CcrA from *B. fragilis* [PDB code: 1ZNB, (32)] and IND-7 from *C. indologenes.*
(A) Active site structure in CcrA; only molecule A is depicted. Zn1 is tetrahedrally coordinated with His116(99). His118(101), His196(162) and Wat1. Zn2 is trigonal-bipyramidally coordinated with Asp120(103), Cys221(181), His263(223), Wat1 and Wat2. Zinc(II) ions and water molecules are presented as orange and red spheres, respectively. Carbon, oxygen, nitrogen and sulphur atoms are shown in grey, red, blue and yellow, respectively. Zn(II)−ligand bonds are shown as yellow dotted lines. The hydrogen bond is shown as a red dotted line. (B) Stereo view of the active site in IND-7. The electron density map (green mesh) is shown contoured at the 1.0σ level in the 2|F_o|−|F_c| map. Zinc(II) ions and water molecules are shown as orange, red spheres, respectively. Carbon, oxygen, nitrogen and sulphur atoms are shown in grey, red, blue and yellow, respectively. Zn(II)−ligand bonds, with the exception of the Zn(II)−Wat2 bond, are shown as yellow dotted lines. The Zn(II)−Wat2 bond is shown as a green dotted line. The hydrogen bond is shown as a red dotted line. The occupancies of Zn1 and Zn2 are refined with 1.0 and 0.7, respectively. Cys221(178) and His263(220) adopted alternative conformations in the IND-7 structure [Cys221A(178) and His263A(220), and Cys221B(178) and His263B(220)]. The occupancies of Cys221A(178) and His263A(220) (magenta sticks) are refined with 0.7 in each case, whereas those of Cys221B(178) and His263B(220) (orange sticks) are refined with 0.3 in each. Zn1 is trigonal-bipyramidally coordinated with His116(96), His118(98). His196(159), Wat1 and Wat2. Zn2 is tetrahedrally coordinated with Asp120(100), Cys221A(178), His263A(220) and Wat1. Unlike the CcrA structure, 'the apical water' Wat3 is absent from the Zn2 site. (C) Alternative conformations of Arg121(101), Arg121A(101) and Arg121B(101). The electron density map (green mesh) of Arg121(101) is shown contoured at the 1.0σ level in 2|F_o|−|F_c| map. Of the two conformations, Arg121A(101) is shown to the left, whereas Arg121B(101) is shown to the right. The former was a Zn2-coordinated form of Cys221(178) and His263(220), whereas the latter was a Zn2-uncoordinated form with no bound Zn2. The occupancies of Arg121A(101) and Arg121B(101) are refined with 0.7 and 0.3, respectively. Carbon, oxygen, nitrogen and sulphur atoms are shown in grey, red, blue and yellow, respectively. Zn(II)−ligand bonds, with the exception of Zn(II)−Wat2, are shown as yellow dotted lines. The Zn(II)−Wat2 bond is shown as a green dotted line. Hydrogen bonds are shown as red dotted lines. The figures were prepared with *PyMol* software (http://pymol.sourceforgen.net/).

occupied in a position equivalent to that of a water molecule in the CcrA structure, forming hydrogen-bonding networks with Wat1 and Wat2 that was coordinated to Zn1, the side chain ND1 of His196(159), and the main chain oxygen atoms of Cys221(181). Interestingly, Wat2 formed two hydrogen bonds with two water molecules, one of which interacted with Wat1 to achieve formation of a hydrogen-bonding network between Wat1 and Wat2—the other was linked to bulk water molecules. This seemed to be one of the factors affecting the structural change in the Zn1 coordination sphere, which was also thought to be closely related to the difference in enzymatic activity.
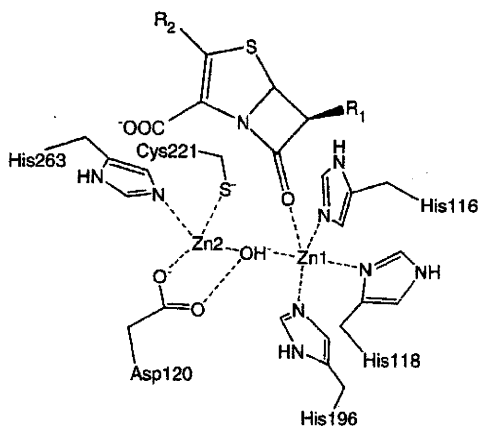
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Fig. 4 A model of the Michaelis complex for β-lactam bound to sub-class B1 metallo-β-lactamase.
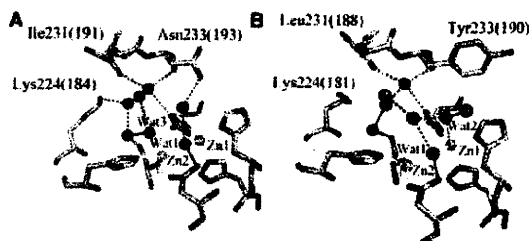

Fig. 5 Comparison of hydrogen-bonding networks between (A) CcrA from *B. fragilis* [PDB code: 1ZNB, (32)] and (B) IND-7 *C. indologenes* in the vicinity of the dinuclear zinc(II) active sites. Carbon, oxygen, nitrogen and sulphur atoms are shown in grey, red, blue and yellow, respectively. Zn(II)—ligand bonds, with the exception of Zn(II)—Wat2, are shown as yellow dotted lines. The Zn(II)—Wat2 bond is shown as a green dotted line. Hydrogen bonds are shown as red dotted lines. The figures were prepared with *PyMol* software (http://pymol.sourceforgen.net/).

Structure-activity relationship between IND-2 and IND-5 deduced from the IND-7 structure

In 2007, Perilli *et al.* (40) isolated IND-5 from a clinical isolate of *C. indologenes* and conducted kinetic studies on the hydrolysis of various β-lactams against IND-5 metallo-β-lactamase. From a comparison of kinetic parameters of IND-5 with those of IND-2, they reported that the catalytic efficiencies (k_{cat}/K_m) of IND-2 for imipenem and meropenem were ~25- and 13-fold higher than those of IND-5, respectively. On the basis of the comparison of the amino acid sequence of IND-5 with those of IND variants, including IND-2, Perilli *et al.* assumed that the residue at position 265 (BBL numbering) might be involved in the drastic reduction in catalytic efficiency of IND-5, compared with that of IND-2 (*39, 40*): Glu265(222) was well conserved in all IND variants with the exception of IND-5 [in this case, Asp265(222)] and was located close to His263(220), which is a Zn2 ligand.

As shown in Fig. 1, IND-7 shared 92% amino acid identity with the IND-5 of sixteen amino acid

substitutions: Ser21(4)Lys, Lys23(6)Tyr, Ile26(9)Met, Ile29(12)Met, Leu30(13)Met, Ser32(15)Ala, Phe34(17)Met, Ala35(*18*)Phe and Ser36(19)Asn in the signal peptide region; and, Met72(54)Val, Val90(72)Ala, Val110(90)Ile, Ala140(119)Ser, Val188(151)Thr, Lys244(201)Ile, Asn247(204)Asp and Glu265(222)Asp. Extrapolating from the determined IND-7 structure, most of the amino acid substitutions of IND-5 were expected to be located either on the surface of the protein or far from the active site. Of these residues, Glu265(222) was located ~8 Å from the dinuclear zinc(II) active site. The backbone CO of Glu265(222) were hydrogen-bonded to the side chain OG of Ser225(182) at distances of 2.6 Å, but the side chain of Glu265(222) was turned to face the surface of the protein. Therefore, the side chain was not likely to influence the catalytic efficiency of IND-5 by Glu-to-Asp substitution at position 265. One possibility affecting the enzymatic activity of the mutant might have been the residue at position 235 (BBL numbering), which was located almost on top of loop 2 at a distance of ~9 Å from Zn1 and Zn2. In IND-1, IND-5 and IND-7, the residue at position 235 was Lys, whereas, for IND-2 the residue at the same position was Gly. Lys235(192) in the IND-7 structure had a strained main chain conformation, with ϕ and ψ angles of −60° and −29°, respectively. However, the Lys-to-Gly substitution at position 235 in IND-2 may have increased the degree of rotation about the ϕ and ψ angles, compared with IND-5, and caused the movement of loop 2 to access the conserved residue in all IND variants, Tyr233(190), of the hydrophobic pocket. Thus, the mutation at position 235 appeared to trigger changes in the mobility of the loop and in the uptake of the substrate to the active site, leading to a difference in the enzymatic activity.

In conclusion, we determined the crystal structure of IND-7 metallo-β-lactamase of *C. indologenes*. As a consequence, the precise coordination mode around the dinuclear zinc(II) active site was ascertained, and its coordination mode appeared to be quite different from those of the well-characterized metallo-β-lactamases, as determined by X-ray crystallography. It was predicted that the hydrogen-bonding interaction between Arg121(101) and Gly262(219) would influence conformational flexibility of His263(220) and, as the result, the fixation of His263(220) to Zn2 by this interaction did contribute to the binding affinity of Zn2 and to the enzymatic performance. Finally, the residue at position 235 of loop 2, rather than Glu-to-Asp substitution at position 265, might be critical to altering the enzymatic activity in the IND variants.

Conflict of interest
None declared.

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High prevalence of mutators among *Enterobacter cloacae* nosocomial isolates and their association with antimicrobial resistance and repetitive detection

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Abstract

We determined the mutation frequencies of 59 nosocomial isolates of *Enterobacter cloacae*, and investigated their association with anti-microbial susceptibility, genotype, and history of exposure to antimicrobials. The frequencies of mutations leading to rifampicin resistance ranged from 5.8×10^{-9} to 8.0×10^{-6} (median, 5.0×10^{-8}). Seven of the 59 (12%) isolates were graded as strong mutators exhibiting a more than 50-fold increase in the mutation frequency relative to that of *E. cloacae* ATCC 13047, and 30 (52%) were graded as weak mutators exhibiting a more than five-fold and not more than 50-fold increase in the mutation frequency. The isolates with higher grade of mutation frequency were resistant to significantly more antimicrobials (medians of two, one and zero agents for strong mutators, weak mutators and non-mutators, respectively; p 0.0078). The 59 isolates were classified into 36 genotypes, and all of the seven strong mutators had distinct genotypes. Mutation frequencies varied more than 10^2-fold within a clone. In patient-based, univariate analysis, intensive-care unit admission, dense antimicrobial exposure (glycopeptide or multiple classes) and repetitive detection of this species were significantly more common among all of the four patients from whom strong mutators were obtained. Strong mutators are highly prevalent in surgical isolates of *E. cloacae*. Higher mutation frequency was associated with antimicrobial resistance and repetitive detection, and may contribute to the adaptability of this species.

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Introduction

Mutators (bacterial strains with increased mutation rates) in bacterial populations are thought to facilitate the bacterium's adaptation to diverse environmental 'stress factors', such as antimicrobial challenge [1,2]. The role of mutation in the acquisition of antimicrobial resistance has been investigated in various species of bacteria. Mutations in the genes encoding the targets of antimicrobials result in resistance to certain agents [3]. Mutations suppressing expression of the OprD porin of *Pseudomonas aeruginosa* reduce the permeability of the cell wall to carbapenems [4]. Genes that regulate the expression of chromosomal type C β-lactamases are also reported to be influenced by mutation, leading to constitutive hyperproduction of the enzyme, which makes the isolates resistant to expanded-spectrum cephalosporins [5].

Only 1% or fewer of natural isolates of *Escherichia coli* and *Salmonella* species display increases in their mutation frequencies of more than 50-fold relative to those of control strains [6,7], and none of environmental *P. aeruginosa* isolates exhibit mutation frequencies 20-fold or greater than that of PAO1 [8]. In clinical settings, more than 50% of *P. aeruginosa* isolates from the lungs of patients with chronic lung diseases were mutators, and their phenotypes exhibited a strong association with multiple drug resistance [9]. Inactivation of the mismatch repair system favours persistence of *P. aeruginosa* in cystic fibrosis mice [10], and accumulation of adaptive mutations plays a major role in the genetic evolution of *P. aeruginosa* isolates obtained from cystic fibrosis patients [11].

Enterobacter cloacae, one of the major causes of nosocomial infection [12], has been reported to show increasing

resistance to multiple antimicrobials. The prevalence of mutators in this species and their clinical impact, however, have not been investigated [13].

In this article, we describe the mutation frequencies of clinical isolates of *E. cloacae* and their association with antimicrobial susceptibility and genotype. We also analysed the characteristics of the patients to find the 'stress factors' associated with detection of mutators in the clinical settings.

Materials and Methods

Study isolates, study period, and clinical background

E. cloacae isolates were collected consecutively from the inpatients of the Department of Surgery at Kyoto University Hospital from January to July 2003. Species identification was performed with the VITEK Gram-Negative Identification Card and API 20E (bioMérieux, St Louis, MO, USA), according to the manufacturer's instructions. MICs of the five selected antimicrobials—piperacillin–tazobactam, ceftazidime, imipenem, gentamicin, and levofloxacin—were determined by the broth microdilution method, according to the CLSI guidelines [14].

Information on characteristics of the patients was collected from their medical records, and included gender, age, history of intensive-care unit (ICU) admission, type of surgery (transplantation or not), and systemic administration of β-lactam, aminoglycoside, fluoloquinolone and glycopeptide antimicrobials in the study period. The isolates obtained from each patient at intervals of 30 days or more were included repeatedly in the study to investigate the relationship between the mutation frequency and repetitive detection of *E. cloacae*.

Determination of mutation frequencies and definition and classification of mutators

Mutation frequencies were determined with the method reported by Oliver et al. [15], with modifications. Briefly, a single colony of *E. cloacae* was resuspended in 20 mL of Mueller–Hinton (MH) broth (Eiken Chemical Co., Ltd, Tokyo, Japan), and grown overnight at 37°C. After centrifugation, the pellet was resuspended in 1 mL of sterile saline. Serial ten-fold dilutions were inoculated onto MH agar plates with and without 100 mg/L rifampicin (Sigma-Aldrich, Inc., St Louis, MO, USA). After 48 h of incubation at 37°C, the colonies were counted and the fractions of CFUs on the rifampicin-containing plates were calculated. To exclude the possibility of a pre-existing rifampicin-resistant subpopulation, approximately 10^7 CFUs of each isolate cultured on a rifam-

picin-free plate were inoculated onto MH agar plates containing 100 mg/L rifampicin, and absence of growth was confirmed. We determined the mutation frequency of *Escherichia coli* ATCC 25922 to confirm the compatibility of our experiment with previous studies [6,7,16].

All experiments were performed in triplicate. We adopted the mean of the triplicate result as the mutation frequency for each isolate, and repeated the experiment when the variation in mutation frequency among the triplicate experiments was more than ten-fold. Each of the isolates was classified as either a 'strong mutator', a 'weak mutator', or a 'non-mutator,' according to the increase in mutation frequency relative to the *E. cloacae* control strain, ATCC 13047. Isolates that exhibited a >50-fold increase in mutation frequency were defined as strong mutators, and were given a score of 3; isolates with mutation frequencies of between >5-fold and ≤50-fold were classified as weak mutators, and were given a score of 2; and the remaining isolates, with ≤5-fold increase in mutation frequency, were classified as 'non-mutators', and were given a score of 1.

Genotype analysis

Pulsed-field gel electrophoresis (PFGE) of genomic DNA digested with *XbaI* (Takara Bio Inc., Otsu, Japan) was performed as previously described [17]. For isolates not typeable with conventional methods, the bacterial pellet was treated with 4% (v/v) formaldehyde in 10 mM Tris-HCl (pH 7.5) and 1 M NaCl for 1 h at 4°C before lysis [18]. The restriction patterns were analysed with an established protocol [19]. A 'clonal group' was defined as a group of isolates consisting of more than one isolate whose restriction pattern differed by no more than three bands. Singletons were defined as the isolates that did not belong to any clonal group.

Enterobacterial repetitive intergenic consensus (ERIC) PCR was also performed [20]. The amplification primers chosen for our assessment were ERIC1 (5′-ATGTAAGCTC CTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTG GGGTGAGCG-3′). Thermocycling was performed with a TaKaRa PCR Thermal Cycler SP (Takara Bio Inc.), and consisted of the following steps: initial denaturation (95°C, 15 min), 40 cycles of denaturation (95°C, 30 s), annealing (50°C, 1 min), and extension (72°C, 1 min), and a single final extension (72°C, 15 min). Isolates were considered to belong to a particular genotypic group on the basis of the criteria described previously [21].

Statistical analysis

The relationships between two categorical variables were analysed with the chi-squared test or Fisher's exact test. The

relationships between the categorical and ordinal variables were determined with the Mann–Whitney U-test. The correlation between two ordinal variables was assessed by Spearman's rank correlation coefficient. Differences in a continuous variable among categories were analysed by Kruskal–Wallis test. p-Values were two-tailed, and were considered to be significant when <0.05. All analyses were performed with StatView version 5.0 (SAS Institute, Cary, NC, USA).

Results

Bacterial isolates

Sixty-two clinical isolates of *E. cloacae* were collected from 42 surgical inpatients at Kyoto University Hospital during the 7-month study period. Forty isolates were obtained from 22 liver transplant recipients, and the others were from 20 patients who had undergone other forms of abdominal surgery. Twenty-nine of the 62 isolates (47%) were obtained from ten patients from whom *E. cloacae* was detected repeatedly.

Mutation frequencies of *E. cloacae*

The mutation frequencies were determined for 59 isolates, because three of the original 62 isolates (one each from a surgical site, sputum, and urine) exhibited resistance to rifampicin at 100 mg/L. The mutation frequency of *Escherichia coli* ATCC 25922 was determined to be 1.7×10^{-8}, which is compatible with previous reports of non-mutator *Escherichia coli* [6,7,16].

The mutation frequencies of the 59 isolates ranged from 5.8×10^{-9} to 8.0×10^{-6}, with a median of 5.0×10^{-8} (Fig. 1). The mutation frequency of *E. cloacae* ATCC 13047 was determined to be 8.0×10^{-9}, so that seven (12%) isolates were classified as strong mutators (i.e. mutation frequencies $>4.0 \times 10^{-7}$), 30 (51%) as weak mutators (i.e.

mutation frequencies $>4.0 \times 10^{-8}$ and $\leq 4.0 \times 10^{-7}$), and 22 (37%) as non-mutators (i.e. mutation frequencies $\leq 4.0 \times 10^{-8}$).

Mutators, antimicrobial resistance, and the sources of recovery

The mutation frequency scores and the MICs of the antimicrobials correlated positively for piperacillin–tazobactam (p 0.02, Spearman's rank correlation), ceftazidime (p 0.007), imipenem (p 0.04), and levofloxacin (p 0.04) (Table 1). The isolates with higher mutation frequency scores were resistant to significantly more antimicrobials (median numbers of antimicrobials to which the isolates were resistant were two among strong mutators, one among weak mutators, and zero among non-mutators; p 0.0078). The isolates resistant to more than two antimicrobials were significantly more frequent among strong mutators (3/7, 43%) than among the other categories (3/52, 5.8%) (p 0.013).

The sources of recovery of the 59 isolates were as follows: 39 surgical site specimens (66%), 11 sputum specimens (19%), five urine specimens (8%), three blood specimens (5%), and one central venous catheter (2%). Four of the seven (57%) strong mutators were recovered from surgical sites, and three (43%) were recovered from sputa. There were no significant differences in the distribution of the sources among strong mutators, weak mutators, and non-mutators.

Genotype and its correlation with mutation frequency

The PFGE patterns of 55 of the 59 *E. cloacae* isolates were classified into 36 pulsotypes. The remaining four isolates showed only smearing on PFGE, even after re-examination with modified pretreatment. These four isolates were analysed only with ERIC PCR, and exhibited four distinct patterns that were different from those of the rest of the isolates. Thus, we classified them as singletons.
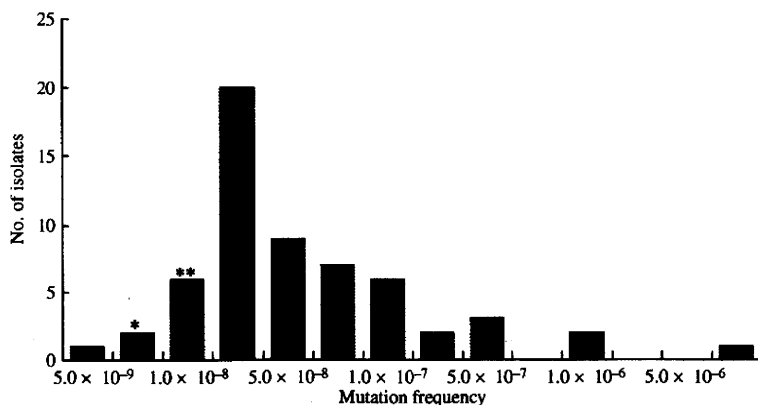

FIG. 1. Distribution of the mutation frequencies of 59 *Enterobacter cloacae* clinical isolates. Range, 5.8×10^{-9} to 8.0×10^{-6}; median, 5.0×10^{-8}. *Mutation frequency of *E. cloacae* ATCC 13047 (8.0×10^{-9}). **Mutation frequency of *Escherichia coli* ATCC 25922 (1.7×10^{-8}).

TABLE I. Comparison of MICs of antimicrobials among the strong mutator, weak mutator and non-mutator isolates of Enterobacter cloacae[a]

Agent	MIC	Overall (n = 59)	Mutators Strong mutators, (n = 7)	Weak mutators, (n = 30)	Non-mutators, (n = 22)	p[c]
TZP	MIC50[b]	16	128	16	2	<0.05
	MIC90	128	128	128	128	
CAZ	MIC50	32	64	32	1	<0.01
	MIC90	64	64	64	64	
IPM	MIC50	0.5	0.5	0.5	0.5	<0.05
	MIC90	1	32	8	4	
GEN	MIC50	1	32	1	1	0.1
	MIC90	32	32	2	32	
LVX	MIC50	2	2	0.25	0.25	<0.05
	MIC90	4	16	2	4	

TZP, piperacillin–tazobactam; CAZ, ceftazidime; IPM, imipenem; GEN, gentamicin; LVX, levofloxacin.
[a]For definition of strong mutators, weak mutators, and non-mutators, see text.
[b]The MIC was determined according to the guidelines of the CLSI [14].
[c]Spearman's rank correlation.

Five clonal groups (groups A, B, C, D, and E) were identified by this genotyping, and the number of isolates within each clonal group ranged from two to eight. The mutation frequencies within each clonal group varied 116-fold in group A (Fig. 2). The differences in mutation frequencies among the five clonal groups were not statistically significant (p 0.28, Kruskal–Wallis test). Each of the seven strong mutators exhibited a distinct genotype.

The characteristics of patients associated with detection of strong mutators

Seven strong mutators were isolated from four patients. On comparison of these four patients with the rest of the 38 patients from whom no strong mutators were obtained, there were four statistically significant characteristics associated with detection of strong mutators: history of ICU admission (2/4 vs. 1/38, p 0.02, Fisher's exact test), administration of glycopeptides (3/4 vs. 6/38, p 0.036), administration of more than one category of antimicrobials (3/4 vs. 8/38, p 0.049), and repetitive detection of E. cloacae (3/4 vs. 1/38, p 0.039).

Mutation frequency and the genotype of the isolates obtained from patients with repetitive detection of the species

More than one isolate of a single genetic group was obtained from three of the ten patients with repetitive detection of E. cloacae. The isolates obtained from two of these three patients included mutators (a strong mutator from one patient, and a weak mutator from the other), but both of these mutators were detected earlier than the non-mutators. There were no patients from whom strong or weak mutators belonging to a specific clonal group were repetitively isolated.

The sources of the 29 isolates obtained from the ten patients with repetitive detection of E. cloacae were as follows: surgical site specimens (21, 72%), sputum (4, 14%), blood (3, 10%), and urine (1, 3%). There were no significant differences in sources between the isolates from these ten patients and the others.

Discussion

To the best of our knowledge, this is the first study to investigate the mutation frequencies of E. cloacae clinical isolates and their clinical significance. Our analysis showed that strong mutators accounted for seven (12%) of 59 nosocomial isolates of E. cloacae. This percentage is almost twice as high

FIG. 2. Distribution of the mutation frequencies within the clonal groups and singletons. Filled circles: strong mutators. Open circles: weak mutators. Crosses: non-mutators.
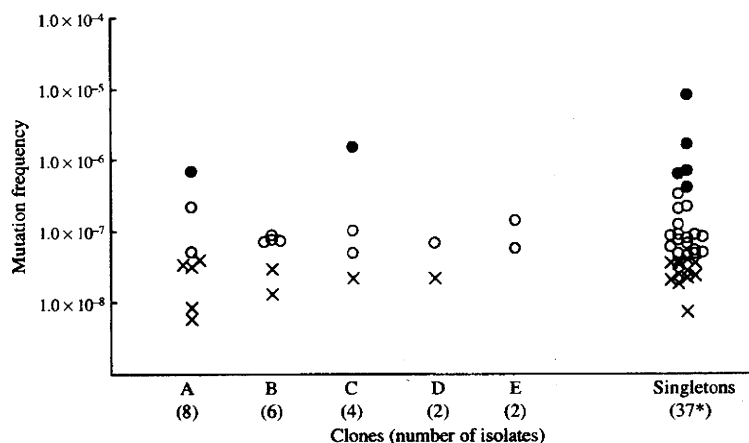
as those reported for *Escherichia coli* and *Salmonella* by LeClerc *et al.* [16], and is 10–20 times as high as the incidence of natural *Escherichia coli* mutators [6,7]. This high prevalence did not come from clonal spread of mutators or repetitive detection of persistent mutator strains from particular patients, because all of the seven mutators belonged to distinct genotypes.

Higher mutation frequency was correlated with higher MICs of antimicrobials and resistance to more antimicrobials. This is consistent with the association of mutator phenotype and antimicrobial resistance reported for *P. aeruginosa* mutators isolated from patients with chronic lung diseases [9].

Two of the four patient background factors associated with detection of strong mutators were related to antimicrobial exposure, which may have provided a favourable microenvironment for the resistant mutators. Consumption of broad-spectrum antimicrobials is much higher among liver transplant patients than among other surgical patients, probably because of the high incidence of infectious complications in liver transplant recipients. In our hospital, most liver transplant surgery is living-donor liver transplantation, in which infectious complications are more frequent than in cadaveric transplantation [22]. It is possible that more resistant mutators were selected in such patients.

Repetitive detection of *E. cloacae* was another factor significantly associated with detection of mutators. However, in contrast to the previous reports of *P. aeruginosa* mutators [9,15], our mutator isolates appeared to have emerged by replacing other clones during the clinical course. This difference may result from the difference between two species and/or of the focus of infection. It is possible that a group of mutators with increased adaptability may play a role in maintaining a favourable microenvironment for this species, leading to prolonged infection with a genotypically heterogeneous population.

It is also possible that ancestral non-mutators evolved into mutators within each patient, as shown for *P. aeruginosa* [15]. The finding that the mutation frequency within a clonal group varied as much as 116-fold is consistent with this hypothesis; however, pairs of a mutator and a non-mutator belonging to the same clonal group were obtained from only two patients, and the detection of the mutator preceded that of the non-mutator in both cases.

Another possibility is that there is an environmental reservoir of permanent mutators in nosocomial settings. An association of a history of ICU admission with detection of strong mutators may support this hypothesis, although the number of such patients was small. Further investigations including environmental isolates would help to verify this hypothesis.

Our study has some limitations. First, the number of isolates was small, and they were obtained only from surgical inpatients. We chose this population because more than two-thirds of all *E. cloacae* isolates in our hospital are derived from these patients. Although the high prevalence of mutators associated with multiple antimicrobial resistance is remarkable, the assessment of the clinical factors affecting mutation frequencies may have been limited.

Second, the molecular mechanisms involved in the higher mutation frequency were not investigated. Our primary aim was to determine the mutation frequencies of *E. cloacae* and their association with antimicrobial resistance and patient factors, so the molecular mechanisms are beyond the scope of this study.

In conclusion, strong mutators are highly prevalent among surgical isolates of *E. cloacae*. The elevated mutation frequency was associated with antimicrobial resistance and repetitive detection, and may contribute to the adaptability of this species in nosocomial settings.

Transparency Declaration

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Regional spread of *vanA*- or *vanB*-positive *Enterococcus gallinarum* in hospitals and long-term care facilities in Kyoto prefecture, Japan

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SUMMARY

Following an outbreak of *vanA*-positive *Enterococcus faecium* in 2005 in Kyoto prefecture, regional surveillance of vancomycin-resistant enterococci (VRE) was initiated. This revealed *vanA*- or *vanB*-positive *Enterococcus gallinarum* in multiple facilities. Eighty-eight *vanA*-positive *E. gallinarum* faecal carriers from 12 facilities and ten *vanB*-positive *E. gallinarum* faecal carriers from eight facilities were found. Pulsed-field gel electrophoresis profiles of the first isolate from each facility showed that 11 of the 12 *vanA* isolates and three of the eight *vanB*-positive *E. gallinarum* isolates belonged to a single clone. This study confirms the clonal spread of *vanA*- or *vanB*-positive *E. gallinarum* in a region and underlines the importance of surveillance of VRE for the presence of vancomycin resistance determinants.

Key words: Antibiotic resistance, *Enterococcus*, infectious disease epidemiology.

INTRODUCTION

Enterococci are important nosocomial infection pathogens with *Enterococcus faecalis* and *E. faecium* being the most prevalent in humans. The spread of vancomycin-resistant enterococci (VRE), i.e. *vanA*- or *vanB*-positive *E. faecalis* or *E. faecium*, in the hospital environment may make it difficult to treat infections and leads to an increased risk of mortality and higher costs associated with prolonged stay of patients in hospitals [1–3].

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The motile enterococci, *E. gallinarum* and *E. casseliflavus/flavescens*, are characterized by the presence of the *vanC* gene cluster and show low-level resistance to vancomycin [4, 5]. However, acquisition of a *vanA* or *vanB* gene cluster results in high-level resistance to vancomycin [6]. *E. gallinarum* and *E. casseliflavus/flavescens* have been shown to colonize the intestinal tracts of both hospitalized and non-hospitalized individuals [7, 8] but they are not considered to be important factors in nosocomial infection control. Since drug susceptibility testing is rarely implemented for isolates from non-sterile sites, they are sporadically detected during surveillance of VRE [9–12]. There are few reports of infections by *vanA*- or *vanB*-positive *E. gallinarum* and little data regarding their clinical importance. Two cases of sepsis due to *vanA*-positive

E. gallinarum have been described [13, 14] and two outbreaks in Argentina and Brazil have been reported [15, 16]. There are no epidemiological reports regarding regional spread of *vanA*- or *vanB*-positive *E. gallinarum*.

In 2005, an outbreak occurred in a hospital located in the southern district of Kyoto City in which more than 100 faecal carriers of *vanA*-positive *E. faecium* were detected. Following this outbreak, we began conducting regional surveillance of VRE annually and promoted screening of clinical faecal samples using selective agar culture in hospitals in Kyoto prefecture. Consequently *vanA*- or *vanB*-positive *E. gallinarum* were detected in multiple hospitals and long-term care facilities (LTCFs) in this region. This paper reports the results of the surveillance exercise and the molecular characterization of the isolates recovered.

MATERIALS AND METHODS

Regional surveillance and sample collection

Kyoto prefecture (population about 2·6 million) is located in the middle of Japan. The capital, Kyoto City, has a population of about 1·5 million inhabitants accounting for 58% of Kyoto prefecture.

Two types of VRE surveillance were performed and prospectively presumptive VRE samples were collected from hospitals and LTCFs in this region. The first was an annual surveillance from 2005 to 2008 in which about 100 hospitals and 40–60 LTCFs (accounting for more than 50% of prefectural hospitals and LTCFs) participated. The number of samples per facility was about 10% of capacity. The second approach was an enhanced VRE screening programme of clinical faecal samples through the collaboration of hospitals and clinical reference laboratories. This programme commenced in 2006 and was performed during the same period as the annual surveillance; about 60% of prefectural hospitals participated but LTCFs were not involved in this programme.

Patients who met more than one of the following criteria were selected: urinary and/or faecal incontinence; had nasogastric feeding tubes or had undergone gastrostomy; presence of urethral catheters; received antimicrobial chemotherapy within the previous 2 weeks, or had undergone surgery within 1 month. Each patient was assigned by facility personnel and not identified by name.

Microbiological methods

In the annual surveillance, rectal swabs or faecal samples were inoculated into 10 ml bile aesculin azide broth containing 15 μg/ml vancomycin (Nissui Pharmaceutical Co. Ltd, Japan). Preliminary experiments had confirmed that multiple strains of *vanB*-positive *E. faecalis*, with minimum inhibitory concentrations (MICs) of 4–8 μg/ml, grew well in this broth. After incubation at 35 °C for 48 h, the broth samples with dark brown or black discoloration were streaked on VRE selective agar® (Nippon Becton, Dickinson and Company, Japan) containing 32 μg/ml vancomycin and *vanA*/*vanB* inducing agents [17]. Samples from the enhanced clinical surveillance were streaked directly on the VRE selective agar.

Presumptive VRE isolates from the selective agar were subcultured to 5% sheep blood agar (Eiken Chemical Co. Ltd, Japan). Seven primer sets targeting the genes *vanA*, *vanB*, *vanC1*, *vanC2*/*C3*, *E. faecalis*-specific, *E. faecium*-specific, and *rrs* (16S ribosomal RNA) were used for multiplex PCR as previously described [18, 19]. The species were identified by a motility test, production of a yellow pigment [5, 20, 21], and multiplex PCR. *E. gallinarum* was confirmed if an isolate was motile and *vanC1*-positive.

Pulsed-field gel electrophoresis (PFGE)

The first isolate from each facility was subjected to typing by PFGE using *Sma*I enzyme (New England Biolabs, USA) as previously described [22, 23]. Electrophoresis was performed in a Genepath System (Bio-Rad, USA) with pulse times increasing from 1·0 to 14·0 s for 18·5 h at 200 V (6 V/cm). Genetic relatedness was analysed with the aid of Gel Compar II (Applied Maths, Belgium). Dendrograms of percentage similarity were calculated using Pearson's correlation coefficient and represented by the unweighted pair-group method with mathematical averages algorithm. A cut-off of 92% similarity was set to cluster strains as belonging to the same clone, according to Morrison *et al.* [24].

As a control for genetically related bacterial strains, six isolates of both *vanA*- and *vanB*-negative *E. gallinarum* detected during this surveillance period were used. Three isolates were recovered in Kyoto prefecture, and the others were each from different prefectures in Japan (Osaka, Miyagi, Fukuoka).

Table 1. *Number of isolates of* vanA- *or* vanB-*positive* E. gallinarum *in Kyoto prefecture*

Facility	No. of patients		Detection of VRE other than *E. gallinarum*	
	vanA (+) *E. gallinarum*	*vanB* (+) *E. gallinarum*	*vanA* (+) *E. faecium*	*vanB* (+) *E. faecium*
A		2		
B	2		+	
C	2		+	
D	2	1	+	+
E	57		+	
F	3		+	
G	10	1		
H		1	+	
I		1		+
J		2		+
K		1		+
L	1			
M	1		+	+
N	1			+
O	5			
P		1		
Q	3		+	
R*	1			

Numbers indicate the number of patients with *vanA-* or *vanB*-positive *E. gallinarum*.
+ Indicates the facilities in which *vanA-* or *vanB*-positive *E. faecium* were concurrently detected during the study period.
* Indicates long-term care facility (facility R).

Antimicrobial susceptibility testing

Isolates were tested for susceptibility (MIC) to ampicillin, erythromycin, vancomycin and levofloxacin (Eiken Chemical Co. Ltd.) using a microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [25].

RESULTS

Regional surveillance and bacterial strains

The number of samples collected during annual surveillance was 2872 in 2005, 2451 in 2006, 2406 in 2007, and 2735 in 2008; for the enhanced clinical laboratories screening programme the number of samples was 11 820 in 2006, 17 184 in 2007 and 14 748 in 2008.

Table 1 shows that 88 patients with *vanA*-positive *E. gallinarum* were found in 11 hospitals and one LTCF and 10 patients with *vanB*-positive *E. gallinarum* were detected in eight hospitals. Multiple patients were identified in ten hospitals: eight hospitals had 2–57 patients with *vanA*-positive *E. gallinarum*, and two hospitals each had two patients with

vanB-positive *E. gallinarum*; both *van* genotypes were found in two hospitals. Seven of the 11 hospitals with *vanA*-positive *E. gallinarum* also harboured patients with *vanA*-positive *E. faecium* and in four of the eight hospitals with *vanB*-positive *E. gallinarum*, *vanB*-positive *E. faecium* was also detected concurrently during the study period.

Figure 1 shows that during the 3 years following an outbreak of *vanA*-positive *E. faecium* at facility E in 2005, carriers of *vanA-* or *vanB*-positive *E. gallinarum* were found in multiple hospitals. The latter genotyopes were not detected in 2005, but in 2006, they began to be recovered in the South district of Kyoto City which includes the hospital where the first outbreak of VRE occurred. Spread of these genotypes was evident through the northern district of Kyoto City in 2007, and subsequently outside the city.

PFGE typing and antimicrobial susceptibility

Figure 2 shows the PFGE profiles of 23 isolates from Kyoto facilities and three controls of *vanA-* or *vanB*-positive *E. gallinarum* from each facility; 12 isolates

Region		2005		2006		2007		2008	
Isolate		Ag	Bg	Ag	Bg	Ag	Bg	Ag	Bg
Northern area									
Kyoto City	Northern district			J(2)		K(1)		P(1)	
Kyoto City	Southern district	B(2) C(2) D(2) E(57) R(1)		A(2) D(1) N(1)	F(3) G(10)	H(1) I(1)		M(1) O(5) Q(3)	G(1)
Southern area						L(1)			

Fig. 1. Regional spread of vanA- or vanB-positive E. gallinarum after first outbreak of vanA-positive E. faecium. ▲, The first outbreak of vanA-positive E. faecium occurred at facility E in 2005. A–R indicates the number of facilities; values in parentheses indicate the number of patients with vanA- or vanB-positive E. gallinarum; Ag, vanA-positive E. gallinarum; Bg, vanB-positive E. gallinarum.

were vanA-positive, eight vanB-positive and six were negative for both elements. Eleven clones were distinguished with the largest single group consisting of 14 isolates (11 vanA-positive, three vanB-positive). The other five vanB-positive isolates fell into four clones. These clones along with three vanA- and vanB-negative isolates (P, S, N) were clearly distinguishable from the predominant clone; two of the latter isolates clustered together on the dendrogram. The control vanA- and vanB-negative isolates from other prefectures were distinct from the major clone.

Eighteen of 20, i.e. all vanA-positive E. gallinarum isolates and six of eight vanB-positive E. gallinarum isolates, showed characteristic susceptibility patterns being susceptible to ampicillin and resistant to both erythromycin and levofloxacin. All vanA- or vanB-positive E. gallinarum isolates were resistant to vancomycin.

DISCUSSION

E. gallinarum organisms carrying vanA or vanB genes have been detected in various environments (e.g. soil, water) and animals (e.g. chickens, other poultry, pigs) [12, 26, 27]. Only a few isolates of E. gallinarum carrying vanA or vanB genes have been identified in human faecal samples during and/or after outbreak surveillance of non-motile VRE [9–12]. To our knowledge, two cases of sepsis due to vanA-positive E. gallinarum [13, 14] and two possible outbreaks

with this genotype have been reported [15, 16]. One described 15 isolates from an intensive care unit (ICU) of a hospital in Argentina comprising two clonal types defined by SmaI PFGE [15] and the other described seven isolates of the same clone from faecal carriers over 3 months in a university hospital in Brazil [16].

This report is to our knowledge the first to document regional spread of vanA- or vanB-positive E. gallinarum. This was an unexpected finding following an outbreak of vanA-positive E. faecium. Despite there being no evidence of gene transfer in this region, previous reports have described vanA genes as being transmissible between E. faecium and E. gallinarum [15, 28].

We have no evidence of the movements of individual VRE-positive patients, since we were not able to follow the course of each individual VRE carrier in this study. However, circumstantial evidence suggests that the spread of VRE from facility E, one of the core hospitals in Kyoto City had occurred as the transfer of patients to or from this facility is routine. Further, in multiple hospitals or LTCFs included in this study, some VRE-positive patients were confirmed to have been transferred from facility E. We acted as infection control consultants in facility E and advised intensive screening and hygienic precautions. However, large-scale, ongoing patient movements in this hospital made it difficult and time-consuming to successfully prevent or even curtail the movements of VRE
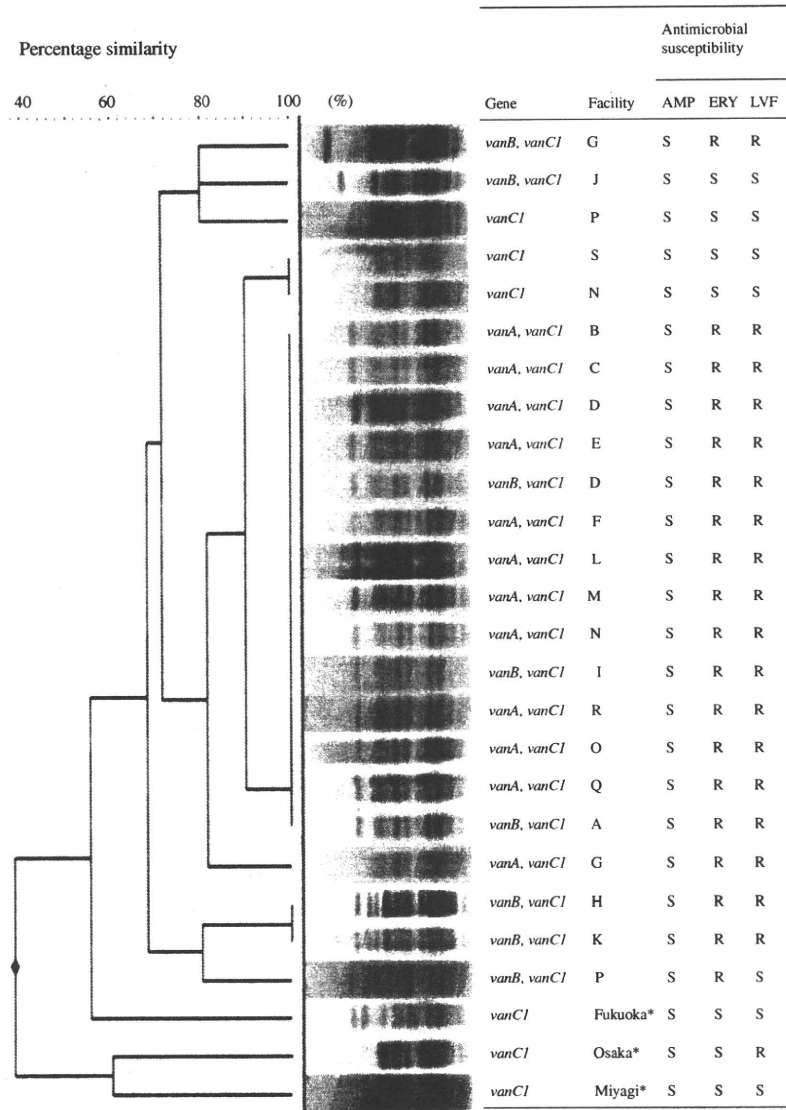
Fig. 2. PFGE profiles and antimicrobial resistance patterns of *Enterococcus gallinarum* isolates included in the study. * Other regions in Japan. AMP, Ampicillin; ERY, erythromycin; LVF, levofloxacin.

carriers, resulting in regional spread from this centre during the surveillance period.

Since *E. gallinarum* has been shown to colonize the intestinal tract, drug susceptibility testing is rarely conducted for isolates from non-sterile sites. The *vanC1* gene is intrinsic to *E. gallinarum* in which it mediates low-level resistance to vancomycin [5]. Even when vancomycin-resistant *E. gallinarum* is detected, we usually do not conduct susceptibility testing or PCR for of resistance genes and therefore may overlook *vanA*- and/or *vanB*-resistance elements in this species.

The motile enterococci account for 3–8% of all enterococcal bacteraemia cases [29, 30] and severe infections of various body sites due to *E. gallinarum*

have been reported, and *vanA*- and *vanB*-positive strains can be relatively difficult to treat [29–33]. Infection control measures are required to stem the spread of these genotypes. Moreover, as *E. gallinarum* may act as a reservoir for the *vanA* or *vanB* gene, motile enterococci should be screened for the presence of these genes, especially in regions or facilities where VRE is endemic.

This study has limitations. We collected faecal samples from anonymous patients, and thus obtained no information about their clinical backgrounds, such as whether any of the cases were carriers or had symptomatic infections. We also could not follow patient transfers between hospitals or LTCFs. Therefore, we were unable to determine whether or not

vanA- or *vanB*-positive *E. gallinarum* were spread by direct transfer. Despite these limitations, this study confirms the regional spread of these organisms and emphasizes the need for their surveillance.

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DECLARATION OF INTEREST

None.

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