IDENTIFYING RESISTANCE™

International Newsletter
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BIOMÉRIEUX

Through the *IDENTIFYING RESISTANCE* Newsletter, bioMérieux's ambition is to contribute to the awareness and progress in the field of resistance to antibiotics.
I hope the information, papers written by worldwide specialists, brings you valuable data to help you in your activities and day-to-day practice.

This new issue deals with a complex resistance mechanism that appeared less than twenty years ago and for which bioMérieux rapidly adapted its offer of tests and software.

This is a perfect illustration of our commitment and continuous effort to bring you a global offer in terms of instruments, reagents, software and expert systems.

We will do our best to deserve your confidence and continue to propose innovative new products to help you in your endeavour.

Dr. Benoît Adelus
Chief Executive Officer

State-of-the-Art

ESBL in Enterobacteriaceae

Karen Bush

Karen Bush, Ph.D., is the Team Leader for the Biology Antimicrobial Agents Research Team at Johnson & Johnson Pharmaceutical Research & Development (Raritan, NJ, USA), where she is responsible for microbiology research in Drug Discovery. Her work on beta-lactamase inhibitors and resistance mechanisms contributed to proposing an updated functional classification scheme for these enzymes.

Development of the "third generation" cephalosporins in the early 1980s was based heavily on the ability of these agents to escape hydrolysis by all the common ß-lactamases in both Gram-positive and Gram-negative bacteria ***(18)***. Broad spectrum ß-lactamases with the ability to hydrolyze the most common penicillins and cephalosporins had been identified in virtually all species of Enterobacteriaceae before 1980 ***(19)***, and had begun to appear in large numbers of *Haemophilus influenzae* and *Neisseria gonorrhoeae* isolates ***(2)***. The broad spectrum TEM-1, its single amino acid variant TEM-2, and the functionally similar SHV-1 enzyme, together with the oxacillin-hydrolyzing OXA-1 enzyme, were the most common plasmid-encoded ß-lactamases in Gram-negative bacteria according to epidemiological surveys in the 1980s ***(13)***. However, the new cephalosporins, cefotaxime, ceftazidime and ceftriaxone, and the monobactam aztreonam exhibited good antibacterial activity against Gram-negative bacilli, in part because of their exceptional stability to the infamous TEM, SHV and OXA enzymes ***(6)***.

ESBL in Enterobacteriaceae

To the dismay of their developers, these agents were challenged by an unexpected set of mutational events shortly after their introduction into clinical medicine. The first extended spectrum ß-lactamases (ESBLs) were reported from Germany in 1983 with the description of three independent *K. pneumoniae* isolates from the same hospital exhibiting transferable cefotaxime resistance ***(10)***. Retrospectively, an even earlier Argentinian *K. pneumoniae* isolate was later shown to produce an ESBL in 1982, the year after the introduction of cefotaxime in the Americas ***(12)***.

Major outbreaks of ESBL-producing Enterobacteriaceae were first reported from France, where 283 cefotaxime-resistant *K. pneumoniae* isolates were detected from 1984 through June 1987, in addition to another 200 isolates of *E. coli, Enterobacter* spp., *Serratia marcescens*, *K. oxytoca* and *Citrobacter freundii* that produced the same ESBL ***(16)***.

Coincidentally, the first ESBL-producing *K. pneumoniae* isolates from the United States were all identified during the first six months of 1987 in Boston, New York City, Chicago and California, but with a ceftazidime-resistant phenotype ***(8, 14, 15, 20)***. In all cases, the producing organisms were multidrug resistant due to large plasmids that usually included aminoglycoside resistance determinants as well as ß-lactamase genes.

Phenotypically, a double disk diffusion assay assessing synergy between cefotaxime (or ceftazidime) and amoxicillin-clavulanic acid was used to identify the presence of early ESBLs in *E. coli* and *K. pneumoniae* ***(9)***. The fact that ESBLs respond to inhibition by either clavulanic acid or tazobactam has served as a distinguishing characteristic of these enzymes throughout their history ***(6)*** and is the basis of the NCCLS protocol for detection of ESBLs in *E. coli* and *K. pneumoniae* ***(17)***.

Characteristics of ESBLs

Data compiled from http://www.lahey.org/studies/webt.stm. (February, 2003).

Enzyme family	TEM	SHV	OXA	CTX-M
Total number in family	118	47	46	26
Number of ESBLs	92	45	11	26
ESBL variants*	73 TEM-1 variants 19 TEM-2 variants*	32 SHV-1 variants 13 SHV-2 variants*	1 OXA-1 variant 3 OXA-2 variants 7 OXA-10 variants	26
Amino acids in enzyme, including leader sequence	286	292	266	290
Number of amino acid positions at which substitutions have been reported from enzymes in clinical isolates	37	32	19	Sequences may differ 20-25%
Maximum number of mutations in a single ESBL compared to parent	6	7	9	Not determined
Most common substitutions in mature protein	E104K (N = 30) R164S or R164H(N=25) M182T (N = 14) E240K (N=10)	L35Q (N = 11) G238S or G238A (N = 17) G238S (N = 26)	OXA-10 series: I10T, G20S, T110S, Y184F, E240G, S258S, E272A (N = 3)# G167D (N = 4)	Not determined

*TEM-1 and TEM-2, differing by a Q39K substitution are not considered to be ESBLs. SHV-1 differs from SHV-2 by a G238S substitution, rendering SHV-2 an ESBL.
#Each substitution appears in 3 enzymes. Different combinations are observed.

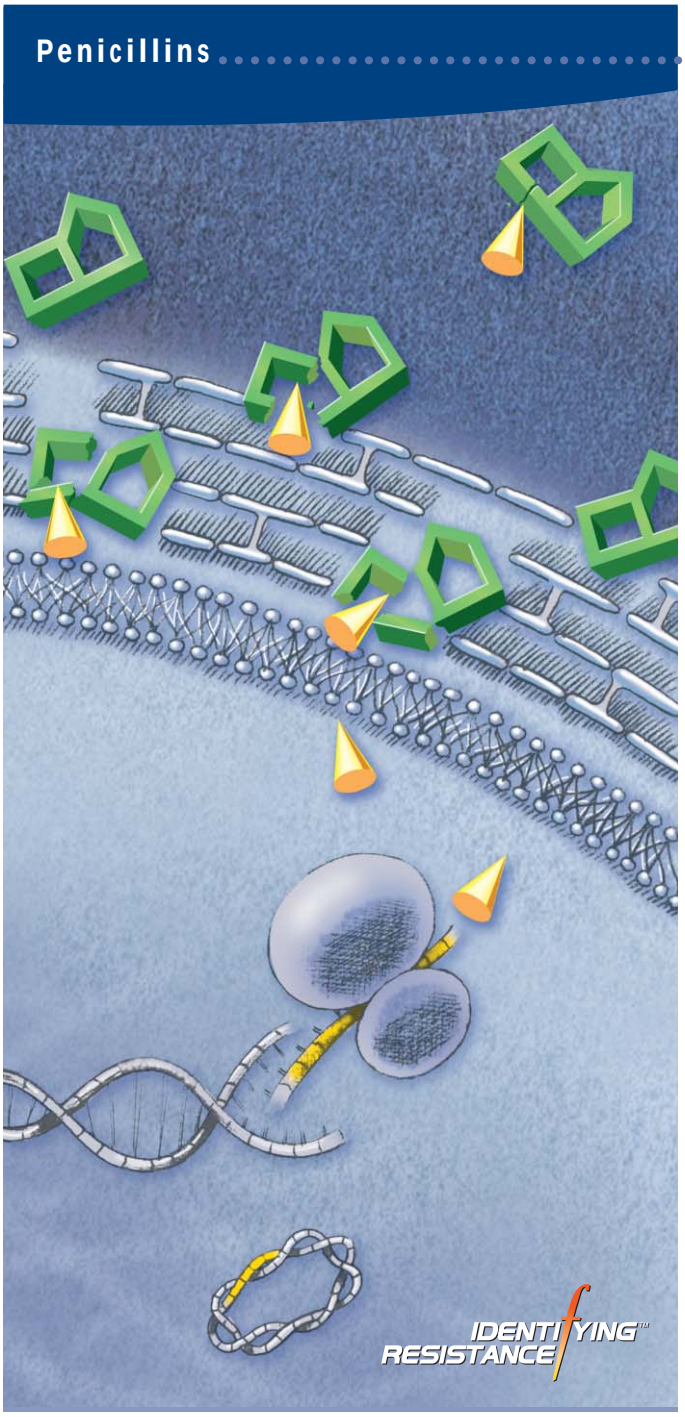

Fig 1.
Resistance by ESBL: enzymatic inactivation of penicillins

As improved molecular techniques became more widely available in the 1990s, sequencing of the responsible genes became routine and is considered to be the "gold standard" for ESBL identification ***(2)***. The original ESBLs from Europe and the United States were all derived from the common TEM-1 and SHV-1 enzymes, usually differing by one to three amino acid substitutions compared to the parent enzyme. In almost all cases, these changes were due to point mutations in the nucleotide sequences. In the United States, almost all the early ESBLs were identified as TEM variants, whereas in Europe a mixture of TEM and SHV ESBLs were characterized ***(12)***.

Comparisons of the geographically diverse ESBLs indicated that two distinct ESBL populations were evident. In Europe, one set of enzymes showed preferential hydrolysis of cefotaxime compared to ceftazidime and were initially named "CTX" enzymes; a second set of enzymes preferentially hydrolyzed ceftazidime and were named "CAZ" enzymes. However, upon sequencing of the producing genes, it was noted that both sets of enzymes were derived from the bla_{TEM-1} gene.

Thus, an early consensus was reached in the ß-lactamase community that the ESBLs would be numbered according to their parent, and not according to their functional status ***(5)***. ESBL nomenclature is currently being monitored on a website managed by G. A. Jacoby and K. Bush (http://www.lahey.org/studies/webt.stm), where amino acid sequences and literature references are provided for all TEM and SHV variants, and for OXA-derived ESBLs (see Table). In addition, references are given for all OXA, CMY-type, IMI-type and CTX-M sequences.

Of assistance to the practicing laboratory enzymologist is a table of all isoelectric points reported for all ESBLs.

Although the majority of ESBLs are associated with either a TEM or SHV heritage, other enzyme families have achieved recognition as they become predominant in their own geographical niches. Extended spectrum OXA-derived enzymes were originally reported in *Pseudomonas aeruginosa* isolates from Turkey ***(7)*** and have now been identified from other European sites ***(2)***.

One of the most rapidly growing new families of ESBLs is the CTX-M family, CTX-M-1 was first identified in cefotaxime-resistant *K. pneumoniae* isolates from Western Europe; CTX-M-2 was then found

in several South American isolates and differed by 16% in its amino acid sequence from CTX-M-1 ***(1)***. These enzymes strongly prefer cefotaxime as a substrate and hydrolyze ceftazidime poorly.

At this time there are over 25 unique members of this family. It is regarded as the most prominent ESBL in South America, and has now been identified with outbreaks in China and the United Kingdom ***(3)***. The producing organisms do not appear to be resistant to ceftazidime in standard susceptibility testing, so detection systems utilizing only ceftazidime will not identify a CTX-M ESBL ***(3)***. As additional families of enzymes continue to be identified, it may be expected that even more narrow spectrum ESBLs will become prevalent.

Resistance to third-generation cephalosporins in *E. coli* and *K. pneumoniae* is often attributed solely to ESBL production; however, other factors must also be considered.

The combined contributions of porin mutations, quantity of enzyme activity, and number of ß-lactamases per strain ***(4, 11)*** will result in elevated MICs for these cephalosporins.

In addition, it is important to note that ESBLs can occur in other Enterobacteriaceae, with their production often masked by the concurrent production of AmpC cephalosporinases ***(4)***. With the promiscuous transfer of ESBL determinants among Gram-negative rods, we can only expect these enzymes to continue to proliferate in the present clinical environment.

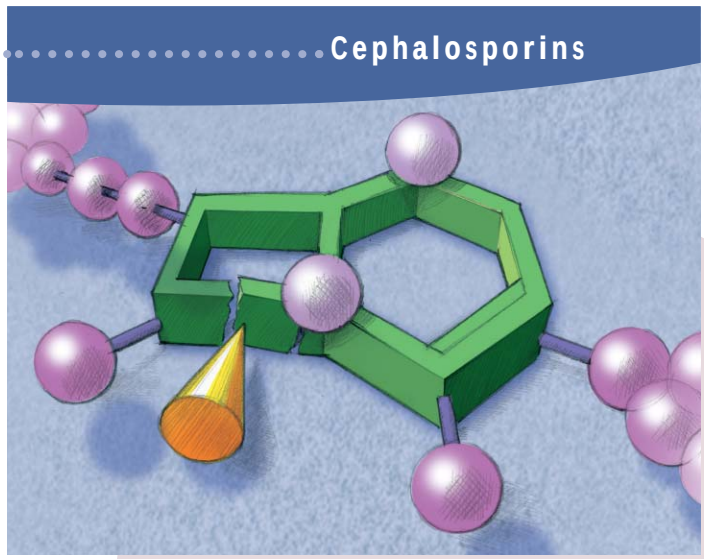

Fig2. Enzymatic inactivation of cephalosporins

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EXPERT SYSTEMS

VITEK2 has been challenged with ESBL in several studies.The broader scope has been published by Livermore et al.

Multicentre Evaluation of the VITEK 2 Advanced Expert System for interpretive reading of antimicrobial resistance tests.
Livermore et al. *Journal of Antimicrobial Chemotherapy* (2002) 49, 289-30010 European centers results were compared final result to final results:

The study was performed by 10 European centers from nine different European countries, and involved around 1000 strains.

	test	agreement	resistant mechanism
E. coli	28	26	TEM/SHV/PER
Klebsiella spp	99	94	TEM/SHV
E. cloacae, C. freundii	6	6	TEM/SHV
Salmonella	3	3	TEM/SHV
E. gergoviae	1	1	CTX-M
total	**137**	**130**	
		disagreement	
E.coli		2	
Klebsiella spp		5	

The only way to evaluate the performance of an expert system is to compare final results.
Here the comparison was made between results after interpretation by the expert system, and genotypic findings or human expert results when using a phenotypic method *(S.pneumoniae)*.

As a consequence, computation of results was similar to that of identification evaluations:

- Agreement when both experts were giving the same result.
- Disagreement when they differed.
- Low discrimination when VITEK2 expert was proposing 2 or 3 answers, one of them being right.

Anne Beal,
Microbiology Laboratory Manager, (Fort Lauderdale, Florida) surrounded by 4 VITEK® 2 XL instruments at Integrated Regional Laboratories (IRL).
This lab serves 13 hospitals in South Florida and processes 2000 samples every day.
VITEK 2 was chosen for routine use at this core lab facility because of its automation, rapid results and the Advanced Expert System.

WEB SITES

http://www.lahey.org/studies

Site of Lahey Clinic, where tables are updated for B-lactamases with amino-acid sequences

120 TEM
50 OXA
12 CMY
13 IMP
6 VIM

http://www.rochester.edu/College/BIO/HallLab/AmpC_Phylo.html

The Hall Laboratory of Experimental Evolution phylogenetic trees

Identifying Resistance News

bioMérieux UK jointly organised a symposium on ***Identifying Resistance***, last February in London, with the Public Health Laboratory Service (PHLS).
One hundred and twenty people attended 8 lectures.

The first part of the meeting addressed the new Health Organisation in this country (replacement of the PHLS by the Health Protection Agency (HPA)), bringing expertise and excellence to the National Health Service (NHS), through a new organisation and a series of reference labs. The focus of the presentations was epidemiology and microbiology and key speakers addressed the audience.

The second part of the meeting concerned the control of antibiotics in hospitals, the role of the microbiology laboratory in detecting resistance and how this can aid infection control by more rapid reporting using VITEK®2.

In the final session, Dr David Livermore discussed *"Green cats"* and the need for interpretive reading of antibiotic results. Dr Jean-Pierre Marcel from bioMérieux, concluded the meeting by discussing the company's experience in developing expert systems and the current developments in DNA Chip technology.

In the UK, authorities are working on Infection Control and Resistance Detection based on the House of Lords white paper *(Path to Least resistance,* 1998) and the Department of Health report "Getting ahead of the Curve", in order to reduce the Socio-Economic Burden of Hospital Acquired Infections.

bioMérieux UK is closely supporting these official bodies, as was the case with this symposium.

Did you know?

nccls recommendations

ref: M100-S13 (M7) January 2003

Table 2A Enterobacteriaceae

Comment 6

Strains of Klebsiella spp and E.coli that produce extended spectrum beta-lactamase (ESBLs) may be clinically resistant to therapy with penicillins, cephalosporins, or aztreonam, despite apparent *in vitro* susceptibility to some of these agents.

Some of these strains will show MICs above the normal susceptible population but below the standard breakpoints for certain extended-spectrum cephalosporins or aztreonam. Such strains should be screened for potential ESBL production by using the ESBL screening breakpoints before reporting results for penicillins, extended-spectrum cephalosporins, or aztreonam.

Other strains may test intermediate or resistant by standard breakpoints to one or more of these agents. In all strains with ESBLs, the MICs for one or more of the extended-spectrum cephalosporins or aztreonam should decrease in the presence of clavulanic acid as determined in phenotypic confirmatory testing.

For all confirmed ESBL-producing strains, the test interpretation should be reported as resistant to all penicillins, cephalosporins, and aztreonam.

The decision to perform ESBL screening tests on all urine isolated should be made on an institutional basis, considering prevalence, therapy, and infection-control issues.

Screening and Confirmatory Tests for ESBLs in *Klebsiella pneumoniae, K.oxytoca,* and *Escherichia coli.*

Medium (CAMHB), antibiotic concentrations, standard broth dilution recommendations for inoculum, incubation conditions, incubation length.

Growth may indicate ESBL production.

Recommended drugs are:

cefpodoxime (4 µg/mL), ceftazidime, aztreonam, cefotaxime, ceftriaxone (1 µg/mL).

ß-lactam antibiotics

Sub-classes of cephem (parenteral) class

cephalosporins I	cefazolin, cephalothin, cephapirin, cephradin
cephalosporins II	cefamandole, cefonicid, cefuroxim (sodium)
cephalosporins III	cefoperazone, cefotaxime, ceftazidime, ceftizoxime, ceftriaxone
cephalosporins IV	cefepime
cephamycin	cefmetazole, cefotetan, cefoxitin
oxacephem	moxalactam

- Cephalosporins I, II, III, IV are sometimes referred to as 1st, 2nd, 3rd, and 4th generation cephalosporins, respectively.
- Cephalosporins III and IV are also referred to as "extended-spectrum cephalosporins". This does not imply activity against ESBL-producing gram-negative bacteria.
- For all confirmed ESBL-producing strains, the test interpretation should be reported as resistant for this antimicrobial class or subclass.

VITEK2 Phenotypes

Extended spectrum ß-lactamase
ESBL + impermeability (cephamycins)

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Practical advice

What is the impact of ESBL?

ESBL-producing bacteria escape treatment by Cephalosporins, including widely used Cephalosporins III and IV.

Why look for ESBL?

Expression of ESBL production is variable in intensity and with substrates. That means that the test result with a drug can be corrected when ESBL production has been demonstrated.

How to suspect an ESBL?

By testing several cephalosporins to contourn substrate specificity. Any non-susceptible result for cefotaxime, ceftazidime, aztreonam or cefpodoxime is a strong indication with some species.

What is the reference method?

Amplification then sequencing of resistant genes is the reference to identify mutations turning some ß-lactamases into ESBL.

Phenotypic confirmatory tests are more widely used: restoration of ß-lactam activity by ß-lactamase inhibitors such as clavulanic acid. These can be performed using the diffusion method (double disk method) or dilution method.

What antibiotics to use for ESBL-producing bacteria?

Alternative drugs are mainly carbapenems (imipenems, meropenem...) or combinations of ß-lactams with inhibitors of ß-lactamase or cephamycins.

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