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International Journal of Antimicrobial Agents 24 (2004) 119–124

INTERNATIONAL JOURNAL OF Antimicrobial Agents

www.ischemo.org

Determining incidence of extended spectrum β-lactamase producing Enterobacteriaceae, vancomycin-resistant *Enterococcus faecium* and methicillin-resistant *Staphylococcus aureus* in 38 centres from 17 countries: the PEARLS study 2001–2002

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Received 18 June 2003; accepted 9 January 2004

Abstract

The PEARLS study prospectively monitored selected nosocomial pathogens from 38 centres in 13 European, three Middle Eastern countries and South Africa during 2001–2002. Extended spectrum β-lactamase (ESBL) production rates among *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter* spp. were 5.4% (142/2609), 18.2% (401/2206) and 8.8% (204/2328), respectively, for all study sites. The overall ESBL production rate for the combined Enterobacteriaceae was 10.5% (747/7143), highest in Egypt, 38.5%, and Greece, 27.4%, and lowest in The Netherlands, 2.0%, and Germany, 2.6%. IEF, PCR and DNA sequencing determined 10.7% false positives among *Enterobacter* spp. when using NCCLS guidelines to screen for ESBL production. The prevalence of nosocomial methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* was 32.4% (294/908) and 8.7% (83/949), respectively. PEARLS provides baseline data against which prospective changes in resistant determinants and outcomes can be measured in this ongoing study.

Keywords: Incidence; Resistance; Nosocomial; Extended spectrum β-lactamase; ESBL; VRE; MRSA; Methicillin; Vancomycin

1. Introduction

Antimicrobial resistance among nosocomial pathogens is a significant problem in most clinical settings adding to the cost of medical care and the morbidity and mortality of patients. The increasing occurrence of infections with antibiotic-resistant microorganisms has required the development of flexible and timely surveillance systems for monitoring these problems. Several previous studies have documented the increasing incidence of various genera of nosocomial pathogens in the past 10 years [1–3]. The Pan-European Antimicrobial Resistance Using Local Surveillance (PEARLS) study began in 2001 and is an ongoing surveillance to examine the resistance determinants and patterns of common nosocomial pathogens in 38 multi-national centres that have agreed to longitudinal follow up.

This study establishes a current baseline prevalence of extended-spectrum β-lactamase (ESBL) producers in selected Enterobacteriaceae, vancomycin-resistant *Enterococcus faecium* (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) throughout participating institutions from 17 countries. Establishment of these baseline data is crucial for follow up intervention programmes to help address the question of why ESBL rates are changing. Several studies offer compelling evidence for the association of expanded-spectrum cephalosporin usage and ESBL production in Enterobacteriaceae [4–7]. Third-generation cephalosporin use, especially oxyimino-β-lactam consumption, are correlated with trends in ESBL infections, multi-drug resistant organisms and mortality [8,9]. Also, there is some limited evidence that suggests that restricting

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0924-8579/$ – see front matter
doi:10.1016/j.ijantimicag.2004.01.010

the use of third-generation cephalosporins, usually in conjunction with barrier protection and other infection control measures, reduces the incidence of methicillin-resistant *S. aureus* infections and glycopeptide-resistant enterococcus carriers [10–12]. These baseline surveillance data will be used to identify selective pressures and determinants affecting the incidence of drug resistance in subsequent phases of the study.

2. Materials and methods

2.1. Study design

The PEARLS study collected isolates from 38 study centres in 17 countries between February 2001 and December 2002. All specimens were from clinically documented nosocomial infections isolated from blood, respiratory tract, urine (limited to no more than 30% of all isolates), skin, wound, fluids, and other well-defined sources. Only one isolate per patient and only species from the selected study group of organisms were accepted: *E. faecium*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae* and *S. aureus*. The handling, processing and testing was performed by a central laboratory, Laboratories International for Microbiology Studies (LIMS, Schaumburg, IL, USA).

2.2. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined at the central laboratory by broth microdilution testing methods as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) using broth microdilution panels purchased from Microscan® (Dade Behring Inc., Sacramento, CA, USA) [13]. Antimicrobial agents tested were: piperacillin/tazobactam, amoxicillin/clavulanic acid, cefotaxime, ceftazidime, ceftriaxone, cefepime, ciprofloxacin, vancomycin, imipenem and levofloxacin. Interpretive criteria followed published guidelines established by the NCCLS [14]. Quality control of Microscan® panels was performed using ATCC strains *E. coli*, ATCC 25922, *Pseudomonas aeruginosa*, ATCC 27853, *S. aureus*, ATCC 29213, and *Enterococcus faecalis*, ATCC 29212.

2.3. ESBL determinations

E. coli and *K. pneumoniae* were screened and confirmed for ESBL activity according to NCCLS guidelines [14]. Preliminary ESBL activity was determined by screening cefotaxime, ceftazidime and ceftriaxone with MICs $\geq$ 1 mg/l using broth microdilution panels. ESBL presence was confirmed by testing the following antibiotic disks: cefotaxime (30 μg), cefotaxime/clavulanic acid (30/10 μg), and ceftazidime (30 μg), ceftazidime/clavulanic acid (30/10 μg). Antibiotic disks were manufactured by Oxoid Inc. (Ogdensburg, New York). Mueller–Hinton agar used in testing was manufactured by Remel Inc. (Lenexa, Kansas). An organism was interpreted as containing an ESBL if there was an increase of $\geq$5 mm in the inhibition zone of the combination disc when compared with that of the cephalosporin alone: cefotaxime/clavulanic acid–cefotaxime $\geq$5 mm or ceftazidime/clavulanic acid–ceftazidime $\geq$5 mm. Quality control of antibiotic disks followed manufactures guidelines (Oxoid) using the following ATCC strains: *Klebsiella pneumoniae*, ATCC 700603 and *Escherichia coli*, ATCC 25922.

2.4. Molecular techniques

Because the NCCLS ESBL screening and confirmatory tests are recommended only for *E. coli* and *K. pneumoniae*, ESBL production among *Enterobacter* spp. was examined more closely. Isoelectric focusing (IEF), PCR and DNA sequencing was performed on a random sample of 48 Enterobacter isolates confirmed ESBL negative or positive in a 2:3 ratio. IEF techniques have been described previously by Bradford et al. [15]. PCR was performed on whole cell lysates from all isolates with visible β-lactamase bands corresponding to TEM or SHV-type enzymes using the PCR Master kit (Roche, Basel, Switzerland) with primers specific for bla_{TEM} and/or bla_{SHV}. Products generated from positive PCR reactions were cloned into the pCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA) and nucleotide sequencing performed using the Applied Biosystems automated DNA sequencing system 3700 (Foster City, CA, USA). Sequence results were analysed using the EditSeq software program (DNASTAR, Madison, WI, USA).

3. Results

3.1. Extended-spectrum β-lactamase (ESBL) producing Enterobacteriaceae

There were 7143 Enterobacteriaceae examined for ESBL production (Table 1). Of the 2609 *E. coli*, 142 (5.4%) were confirmed positive for ESBL from all centres. Overall ESBL production for *K. pneumoniae* was seen in 401 of 2206 (18.2%) isolates. Initial NCCLS screening resulted in 204 (8.8%) positive ESBL producing isolates of *Enterobacter* spp. NCCLS screening is not recommended for *Enterobacter* spp. because of the high number of false positive results. Because of this, it was necessary to perform additional confirmatory tests using molecular techniques for a subset of Enterobacter isolates to determine the incidence of false positives in this group. IEF detected β-lactamases in 46 of the 48 (96%) NCCLS screened isolates. Nineteen of these were found to express only the AmpC β-lactamase that is naturally occurring in *Enterobacter* spp. and were not positive in either the ESBL screening or confirmatory tests. For the strains where IEF showed β-lactamase bands compatible

Table 1
Incidence (%) of extended-spectrum β-lactamase (ESBL) producing Enterobacteriaceae from 7143 isolates collected from 38 centres in 17 countries

Organism	ESBL producers (*n*)[a]	Total *N*	% Total
Escherichia coli	142	2609	5.4
Klebsiella pneumoniae	401	2206	18.2
Enterobacter spp.	204	2328	8.8[b]
Enterobacteriaceae spp. (total)	747	7143	10.5

[a] Using the antimicrobial disk Phenotypic Confirmatory Tests for ESBLs as described for *Klebsiella pneumoniae* and *Escherichia coli* in NCCLS documents M100-S12, Table 2A [14].

[b] Results of sampling and DNA sequencing revealed 10.7% of *Enterobacter* ESBL producers to be false positives and, if extrapolated to all Enterobacter isolates tested, would yield a lower % total of 7.8% for this species.

with TEM or SHV-type enzymes, PCR testing resulted in 14 isolates positive for TEM type, eight isolates positive for TEM plus SHV types and five isolates positive for SHV type β-lactamases. In addition, DNA sequencing identified the following SHV and TEM type enzymes: SHV-5, SHV-12, TEM-1, TEM-4, TEM-12, TEM-24 and TEM-26. Of the 28 strains confirmed as ESBL producers by NCCLS methodology, 25 were confirmed to be positive by molecular techniques resulting in three (10.7%) false positives using the NCCLS criteria. There were no false negatives in this subset of isolates. The observed ESBL production rate of 8.8% could be crudely estimated to be 7.8%, assuming the false positive rate determined in this small subset were consistent in all the isolates of *Enterobacter* spp. The overall ESBL production rate for the Enterobacteriaceae in this study was 10.5%. The breakdown of these data by individual countries is presented in Table 2. ESBL rates varied from a low of 2% in The Netherlands to a high of 38.5% in Egypt and were approximately three-fold higher in southern Europe (13.5%) than northern Europe (4.7%).

3.2. Vancomycin-resistant *E. faecium* (VRE)

Vancomycin resistance was detected in 83 of the 949 (8.7%) *E. faecium* in this study (Table 3). The highest rate of VRE, 59.0%, was seen in the two centres from Portugal. Further analysis revealed no significant difference between the two centres: centre 1, 14/25 (56.0%); centre 2, 22/36 (61.1%). Five countries had ≤1% VRE, but they contributed fewer than 10 strains each. The incidence of VRE, excluding the countries with <10 isolates, ranged from 1.0% in France to 59.0% in Portugal.

3.3. Methicillin-resistant *S. aureus* (MRSA)

Of the 908 *S. aureus* reported in this study, 294 (32.4%) were methicillin-resistant (Table 3). Portugal (two centres) had the highest incidence of MRSA at 88.5% (46/52). Two countries, Lebanon (1/25 strains) and The Netherlands (1/50 strains), reported one isolate each. One centre in Saudi Arabia had none. Excluding the extremes of these six centres,

Table 2
Incidence (*N*/total (%)) of extended-spectrum β-lactamase (ESBL[a]) producers in 7143 isolates of Enterobacteriaceae

Country	*N*/total (%)				Totals
	Enterobacter aerogenes	*Enterobacter cloacae*	*Escherichia coli*	*Klebsiella pneumoniae*	
Northern Europe	64/371 (17.3)	14/643 (2.2)	14/1016 (1.4)	43/827 (5.2)	135/2857 (4.7)
Austria	2/60 (3.3)	1/81 (1.2)	3/152 (2)	7/109 (6.4)	13/402 (3.2)
Belgium	29/88 (28.4)	4/99 (4)	3/152 (2)	15/125 (12)	51/464 (10.1)
France	31/101 (26.7)	3/119 (2.5)	0/214 (0)	1/158 (0.6)	35/592 (5.2)
Germany	1/55 (1.8)	2/206 (1)	4/272 (1.5)	14/235 (6)	21/768 (2.6)
Switzerland	0/6 (0)	2/40 (5)	0/73 (0)	4/58 (6.9)	6/177 (3.4)
The Netherlands	1/61 (1.6)	2/98 (2)	4/153 (2.6)	2/142 (1.4)	9/454 (2)
Southern Europe	83/476 (17.4)	25/671 (3.7)	83/1265 (6.6)	281/1095 (25.7)	472/3507 (13.5)
Croatia	3/43 (7)	1/66 (1.5)	12/181 (6.6)	49/144 (34)	65/434 (15)
Greece	8/53 (13.2)	6/65 (7.7)	30/124 (24.2)	52/101 (51.5)	96/343 (27.4)
Italy	20/148 (12.2)	13/198 (5.6)	30/304 (9.9)	77/301 (25.6)	140/951 (14.3)
Portugal	47/83 (49.4)	2/86 (2.3)	5/162 (3.1)	23/128 (18)	77/459 (15.5)
Slovenia	0/9 (0)	0/54 (0)	0/74 (0)	16/72 (22.2)	16/209 (7.7)
Spain	3/106 (2.8)	1/183 (0.5)	3/289 (1)	33/240 (13.8)	40/818 (4.9)
Turkey	2/34 (5.9)	2/19 (10.5)	3/131 (2.3)	31/109 (28.4)	38/293 (13)
Other countries	4/25 (16)	14/142 (9.9)	45/328 (13.7)	77/284 (27.1)	140/779 (18)
Egypt	1/2 (50)	0/2 (0)	6/14 (42.9)	3/8 (37.5)	10/26 (38.5)
Lebanon	1/8 (12.5)	2/25 (8)	16/82 (19.5)	14/66 (21.2)	33/181 (18.2)
Saudi Arabia	1/3 (33.3)	5/30 (13.3)	16/81 (19.8)	12/69 (17.4)	34/183 (18.6)
South Africa	1/12 (8.3)	7/85 (7.1)	7/151 (4.6)	48/141 (34)	63/389 (15.9)
Totals for all countries	151/872 (17.3)	53/1456 (3.6)	142/2609 (5.4)	401/2206 (18.2)	747/7143 (10.5)

[a] Using the antimicrobial disk Phenotypic Confirmatory Tests for ESBLs as described for *Klebsiella pneumoniae* and *Escherichia coli* in NCCLS documents M100-S12, Table 2A [14].

Table 3
Incidence (%) of vancomycin-resistant *Enterococcus faecium* (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA)

Country	*N*/total (%)	
	VRE	MRSA
Northern Europe	26/386 (6.7)	89/352 (25.3)
Austria	9/62 (14.5)	12/50 (24)
Belgium	1/29 (3.4)	21/50 (42)
France	1/97 (1)	14/49 (28.6)
Germany	9/128 (7)	12/102 (11.8)
Switzerland	3/18 (16.7)	29/51 (56.9)
The Netherlands	3/52 (5.8)	1/50 (2)
Southern Europe	57/530 (10.8)	182/443 (41.1)
Croatia	2/38 (5.3)	8/66 (12.1)
Greece	7/42 (16.7)	28/50 (56)
Italy	8/201 (4)	45/100 (45)
Portugal	36/61 (59)	46/52 (88.5)
Slovenia	0/43 (0)	3/26 (11.5)
Spain	3/130 (2.3)	31/109 (28.4)
Turkey	1/15 (6.7)	21/40 (52.5)
Other countries	0/33 (0)	23/113 (20.4)
Egypt	0/2 (0)	4/9 (44.4)
Lebanon	0/7 (0)	1/25 (4)
Saudi Arabia	0/3 (0)	0/25 (0)
South Africa	0/21 (0)	18/54 (33.3)
Totals for all countries	83/949 (8.7)	294/908 (32.4)

the incidence of MRSA ranged from 11.5 to 56.9% with an average of 32.5%. The 41.1% incidence of MRSA seen in the southern European countries was higher than the 25.3% seen in strains from northern European countries.

4. Discussion

This study establishes baseline data in selected centres for future assessment of prospective interventions which effects changes in antimicrobial resistance. These data document current incidence rates during a 2-year period from 2001 to 2002 of five common nosocomial pathogens: ESBL producing *K. pneumoniae*, *E. coli* and *Enterobacter* spp., vancomycin-resistant *E. faecium* (VRE) and methicillin-resistant *S. aureus* (MRSA).

Previous reports from 1994 to 1999 place the incidence of ESBL producing *Klebsiella* spp. in Europe between 23 and 25%, and 5.4% for *E. coli*, with wide variance among geographical locations [1,16,17]. The initial screening for ESBL (cefotaxime, ceftazidime, or ceftriaxone MICs $\geq$ 1 mg/l) concurred with the NCCLS phenotypic confirmatory test (ceftazidime or cefotaxime with and without clavulanic acid) for *E. coli* and *K. pneumoniae*. The application of this methodology to Enterobacteriaceae other than *E. coli* and *Klebsiella* spp. remains controversial and is not recommended by the NCCLS [18]. Using this screening and confirmatory methodology, the rate of ESBL producers for *Enterobacter* spp. was 8.8% (204/2328). However, the NCCLS screening and confirmatory tests are sensitive but not specific for ESBLs in *Enterobacter* spp. thereby giving false positive indications for ESBL in this species. By using molecular techniques of IEF, PCR and DNA sequencing, it was determined the NCCLS confirmatory criteria resulted in 3/28 (10.7%) of *Enterobacter* spp. gave a false positive test for ESBLs. Using these data derived from this subset of strains, the ESBL rate for *Enterobacter* isolates in this study could be crudely estimated to be 7.8%. No false negative ESBLs were detected in the sampled subpopulation.

Since the first reports of vancomycin-resistant enterococci (VRE) that began to appear in the late 1980s, VRE now ranks third among antimicrobial resistant nosocomial infections [2,19]. The geographical distribution and the importance of VRE as a nosocomial pathogen have steadily increased in the intervening 20 years. The incidence of VRE in the United States has increased from 0.3 to 10.8% in intensive care units from 1989 to 1995 [20] and up again from 14% in 1977 to 17% in 1999 [3]. Rates of VRE in nosocomial infections are typically lower in Europe than the United States while community reservoirs of VRE strains in humans and animals are quite common [21]. Documented rates of VRE in Europe have ranged between <1 and 3% from 1995 to 1999 [3,22–24]. The higher VRE rate of 8.7% seen in this study is due in part to the disproportionately high incidence of VRE from the two centres in Portugal (59%). The 2001 European Antimicrobial Resistance Surveillance System (EARSS) reports a 21% (7/34) VRE rate for Portugal that, although not as high as the 59% reported here, is similarly disproportionate to that of other European centres [17]. Even after excluding the data from Portugal, the VRE rates of 6.7% in northern European and 4.5% in southern European (minus Portugal) centres represent a significant increase in VRE rates in Europe since 1999.

Since first reports of methicillin resistance were published from Boston in 1968, methicillin-resistant *S. aureus* has been reported as the most frequent cause of nosocomial infections in America [2,25]. Like the ESBL producing Enterobacteriaceae and VRE, the prevalence of MRSA varies greatly from one geographical location to another and between institutions within a given location [26]. Typically, 50% of all *S. aureus* recovered in nosocomial infections are methicillin-resistant [2]. European MRSA rates have likewise increased steadily over the last decade from 9.1% in 1992 to 25% in 2000 [27–30]. This study demonstrates a current MRSA rate of 32.4%. MRSA rate varies widely among the individual countries but were higher in southern European countries than northern European countries.

The PEARLS study is unique in that it is providing useful information on resistant determinants at local, regional and national levels. Previous ESBL, VRE and MRSA studies have been limited to single unit outbreaks, individual medical centres or a few centres within a limited geographical area. The PEARLS study is the largest prospective intervention study of this type to date. This work in progress will determine the effects of uni- and multi-variables on the expression of selected resistance mechanisms.

Acknowledgements

We acknowledge our appreciation for the contributions of the following investigators: Kristen Focht, M. Lizarazo, Prof. Dr. A. Georgopoulos, Austria; Prof. F. Allerberger, Austria; Prof. M. Struelens, Belgium; Prof. H. Goossens, Belgium; Prof. Dr. S. Kalenic, Croatia; Prof. Dr. V. Punda-Polic, Croatia; Dr. S. Abdul Latif, Egypt; Dr. J. Nguyen, France; Dr. D. Talon, France; Dr. H. Jean Pierre, France; Prof. Dr. H. Geiss, Germany; Dr. M. Horn, Germany; Dr. S. Ziesing, Germany; Prof. Dr. H. Seifert, Germany; Prof. H. Giamarellou, Greece; Prof. P. Nikolaidis, Greece; Prof. G. Schito, Italy; Prof. G. Fadda, Italy; Dr. F. Luzzaro, Italy; Prof. D. Cirillo, Italy; Dr. G. Araj, Lebanon; Dr. J. Correia da Fonseca, Portugal; Dr. D. Costa, Portugal; Dr. Z. Memish, Saudi Arabia; Dr. M. Muller-Premru, Slovenia; Prof. L. Liebowitz, South Africa; Prof. H. Crewe-Brown, South Africa; Dr. R. Martin, Spain; Dr. M. Gobernado, Spain; Dra. E. Cercenado, Spain; Dr. J. Aznar, Spain; Prof. J. Garbino, Switzerland; Prof. J. Verhoef, The Netherlands; Prof. Dr. J. Hoogkamp-Korstanje, The Netherlands; Prof. Dr. S. Unal, Turkey; Dr. V. Korten, Turkey; Dr. M. Yilmaz, Turkey; Prof. Dr. M. Ozinel, Turkey. This study was sponsored by a grant from Wyeth Pharmaceuticals.

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