

laboratories around the world routinely sequence a region of ORF2, which encodes the major capsid protein. Increased outbreak activity or virus sequence variation that might signal the emergence of a new pandemic strain, which can then be communicated via the global network NoroNet as an alert for health authorities.

Genotyping of HIV

Patients infected with HIV are frequently on long-term, highly active antiretroviral therapy (HAART). These drugs typically act by binding to and blocking the function of viral proteins, inhibiting viral replication. The high frequency of mutation which occurs during HIV replication can result in therapy escape. This is partly countered by the concurrent use of multiple drugs. By monitoring the efficacy of therapy, when viral escape is detected by a rise in the patient's viral load, the patient's HIV virus can be sequenced to determine which mutations are accumulating, especially those that will cause an amino acid substitution that inhibits the binding of an antiretroviral drug. There is extensive, ongoing research into which mutations, either singly or in combination, will critically influence the efficacy of each antiretroviral drug. Within the laboratory, the genes encoding the proteins targeted by the patient's HAART, most frequently the Protease and Reverse Transcriptase enzymes, are amplified and sequenced. The sequence electropherograms are examined as mixed bases and occasionally INDELS can be present, arising from the various mutationally-derived HIV strains present in the patient. Once corrected, the interpretation of the

predicted 'virtual' resistance is electronically performed using web-based drug resistance predicting algorithms available either commercially (e.g. Virco, <http://www.vircolab.com>) or publicly (e.g. Stanford University's HIVdb) to guide the choice of HAART drugs.

Conclusion

Using sequence for the information it contains is now common. The above examples detail a variety of such uses and demonstrate the increased resolution afforded by a sequence-based approach. The latest generation of sequencers which permit direct mass sequencing will only enhance the availability, resolution and thus the use of sequence-based data as a laboratory tool.

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Biography

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A pragmatic approach to screening for transmissible resistance in the *Enterobacteriaceae*

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Determining the origin, spread and characteristics of important resistance genes and plasmids in Gram-negative bacteria may be more informative than determining the epidemiology of bacteria themselves. Infection control resistance surveillance and containment efforts should focus on the transmission characteristics of the resistance rather than those of the index organism at the point of recognition.

Detection of transmissible antibiotic resistance in Gram-negative bacteria

The predictable relationship between the antibiotic-resistant phenotype and the presence of the responsible gene in Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) allows confident screening on phenotype or genotype in healthcare facilities. Gram-negative bacteria adapted to a polymicrobial environment such as the gut may have one or more of a large number of resistance mechanisms, many of which are interdependent and/or duplicative and some of which may be 'silent', that is they are not expressed or require cofactors to provide the resistance phenotype. Even those in which single-gene mechanisms are responsible may have any one of hundreds of different genes. These may be acquired from a number of different bacteria and their nomenclature and/or identification is often disorganised and confusing. However, new opportunities are now available with coincident advances in bioinformatics,

genomics and biotechnology. In this article, we will focus on surveillance of resistance to the β -lactam and aminoglycoside antibiotics in the *Enterobacteriaceae*, in particular, as these form the cornerstones of treatment for life-threatening sepsis.

Effective treatment must be instituted long before data about cultured bacteria are available

The provision of successful and cost-effective life support for the critically ill has meant that antibiotic resistance has once more risen to prominence as a public health threat requiring urgent intervention. Approximately 12% of patients admitted to an Australian ICU develop severe sepsis and 27% of these will die in ICU¹.

Failed early treatment due to antibiotic resistance greatly increases the risk of death, with a mortality cost of ~7% per hour of delay early on². Antibiotic-resistant infections are linked to excess health costs and mortality overall and the urgency to institute effective antibiotic therapy is made more problematic by the emergence of antibiotic resistance, which may arise in the form of a life-threatening superinfection³ or when the original pathogen elaborates acquired resistance after brief antibiotic exposure⁴.

Causes of life-threatening sepsis

A retrospective analysis of 2,700 cases revealed that although Gram-positive bacteraemias (e.g. *S. aureus*) are more common, Gram-negative bacteraemias (e.g. *E. coli*) are more likely to result in the most severe physiological manifestation of infection, septic shock². Similarly, more than half of 140 consecutive episodes of severe sepsis in a paediatric haematology unit were due to Gram-positive bacteria, but 83% of the episodes requiring ICU admission were due to Gram-negative bacteraemias⁵. Some bacteria may be more aggressive and others simply more inflammatory, for example, LPS-rich *E. coli* elicited stronger inflammatory

responses than *S. aureus* in a rat model⁶. Epidemiological studies of septic shock are confused by the common presence of coagulase-negative staphylococci and yeasts as contaminants, co-infections and superinfections in patients treated with broad-spectrum antibacterials. Studies from the 1980s and 1990s reflect the predominance of Gram-positive pathogens, particularly *S. aureus*^{7,8}. However, members of the *Enterobacteriaceae* such as *E. coli* and *Klebsiella pneumoniae* are now responsible for probably around 40–50% of cases of septic shock presenting to ICU, as compared to the much more common infective agents *Streptococcus pneumoniae* (~15–20%) or *S. aureus* (~15–20%) (Figure 1)⁹.

The predominance of Gram-negative bacteria among life-threatening infections may reflect increasing critical care and immune-compromised populations in hospitals but is important because the acquisition and expression of resistance is more complex and more quickly changeable.

Surveillance of transmissible resistance in Gram-negative bacteria

The risks of unexpected antibiotic failure can be anticipated to some extent by surveillance for the presence of resistant bacteria. Similarly, surveillance for transmissible resistance can allow timely and potentially lifesaving intervention^{4,10}. Screening by phenotype is practical for those with stable phenotypes (e.g. MRSA, carbapenem-resistant *Acinetobacter* spp.) but is seriously compromised by natural variation in the phenotype encoded by transmissible genes moving among the *Enterobacteriaceae*¹¹.

The aminoglycoside antibiotics

Aminoglycosides bind to the 16S rRNA of bacterial 30S ribosomal units and inhibit protein synthesis. Major resistance mechanisms include modification of the target (16S rRNA), modification of the antibiotic and reduction of intracellular accumulation through reduced uptake and/or enhanced efflux^{12,13}. Aminoglycoside-

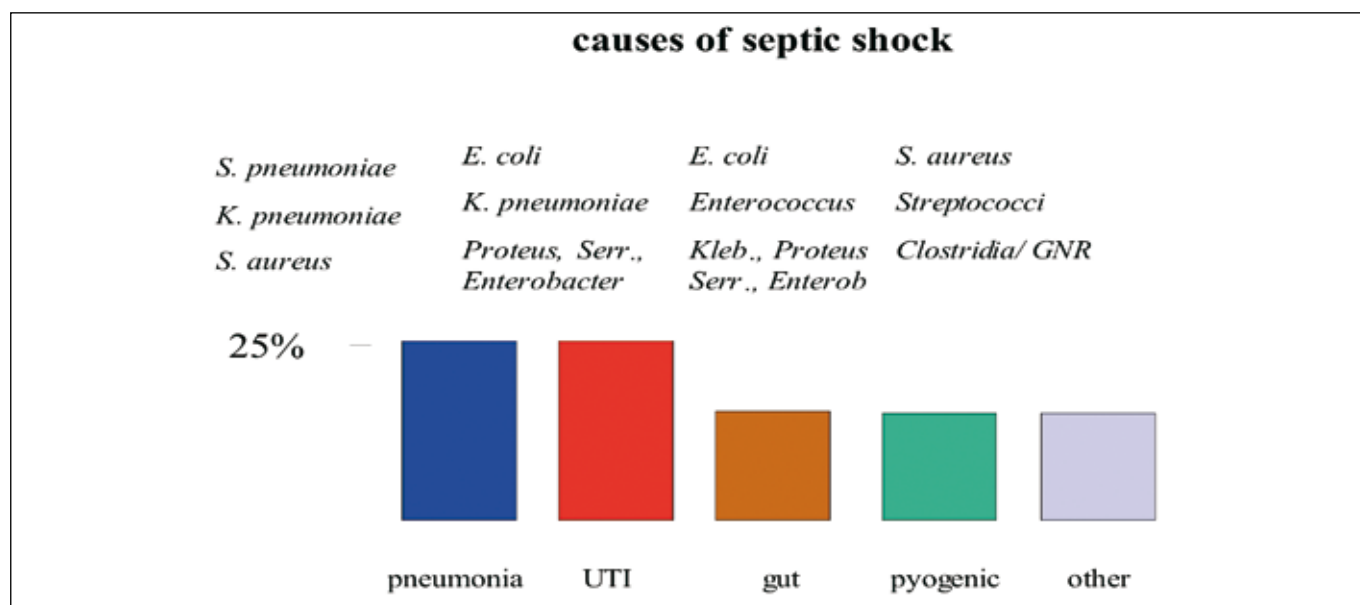


Figure 1. Pneumonias and urinary tract infections each contribute about a quarter of all causes of septic shock, but the dominant pathogens overall are the Gram-negative bacteria *E. coli*, *K. pneumoniae*, and other *Enterobacteriaceae*, and *Pseudomonas aeruginosa*.

modifying enzymes are the most common mechanism of resistance in the *Enterobacteriaceae* and are associated with a variety of genes, encoding acetyltransferases (*aac*), adenyltransferases (*aad*, also known as nucleotidyltransferases, *ant*) and phosphotransferases (*aph*)^{13,14}. Expression of these enzymes in *E. coli* certainly results in MICs well above the clinical breakpoint for resistance, but methylation of target 16S rRNA causes much higher-level and more comprehensive aminoglycoside resistance.

Since the antibiotic-modifying enzymes tend not to cover all of the partially related aminoglycoside antibiotics, the presence of high-level resistance to aminoglycosides less commonly affected (e.g. here, amikacin) is a strong hint as to the presence of an acquired 16S rRNA methylase and is probably a useful screen (e.g. gentamicin and/or amikacin 128 µg/mL). The 16S rRNA methylases are encoded by *armA* and *rmtB* (most common), and *rmtA*, *rmtC*, *rmtD* and *npmA* genes. These enzymes are important because they confer broad aminoglycoside resistance to the organism, and are often found in isolates carrying additional resistance genes, including those encoding β -lactamases^{15,16}. These are well-described in Asia and, therefore, many are also present in Australia, including at least *ArmA*, *RmtB*, and *RmtC* (unpublished data;¹⁷).

In some organisms, the gene may not be expressed because it is too 'distant' from the promoter (e.g. a gene cassette in tandem array behind several others). In this circumstance, a genetic screening will yield a falsely positive result. However, screening for the most relevant expressed genes is simple because the phenotype is generally consistent.

Options include . . .

- Breakpoint screening: for GEN/TOB (and AMK) resistance.
- Point-prevalence comprehensive screening to detect all known or likely genes associated with the phenotype.

- More frequent screening using a set which covers 'local' genes and iterative inclusion of those phenotypes not explained.

The β -lactams and related antibiotics

β -lactam antibiotics inhibit the synthesis of the bacterial cell wall by targeting the penicillin binding proteins that lie on the cytoplasmic membrane¹⁸. β -lactamases with varying hydrolysing activity catalytically disrupts the lactam bond of the β -lactam nucleus to form an acyl enzyme complex. During deacylation, water molecules hydrolyse the ester linkage, releasing the penicilloyl and cephalosporin moiety^{19,20}. Extended-spectrum β -lactamases (ESBLs) confer bacterial resistance to the penicillins, first-, second- and third-generation cephalosporins, aztreonam and are inhibited by β -lactamase inhibitors^{20,21}. CTX-M β -lactamases are the most prevalent class of ESBLs worldwide and most preferentially hydrolyse cefotaxime²².

Plasmid-mediated AmpC β -lactamases (plasmid AmpCs) are the most under-rated of all the β -lactamases. Although these enzymes are less common than ESBLs, they are important to recognise since they provide an even broader spectrum of resistance, including the cephamycins, and are not inhibited by β -lactamase inhibitors^{23,24}.

Metallo β -lactamases (MBLs) confer resistance to virtually all β -lactam antibiotics, including the carbapenems, which are the antimicrobials of last resort for the treatment of multidrug-resistant gram-negative bacteria. MBLs are not inhibited by serine β -lactamase inhibitors^{25,26}. Other carbapenemases exist with varying ability to hydrolyse the carbapenems. Class A carbapenemases include the KPC, IMI, SME, NMC-A and some GES enzymes. They are produced by members of the *Enterobacteriaceae* family but have recently been detected in isolates of *P. aeruginosa* and *A. baumannii*²⁷. Most class

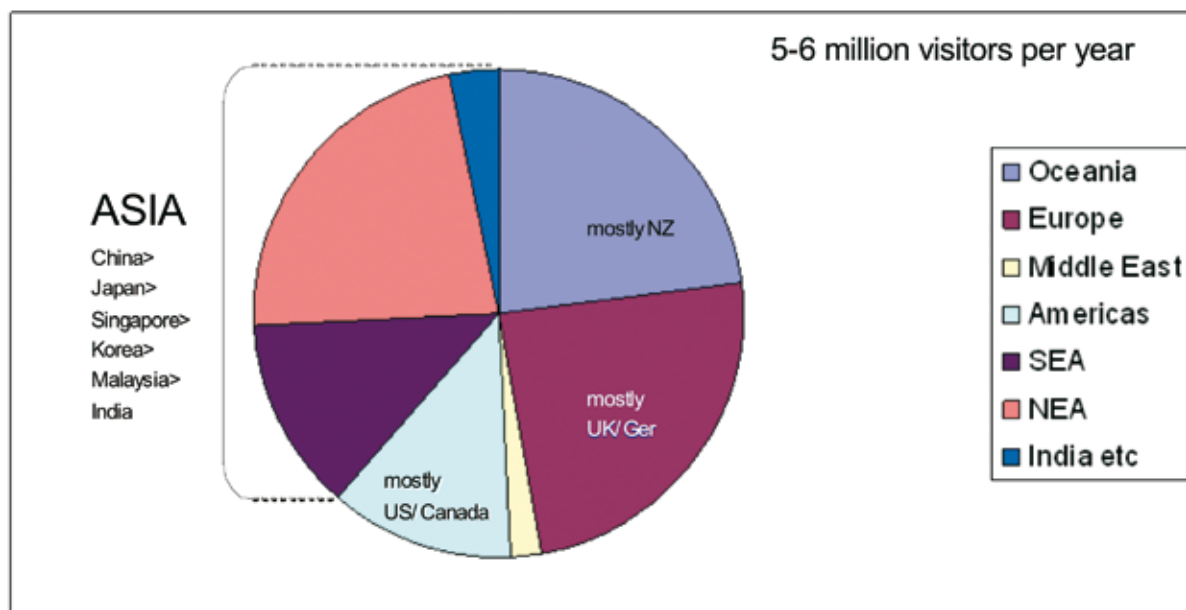


Figure 2. Australia has >5 million visitors every year, bringing microflora from North-East Asia (NEA) and South-East Asia (SEA), as well as from Western Europe and North America. Data from <http://www.tourism.australia.com>

D carbapenemases hydrolyse carbapenems weakly and are inhibited poorly by clavulanate. They belong to the OXA family and are most commonly produced by *Acinetobacter* spp. but have also been reported in some *P. aeruginosa*, *K. pneumoniae*, and *E. coli* strains²⁷.

The plasmids

In countries such as India, the prevalence of transmissible resistance to the modern extended-spectrum β -lactam antibiotics in *E. coli* may exceed 70%²⁸, while it is still <5% here in Australia. It is feared that the prevalence of antibiotic resistance may therefore rise in Australia – we are now seeing transferable mechanisms of resistance which were first described in India and Asia. Many of the most important genes are carried on multiple plasmids between different genera in the *Enterobacteriaceae*. Plasmids are extra-chromosomal circular genetic elements that replicate autonomously in host cells²⁹, which vary in size (ranging from a few kilobases to >150 kb of DNA), modes of replication and transmission, host range and the genes that they carry³⁰. The ability of plasmids to acquire and transfer antibiotic resistance determinants from a variety of sources provides the ideal platform for the evolution of new multi-resistance plasmids³¹. The extent to which the plasmids themselves are evolving with the antibiotic resistance gene pool is ill-defined and is an important topic for future research.

Practical approaches to surveillance

Real-time multiplex PCR assays for the rapid detection and identification of β -lactamase resistance genes are practical solutions for larger laboratories. These assays at present need to include the detection of IMP-, VIM-, SPM-, SIM-, AIM- and NDM-type MBLs, KPC-, GES- and OXA-type carbapenemases, CTX-M, SHV and VEB-type ESBLs and plasmid-mediated AmpC -lactamases (DHA-1-like, CMY-2-like, ACT/MIR, MOX/CMY-1-like, ACC and FOX-types) in non-ESCAPPM organisms, but this list is in constant flux and needs to be reviewed at least yearly in working laboratories providing such services. Larger platforms such as

reverse line blot multiplex assays³² are useful for the detection of a greater diversity including less common resistance genes. Like all Southern hybridisation methods, it can be reduced in stringency to detect minor variants, and the infrequent sampling of larger numbers of strains allows efficient development of more expensive fluorometric ‘real-time’ multiplexed assays, whether they be based on conventional PCR or on mass spectroscopy-based platforms.

A simple screening algorithm in clinical laboratories should target the most common and (infection control) problematic genes in *E. coli* and *Klebsiella (pneumoniae)* indicator organisms, as these are the most important pathogens. This can be done with a small set of targets, the intention being to minimise the number for which no explanation is apparent and further screening for a much larger set is required. An example relevant to the current NSW microflora would be:

CAZ/CTX >1 ug/mL FOX >8 ug/mL: consider IMP, VIM, CMY, DHA, NDM

CAZ/CTX >1 ug/mL FOX ≤8 ug/mL: consider CTX-M (-1/-9 groups), SHV (-5/-12), VEB, KPC, GES (-5)

GEN>200 or GEN+TOB+AMK>8 ug/mL: consider armA, rmtB

Conclusion

Systematic antibiotic resistance surveillance and reporting networks are active all over the world (e.g. AGAR and SENTRY), but reporting of resistance, without addressing the mechanisms and routes of spread of resistance genes, is of limited utility. For example, TEM and SHV variants were the most important targets for ESBL assays a decade or so ago, but these have been largely displaced by various CTX-M variants, including some which may be quite geographically localised. This local knowledge of antibiotic resistance is no less important than it ever was, but must be supplemented by knowledge of companion genes and of transmissibility/host range to be truly useful and to allow us to move to a more proactive surveillance role.

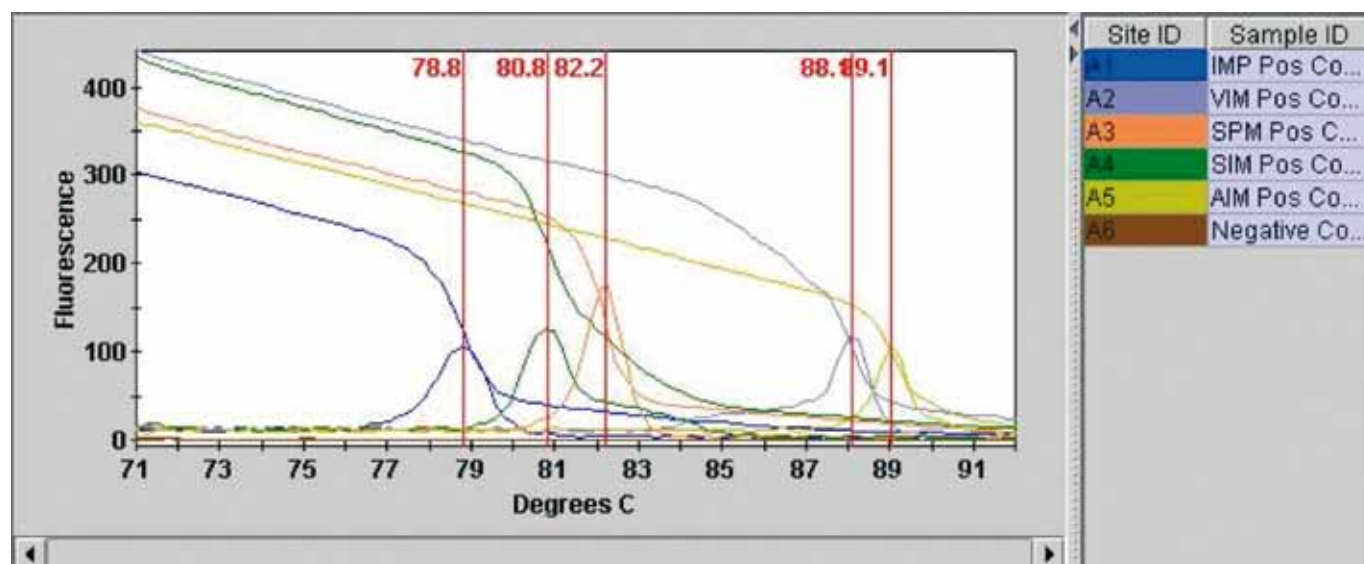


Figure 3. A ‘real-time’ multiplex PCR assay for some of the Australian causes of transmissible carbapenem resistance.

There are a few pointers to remember when recognising the troublesome emerging transmissible resistance genes in *Enterobacteriaceae* in the diagnostic laboratory:

- Automated systems have been unreliable and will probably always be troublesome, simply because computerised 'expert' algorithms often cannot account for recent and/or regional epidemiological variations or for complex β -lactam phenotypes.

With respect to screening for transmissible high-level aminoglycoside resistance due to the 16S methylases (e.g. encoded by *armA*, *rmlB*), there are two reliable predictable features:

- Broad-spectrum: that is, gentamicin, tobramycin, and Amikacin are generally all affected.
- High-level resistance: that is, greater than 128 ug/mL.

With respect to screening for the transmissible carbapenemases:

- The genes common in Asia can be expected to be found locally.
- The common IMP and VIM variants (metalloenzymes) KPC (an Ambler class A enzyme) has not yet been recognised but can be expected to now be present in the Australian microflora.

For the MBLs, ceftioxin resistance (>8 ug/mL) is a reliable marker (but is also a marker of AmpC enzymes, both chromosomal and plasmid-borne). The potential for high-level carbapenem resistance is not well understood – but at least in *Klebsiella*, is related to permeability changes (e.g. porins) – and may be inducible, perhaps more efficiently than the 'ESCAPPEM' phenomenon.

With respect to specific identification of the relevant resistance genes:

- Once- or twice-yearly comprehensive screening (e.g. in conjunction with a specialised laboratory that is monitoring international trends and reports and can characterise any novel resistance genes which emerge) informs the content of diagnostic tests.
- Modern multiplexed methods allow a phenotype-based screen containing several resistance genes, for example, ESBLs, MBLs, aminoglycoside resistance genes, and some of these methods may become useful in on-site platforms in regional laboratories serving the critically ill.

Ideally, the epidemiology of the transmitting vehicle and its host range (ability to spread into different species) is a crucial part of national infection control policy which is presently neglected.

The networking of laboratories, for example, at a state or even national level, to do this most efficiently, is an important future priority, if we are to control the spread of transmissible resistance

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