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ORIGINAL ARTICLE

Increasing resistance rate to carbapenem among blood culture isolates of *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* in a university-affiliated hospital in China, 2004–2011

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The objective of this study is to investigate the profile of antimicrobial resistance of Gram-negative bacteria in blood cultures from 2004–2011. Pathogens from positive blood cultures were subcultured, and identified in the First Affiliated Hospital of Nanjing Medical University from January 2004 to December 2011. The antibiotic resistance pattern was analyzed by WHONET 5.4. A total of 1224 cases of Gram-negative bacterial isolates were documented, accounting for 38.6% of the total pathogens isolated from positive blood cultures in the 8-year period. The isolation rates of *Klebsiella pneumoniae* and *Acinetobacter baumannii* increased nearly three times over the same time span. Most Gram-negative bacteria isolates, except the isolates of *Pseudomonas aeruginosa*, showed a significantly increased resistance rate to cephalosporins (in particular third/fourth generation cephalosporins). Noteworthy, the antimicrobial resistance of *K. pneumoniae*, *A. baumannii* and *P. aeruginosa* isolates to carbapenem (imipenem and meropenem) was significantly increased and the resistant rate to carbapenem was >80.0% in *A. baumannii* in 2011. The results from PCR detection for carbapenemases were as follows: 82.8% (24/29) isolates of *K. pneumoniae* carried the *kpc-2* gene; only three metallo-beta-lactamase-positive *P. aeruginosa* isolates were detected; and 93.1% (67/72) *A. baumannii* isolates were *blaOXA-23* positive. The antimicrobial resistance rate of Gram-negative bacteria isolated from blood cultures significantly increased from 2004 to 2011, with significant resistance to the third/fourth generation cephalosporins and carbapenem.

INTRODUCTION

Bloodstream infection (BSI) is a systemic infectious disease. Severe BSI can cause septic shock, disseminated intravascular coagulation and multiple organ dysfunction syndrome. BSI accounts for 10.0–20.0 mortality% of all nosocomial infections and is the eighth leading cause of mortality (15.0%) in the United States.[1–3] Blood culture is the most direct method for the detection of BSI in patients.

The epidemiology of invasive pathogens has changed dramatically over the years[4–6] with an increase in resistance to many antibiotic drugs[4] resulting in a reduction in therapeutic options.[5] The emergence of antibiotic resistance has become a global public health problem, especially for Gram-negative bacteria, as there is a lack of novel antibiotics directed against these organisms. For example, carbapenem, the agents of last resort against multidrug-resistant Enterobacteriaceae, are under threat with a growing incidence of pan-resistant isolates.[7,8] A study has observed that inadequate therapy is the common reason for antimicrobial resistance.[9] Information about the detection of pathogens and antibiotic resistance pattern are now seen as decisive for optimizing treatment.[10] Thus, surveillance of BSI pathogens along with their antimicrobial resistance patterns is the key for prevention.

In this study, the profile and the antibiotic resistance patterns of Gram-negative bacteria, isolated from the blood culture in the First Affiliated Hospital of Nanjing Medical University from January 2004 to December 2011 were retrospectively reviewed to provide information on changes in antibiotic resistance patterns over time, and then it would provide evidence for clinical therapy.

MATERIALS AND METHODS

Patients

Our study was undertaken in a general teaching hospital with 2200 hospital beds. All the blood culture specimens were collected between 1 January 2004 and 31 December 2011 (single bottle aerobic culture for most specimens). At least 5 ml of blood was inoculated into each culture bottle. The blood culture was collected from patients with symptoms or signs of bacteremia before antibiotic therapy and sent immediately to the microbiology laboratory for

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processing. If the same organism was repeatedly isolated from the same patient, only the first isolate was included in the analysis. Collection and culture of the blood specimens were performed according to standard operating procedures, following the Clinical and Laboratory Standards Institute guidelines.[11]

Bacterial identification

The Bactec Fluorescent series 9240 (Becton Dickinson, Franklin Lakes, NJ, USA) instrument was used for blood culture analysis. When a positive alert was detected, the particular bottle was removed and Gram stain and subculture were on blood agar and chocolate agar plates, respectively. Specifically for anaerobic cultures, an additional anaerobic blood agar was performed. Bottles with no or negative alert were kept in the instrument for 7 days and subsequently subcultured to confirm the absence of bacterial organisms before being regarded as negative bottles. Gram-negative bacterial isolates were confirmed by API system (Mérieux, Lyon, France) and the VITEK-2 compact automatic identification system (Mérieux).

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was performed predominantly by Kirby-Bauer disk diffusion method, following the Clinical and Laboratory Standards Institute guidelines.[11] Susceptibilities to the following antibiotics were tested: cephalosporins (cefazolin, cefuroxime, ceftazidime, cefotaxime and cefepime), monobactams (aztreonam), carbapenem (imipenem and meropenem), compound agents (amoxicillin/clavulanate, piperacillin/tazobactam and cefoperazone/sulbactam), aminoglycosides (amikacin) and fluoroquinolones (ciprofloxacin and levofloxacin). Microbiologic data were extracted from the laboratory information system and converted into a standard format using WHONET 5.4 (WHO, Geneva, Switzerland), with duplicates eliminated according to the guidelines of the Clinical and Laboratory Standards Institute guidelines. The control strains included were as follows: *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 700603 and *Pseudomonas aeruginosa* ATCC 27853.

PCR-based detection of genes encoding carbapenemases

All of the 130 carbapenem-resistant isolates, which includes one *E. coli* isolate, 29 *K. pneumoniae* isolates, 28 *P. aeruginosa* isolates and 72 *Acinetobacter baumannii* isolates, were subjected to PCR analysis to detect genes encoding carbapenemases. These genes include *blaOXA-23*, *blaOXA-24*, *blaOXA-48*, *blaIMP*, *blaVIM*, *blaNDM* and *blaKPC*. PCR was performed on a PTC-100-type PCR instrument (MJ Research Inc., USA). DNA was extracted as previously described.[12] The reaction conditions were as follows: pre-denaturation at 94 °C for 4 min, followed by 30 amplification cycles of 94 °C

for 1 min, 55 °C for 1 min and 72 °C for 1.5 min, with a final extension step of 72 °C for 10 min. Primers used in this study were listed in Table 1.[13–17] Purification of PCR products and DNA sequencing were performed by GenScript Co., Ltd Similarity and alignment searches for the nucleotide sequences were analyzed using BLAST (http://www.ncbi.nlm.nih.gov/BL AST).

Statistical analysis

Statistical analysis was accomplished by WHONET 5.4, the microbiology laboratory data management software recommended by World Health Organization. If the number of isolates was <10, the antimicrobial susceptibility data were not included in the overall analysis.

RESULTS

Pathogens distribution

A total of 18 745 blood culture samples were collected over a period of 8 years, with a pathogen-positive rate of 16.9% (3168/18 745). Of those pathogens, 38.6% (1224/3168) were Gram-negative bacteria. An increased trend in the isolation rate of Gram-negative bacteria, from 32.2% (94/292) in 2004 to 47.5% (286/602) in 2011, was observed, greater than that of Gram-positive bacteria (45.0%) in 2011. *K. pneumoniae* and *A. baumannii* were found increased observably in this study. The top nine frequently detected bacterial species or groups were: coagulase-negative staphylococci, *E. coli*, *Staphylococcus aureus*, *K. pneumoniae*, fungi, *Streptococcus*, *Enterococcus*, *A. baumannii* and *P. aeruginosa* (Figure 1).

Antimicrobial resistance of Enterobacteriaceae

An increased resistance rate to cephalosporins was seen among most Enterobacteriaceae species. In *E. coli*, the resistant rate was >75.0% to all cephalosporins in 2011, and significantly increased trends were observed to cefuroxime, ceftazidime, cefotaxime and cefepime (Table 2). Resistance to cephalosporins was also seen for *K. pneumoniae* isolates during the study period. For example, the resistance rate to ceftazidime was 15.4% in 2005, but increased significantly to 62.5% in 2011. The same trend was also observed in terms of resistance to other cephalosporins (Table 3).

Resistance to antibiotics containing β-lactamase inhibitors such as amoxicillin/clavulanate, cefoperazone/sulbactam and piperacillin/tazobactam also showed increasing trends in both *E. coli* and *K. pneumonia* isolates. Although *E. coli* isolates retained high susceptibility to carbapenem (imipenem and meropenem), *K. pneumoniae* isolates showed significantly increased resistance rates to imipenem and meropenem over the 8-year period, from 0.0% in 2005 to almost 30.0% in 2011 (Tables 2 and 3). No obvious increasing trend was found for resistance to aztreonam, amikacin and levofloxacin in either *E. coli* or *K. pneumonia* isolates. The resistance rate to amikacin was relatively low compared to the three antibiotics mentioned above for both *E. coli* and *K. pneumonia* isolates.
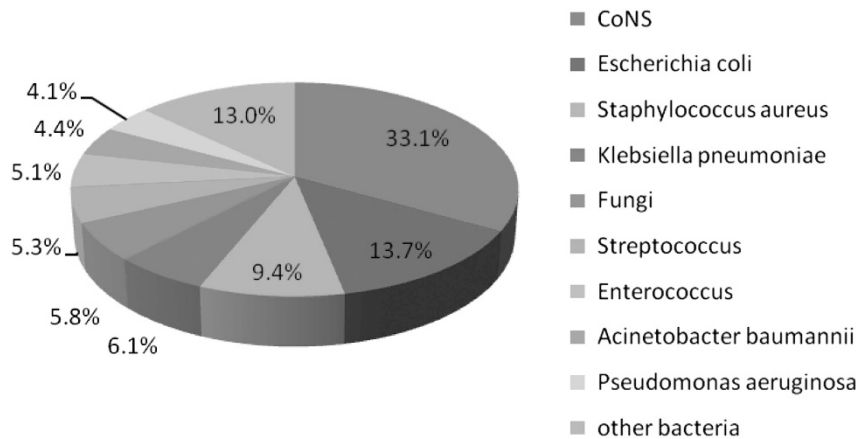
Antimicrobial resistance of non-fermenting bacteria

Extremely high levels of cephalosporin resistance were observed for *A. baumannii*. In 2011, the resistance rates to ceftazidime, cefotaxime and cefepime were 95.6, 100.0 and 91.3%, respectively. For *P. aeruginosa* isolates, the resistance rates to ceftazidime and cefepime were <40.0%, however, a resistance rate of 85.7% was observed to cefotaxime (Tables 4 and 5). Compared to *A. baumannii* isolates, *P. aeruginosa* isolates showed relatively lower resistance rate to compound agents (<45.0%). Strikingly, both *P. aeruginosa* and *A. baumannii* isolates showed significant increase in carbapenem resistance during the study period. In 2008, the resistance rates to imipenem and meropenem for *P. aeruginosa* were 7.1 and 7.6%,

Table 1 Primers used in this study

Primer	Sequence (5′–3′)	Length	Reference
OXA-23	F:GATGTGTCATAGTATTCGTCG	1067	1
	R:TCACAACAACTAAAAGCACT		
OXA-24	F:GTACTAATCAAAGTTGTGAA	1024	1
	R:TTCCCCTAACATGAATTTGT		
OXA-48	F:TTGGTGGCATCGATTATCGG	744	2
	R:GAGCACTTCTTTTGTGATGGC		
KPC	F:CGTCTAGTTCTGCTGTCTTG	798	3
	R:CTTGTCATCCTTGTTAGGCG		
NDM	F:ATGGAATTGCCCAATATTATGC	490	4
	R:CGAAAGTCAGGCTGTGTTG		
VIM	F:GTTTGGTCGCATATCGCAAC	382	5
	R:AATGCGCAGCACCAGGATAG		
IMP	F:GAAGGCGTTTATGTTCATAC	587	5
	R:GTATGTTTCAAGAGTGATGC		

Abbreviations: IMP, Imipenemase; KPC, Klebsiella pneumoniae Carbapenemase; NDM, New Delhi metallo-beta-lactamase; OXA, oxacillinase; VIM, Verona imipenemase.

- CoNS
- Escherichia coli
- Staphylococcus aureus
- Klebsiella pneumoniae
- Fungi
- Streptococcus
- Enterococcus
- Acinetobacter baumannii
- Pseudomonas aeruginosa
- other bacteria

Figure 1 The distribution of top nine pathogens in blood cultures from 2004 to 2011 in the First Affiliated Hospital of Nanjing Medical University. CoNS is coagulase-negative staphylococci. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Table 2 Resistance of *Escherichia coli*, 2004 to 2011

Antibiotics	2004 (n = 19) (r = 6.5%)	2005 (n = 41) (r = 13.5%)	2006 (n = 46) (r = 12.5%)	2007 (n = 64) (r = 18.2%)	2008 (n = 60) (r = 15.2%)	2009 (n = 71) (r = 16.2%)	2010 (n = 53) (r = 12.7%)	2011 (n = 79) (r = 13.1%)
Amoxicillin/clavulanate	10.5	7.3	23.6	17.2	22.4	26.6	34.6	26.9
Cefoperazone/sulbactam	5.2	2.4	14.8	6.2	8.3	11.3	22.9	19.2
Piperacillin/tazobactam	0.0	4.9	13.5	18.3	11.9	10.3	16.7	10.1
Cefazolin	66.7	68.4	79.2	62.9	70.7	76.4	64.6	75.9
Cefuroxime	61.8	69.4	73.1	62.3	70.7	79.7	74.5	77.3
Ceftazidime	31.6	16.8	16.4	22.2	71.2	78.8	66.7	75.9
Cefotaxime	31.4	45.5	66	53.1	71.2	72.1	68.8	78.9
Cefepime	47.4	26.8	37	41.9	70.2	77.9	70.6	75.9
Aztreonam	57.9	23.7	27.8	31.1	70.7	75.4	70.6	75.9
Imipenem	0.0	0.0	0.0	0.0	0.0	0.0	2.8	0.0
Meropenem	0.0	0.0	0.0	0.0	0.0	0.0	2.8	0.0
Amikacin	11.1	23.7	3.7	15.6	11.7	17.2	22.4	30.4
Levofloxacin	72.2	57.5	66.7	79.7	71.4	82.8	55.3	60.3

respectively; these rates increased rapidly to 45.9 and 46.5% in 2011. In *A. baumannii* isolates, the resistance rates to imipenem and meropenem were both only 10.0% in 2008, which raised to 83.5 and 82.1%, respectively, in 2011.

Molecular characterization

No genes encoding carbapenemases were found in *E. coli* isolates. Among the 29 isolates of *K. pneumoniae*, 82.8% (24/29) carried the *kpc-2* gene and no other carbapenemase-encoding gene was detected. Among the 28 carbapenem-resistant *P. aeruginosa* isolates, three metallo-beta-lactamase-positive strains were confirmed by PCR and DNA sequence analysis, including two strains positive for VIM-2 and one strain positive for *IMP-1*. Among the 72 isolates of *A. baumannii*, there were 67 isolates harboring an *OXA-23*-like gene and one strain harboring an *OXA-24* gene. Neither *blaOXA-48* nor *blaNDM* was amplified in any of these isolates.

DISCUSSION

In this study, *E. coli* remained the primary Gram-negative pathogen causing BSI in the recent 8-year period. In fact, Gram-negative bacteria have been encountered more frequently in blood culture than Gram-positive bacteria in studies conducted in Iran (52.4% vs 47.6%) and in Northeastern Malaysia (55.2% vs 42.1%).[18,19] Because of the long-term use of antibiotics[13,14] the incidence of antibiotic-resistant pathogens, such as *A. baumannii* and *K. pneumoniae*, have gradually increased. A substantial increase in the isolation rate of *K. pneumoniae* and *A. baumannii* was found during this study. The isolation rate of *A. baumannii* significantly increased from 1.8% (2005) to 7.6% (2011), more than that of *P. aeruginosa* since 2009. *A. baumannii* generally infects patients with low immunity, old age or immunosuppressive medications. In this study, most blood samples positive for *A. baumannii* came from departments of respiratory medicine, hematology, neurology, burn and intensive care units, because the patients in these sections were all in critical condition,

Table 3 Resistance of *Klebsiella pneumoniae*, 2005–2011

Antibiotics	2005 (n = 12) (r = 3.7%)	2006 (n = 19) (r = 5.6%)	2007 (n = 13) (r = 3.7%)	2008 (n = 14) (r = 3.5%)	2009 (n = 22) (r = 5.0%)	2010 (n = 44) (r = 10.5%)	2011 (n = 65) (r = 10.8%)
Amoxicillin/clavulanate	16.7	5.6	25	21.4	15.8	55.6	45.2
Cefoperazone/sulbactam	7.7	5.9	15.4	7.1	7.1	37.1	37.7
Piperacillin/tazobactam	15.4	5.9	23.1	7.7	19	36.8	50.8
Cefazolin	50.0	52.2	62.5	28.6	28.6	71.1	64.5
Cefuroxime	55.6	54.5	62.5	35.7	38.1	71.4	64.5
Ceftazidime	15.4	13	12.5	28.6	23.8	73.7	62.5
Cefotaxime	41.7	45.5	37.5	28.6	23.5	70.6	61.3
Cefepime	23.1	18.2	20.0	28.6	23.8	73.7	66.7
Aztreonam	33.3	21.7	20.0	30.8	23.8	73.7	64.1
Imipenem	0.0	0.0	0.0	0.0	4.8	10.8	30.0
Meropenem	0.0	0.0	0.0	0.0	4.8	8.7	35.5
Amikacin	7.7	11.1	30.8	7.1	30.0	26.3	23.4
Levofloxacin	15.4	23.5	23.1	20	13.3	42.1	47.6

Table 4 Resistance of *Pseudomonas aeruginosa*, 2008–2011

Antibiotics	2008 (n = 17) (r = 4.3)	2009 (n = 17) (r = 3.9)	2010 (n = 13) (r = 2.9)	2011 (n = 37) (r = 6.2)
Cefoperazone/sulbactam	0.0	11.1	44.4	33.3
Piperacillin/tazobactam	12.5	29.4	44.4	32.4
Ceftazidime	5.9	5.9	33.3	37.8
Cefotaxime	82.4	100	77.8	85.7
Cefepime	17.6	17.6	33.3	27.8
Aztreonam	5.9	12.5	55.6	21.6
Imipenem	7.1	17.6	44.4	45.9
Meropenem	7.6	18.8	45.6	46.5
Amikacin	5.9	18.2	20	45.9
Ciprofloxacin	0.0	0.0	57.1	44.8
Levofloxacin	0.0	7.1	71.4	43.2

'r' means the percentage of *P. aeruginosa* strains among all strains isolated from blood culture. In 2004–2007, the percentage were 7.2% (21/292), 4.0% (13/327), 2.9% (10/342) and 0.9% (3/352), respectively.

Table 5 Resistance of *Acinetobacter baumannii*, 2008–2011

Antibiotics	2008 (n = 11) (r = 2.8%)	2009 (n = 26) (r = 5.9%)	2010 (n = 28) (r = 6.7%)	2011 (n = 46) (r = 7.6%)
Amoxicillin/clavulanate	81.8	83.3	90.5	94.7
Cefoperazone/sulbactam	9.1	26.7	71.4	76.3
Piperacillin/tazobactam	54.5	80.8	85.7	95.7
Ceftazidime	63.6	84.0	95.0	95.6
Cefotaxime	72.7	96.0	100.0	100.0
Cefepime	54.5	76.0	76.2	91.3
Aztreonam	40.0	96.2	81.0	100
Imipenem	10.0	47.6	76.2	83.5
Meropenem	10.0	44.0	76.2	82.1
Amikacin	72.7	53.8	76.2	56.5
Levofloxacin	45.5	69.2	76.2	69.6

'r' means the percentage of *A. baumannii* strains among all strains isolated from blood culture. In 2004–2007, the percentage were 3.1% (9/292), 1.8% (6/327), 2.0% (7/342) and 2.6% (9/352), respectively.

showing tracheostomy, low immunity, long-term hospitalization or suffering from various underlying diseases.

The resistance rate of Enterobacteriaceae to cephalosporins increased significantly, especially to third/fourth generation cephalosporins. The resistance rates of *E. coli* and *K. pneumoniae* to cephalosporins were all >60.0% in 2011. Similar results were also reported in India,[20] which was different from that in Europe and America.[21,22] Our results also indicated that Enterobacteriaceae retained a high susceptibility to carbapenem, compared with that of non-fermenting bacteria. Nevertheless, 30.0% of *K. pneumoniae* isolates were imipenem-resistant in 2011 compared with 0.0% in 2004, and the number was only 4.3% in the USA.[23] This discrepancy is possibly related to the prevalence of Klebsiella pneumoniae Carbapenemase (KPC) carbapenemase.[24] The results from PCR analysis indicate that the *blaKPC-2* gene is the main reason for carbapenem resistance in *K. pneumoniae* isolates in our hospital.

P. aeruginosa is a common Gram-negative nosocomial pathogen. Because of the wide use of broad-spectrum antibiotics, the emergence of multidrug-resistant *P. aeruginosa* has continually increased, and the stains were often carbapenem-resistant, which can severely limit therapeutic options.[25] In this study, only the resistance rate of *P. aeruginosa* to carbapenem showed an increasing trend from 0.0% in 2004 to >30.0% in 2011. Moreover, carbapenem-resistant *P. aeruginosa* has also been observed more frequently in Europe and North America.[26,27] Li et al.[28] found that the efflux pump on the cell membrane of *P. aeruginosa* is one of the main reasons for its multidrug resistance and the outer membrane protein OprM is the most common one among six efflux pumps. A study from Korea demonstrated that the major factor leading to carbapenem resistance in *P. aeruginosa* isolates is a result of a loss or decreased levels of OprD that leads to cell membrane impermeability.[29] Moreover, only three *P. aeruginosa* isolates were shown to carry the genes coding for

carbapenemases, which indicated that carbapenemases were not the main mechanism for carbapenem resistance in *P. aeruginosa* in this study.

Noteworthy, the antibiotic resistance of *A. baumannii* has become increasingly serious especially in terms of resistance to carbapenem (imipenem and meropenem), with the resistance rate rising from 10.0% in 2008 to >80.0% in 2011. Similar resistance rates were also found in Londrina, Brazil.[30] Earlier reports indicated that *A. baumannii* was the cause of infection outbreaks in hospitals,[31,32] and the multidrug resistance patterns among *A. baumannii* isolates often left carbapenem as the only effective treatment for severe infections.[32] Today, carbapenem-resistant *A. baumannii* stains are an emerging threat worldwide and have been observed in several countries,[30,31,33–37] although rare in Canada.[38] Once carbapenem-resistant *A. baumannii* emerged, a pan-drug resistant *A. baumannii* appeared; consequently, an infected patient has little chance of an effective therapy.[39] As carbapenemases are probably the major contributor to carbapenem resistance,[40] the resistance of *A. baumannii* to carbapenem is most concerning.[41] The major mechanism of carbapenem resistance in *A. baumannii* is the production of OXA β-lactamases, primarily OXA-23, OXA-66 and OXA-58.[24] In our study, most carbapenem-resistant *A. baumannii* isolates were positive for blaOXA-23, which indicated that the presence of *blaOXA-23* gene was the main factor associated with carbapenem resistance in *A. baumannii*.

In recent years, the emergence of *blaNDM* was reported in some provinces of mainland China,[42–44] with an outbreak of *NDM-1*-producing *K. pneumonia* having been reported.[45] In our study, we did not find any *NDM*-positive isolate. The carbapenem resistance is showing an increasing trend worldwide that could be due to the following factors.[46] First, the transmission mode of genes encoding carbapenemases accelerates the spread of carbapenem resistance.[47] Most genes are located on mobile gene structures and can transmit on a high speed among various bacterial species. Second, the overprescribing of carbapenem is an important factor leading to carbapenem resistance. The prevalence of extended-spectrum β-lactamase (ESBL)-producing bacteria makes carbapenem a conventional choice in the treatment of multidrug-resistant bacteria, and the long-term use of carbapenem is the main factor causing carbapenem-resistant bacterial infection in some inpatients. Other factors such as international travel,[48] inefficient measures to control hospital infection and frequent use of invasive medical devices may also be involved in the spread of carbapenem-resistant bacteria.

Our study indicated that Gram-negative bacteria have become a more significant cause of BSI, and that antimicrobial resistance is severe in this hospital. This is likely due to several factors with the major factor appearing to be excessive use of antibiotics.[49] The total annual consumption of carbapenem markedly increased, 192 times more in 2009 than in 2001 in our hospital.[50] An association between the amount of carbapenem consumption and the resistance rate to carbapenem have been reported.[20,51,52] Now a related policy has been formulated in our hospital, which restricted the right of prescribing certain antibiotics including carbapenems to specified senior physicians.

In conclusion, this 8-year study highlights the increase in the isolation rate of Gram-negative bacteria causing BSI and confirms the increase in resistance to certain antibiotics. Reducing antibiotic consumption by proper prescription should be the primary step to minimizing resistance, but antibiotic surveillance is needed in order to enhance the effectiveness of medication.

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