BREAK POINT

2014 - ISSUE 08

FROM THE NEWSLETTER EDITOR'S DESK

Welcome to another edition of Breakpoint. In this issue, we are blessed with a short article each from four of the 2014 ASA Travel award recipients, describing their work. Janet Montgomery, Austin Health, provides us with further insights into the detection of carbapenem-resistant Gram negative bacilli using phenotypic detection methods. An acceptable screen could include the combined use of chromID ESBL agar and the Carba NP test…. read on. Elizabeth Witherden, University of Tasmania then updates us on resistant *ftsI* genes in a number of *Haemophilus* species. In the third article, Hanna Sidjabat cautions us against the increasing emergence of IMP-4 producing *Enterobacteriaecae* in Queensland hospitals. The report by Wei Gao concludes the issue by discussing the genomics of persistent *Staphylococcus* infections.

Notices and a conference calendar follow. As always, feedback is warmly welcome.

Sharon Chen

ASA Breakpoint Editor,

On behalf of the ASA committee

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IN THE NEWS

Scrolling through the list of new publications in "Antimicrobials" always reminds us of the rapidity of expansion, almost by magic, of knowledge in this field.

Useful articles of clinical relevance include a new review on the use of COLISTIN in multi-drug resistant *Pseudomonas aeruginosa* blood-stream infections (Journal of Infection 2014; http://dx.doi.org/10.1016/jinf.2014.03.001. This polymixin's nephrotoxicity and neurotoxicity could be argued to have been over-estimated. The paper discusses upgrading its current status as "salvage therapy" against multi-drug resistant *P. aeruginosa* to incorporate its use as a first line antibiotic in combination with other anti-pseudomonal antibiotics to produce more effective strategies.

The introduction of intravenous posaconazole to treat and protect against fungal infections is long awaited. Maertens *et al.* (Antimicrob Agents Chemother 2014 14 April 2014) provide pharmacokinetic and safety data that support substantially improved the pharmacokinetics of the intravenous formulation over the oral suspension in high risk patients at a single daily dose of 300 mg in a prophylaxis study. Its introduction in Australia is not far off.

PHENOTYPIC DETECTION OF CARBAPENEM-RESISTANT GRAM NEGATIVE BACILLI. WHICH METHOD IS BETTER?

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Background

Infections due to carbapenem-resistant Gram Negative Bacilli (CRGNB) are increasingly reported worldwide[1,2]. Australians are returning from overseas travel to relatively high prevalence regions with infections due to multi-drug-resistant bacteria[3]. These organisms are a cause of therapeutic failures. Rapid laboratory detection of these organisms, in both clinical infections and carriage, thus are of major importance. Many diagnostic laboratories do not have rapid genotypic methods available and detection by phenotypic methods remains the cornerstone of detection.

This study compared a number of phenotypic tests that are readily available for accurate and rapid screening methods, for detection of these enzymes in clinically significant isolates of CRGNB.

Methods and Materials

One hundred and five non-consecutive clinical isolates of Gram negative bacilli were recovered from storage in glycerol broth at -80°C. Of these 66 were *Enterobacteriaceae*, 9 were *Acinetobacter baumannii* and 30 *Pseudomonas aeruginosa*. Fifty three were non-carbapenemase producing using phenotypic methods only. Fifty-two were confirmed to be CRGNB by moeluclar methods either for IMP, VIM, KPC, NDM, OXA-23-like (8), OXA-24-like (1), OXA-48-like (1) or OXA-51-like (1) genes. The PCR positive organisms were: IMP *K. pneumoniae* (6), *S. marcescens* (5), *C. freundii* (3), *E. coli* (2), *E. cloacae* (2) and *Ps. aeruginosa* (2); VIM were *P. aeruginosa* (3) and *Pr. mirabilis* (1); KPC's were *K. pneumoniae* (11) and *E. coli* (1); NDM's included *K. pneumoniae* (2), *E.coli* (1) and *M. morganii* (1). There was one AIM from a *Ps. aeruginosa.* There were eight OXA-23-like *A. baumannii*, one OXA-24-like *A. baumannii* and one OXA-51-like was *E. coli*.

The phenotypic screens performed were the Carba NP test[4] (CNP), Modified Hodge Test[5] (MHT) and Metallo-ß-lactamase screen using EDTA (MBL)[6]. The chromogenic agars included chromID ESBL (ESBL), chromID Carba (CARBA) and brilliance CRE (CRE). All OXA-like organisms had the CNP test repeated using a heavier inoculum suspension of >2.0 McFarland.

Results

The results for the phenotypic methods are shown in the Table 1 and 2. If the OXA-like enzymes were excluded from the analysis the sensitivity of the Carba NP improved significantly to 95.4%. One *P. aeruginosa* was repeatedly Carba NP test positive though no carbapenemase enzyme was detected by the molecular methods used. The modification of the Carba NP test using a heavier inocula, for the detection of the OXA-like enzymes which tested negative, still did not detect these enzymes. The MBL test is designed to detect metallo-beta-lactamases with 17 of the 29 (58.6%) detected.

Conclusions

Carbapenem Resistant Gram Negative Bacilli detected by PCR were not comprehensive in this study and some may have been missed.

The chromID ESBL detected the most isolates on the chromogenic agars tested but had poor specificity. The Carba NP test had excellent sensitivity if OXA-like enzymes were not included in the analysis. This method did not detect any of the OXA-like enzymes included. Further work is required on these isolates using recently published amendments to the method. However the specificity was excellent with one false positive detected in *Pseudomonas aeruginosa* despite repeated testing.

PHENOTYPIC DETECTION OF CARBAPENEM-RESISTANT GRAM NEGATIVE BACILLI. WHICH METHOD IS BETTER? CONT'D

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In the routine laboratory where PCR is not readily available, no one phenotypic method will detect all carbapenemase-resistant GNB producing organisms. An acceptable screen could include the chromID ESBL agar and the Carba NP test. Awareness of local susceptibility patterns and presence of OXA-like enzymes can also aid in detection of these organisms, and the requirement for phenotypic screening and further testing and referral for molecular testing.

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Table 1: Performance of Chromogenic agars

Enzyme	No	chromID ESBL	chromID Carba	Brilliance CRE
OXA	**11**	11	6	8
KPC	**12**	12	12	10
NDM	**4**	4	4	4
IMP	**20**	20	17	17
VIM	**4**	3	3	3
AIM	**1**	1	1	1
TOTAL	**52**	**51**	**43**	**43**
Sensitivity		98.2	85.2	85.2
Specificity		68.8	74.6	73.6
Positive Predictive Value		68.4	74.2	73.2
Negative Predictive Value		98.8	85.5	85.5

Table 2: Phenotypic Test Results

Enzyme	No	carbaNP	MHT	MBL
OXA	**11**	0	7	1
KPC	**12**	12	12	0
NDM	**4**	4	0	4
IMP	**20**	19	17	9
VIM	**4**	3	0	3
AIM	**1**	1	0	0
TOTAL	**52**	**39**	**36**	**17**
Sensitivity		80	76.5	72.5
Specificity		98.1	88.3	80.3
Positive Predictive Value		98.1	88.1	69.0
Negative Predictive Value		80.3	76.8	82.8

THE ROLE OF INTER-SPECIES RECOMBINATION

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Background

Haemophilus influenzae is a significant opportunistic pathogen that causes a range of respiratory infections, including community-acquired pneumonia (CAP), acute exacerbations of chronic obstructive pulmonary disease (COPD) and acute otitis media (AOM). These infections frequently require antibiotic therapy for management, with a β-lactam such as amoxicillin, cefaclor, and amoxicillin-clavulanate historically used as first line therapies. However, the efficacy of these antibiotics is threatened by the increasing prevalence of β-lactam resistance mediated by specific mutations in the ftsI gene that produce an N526K substitution in the encoded penicillin-binding protein 3 (PBP3). This type of resistance, termed β-lactamase-negative ampicillin-resistance (BLNAR), is difficult to detect in the diagnostic laboratory, as many BLNAR isolates do not actually show an ampicillin resistant phenotype using standard susceptibility testing methods. As a result genotypic testing methods are being increasingly adopted for BLNAR detection. Furthermore, the recent recognition of *Haemophilus haemolyticus*, a close non-pathogenic relative of *H. influenzae*, in diagnostic specimens from the respiratory tract, has compounded the issue. This is because *H. haemolyticus* isolates are frequently mis-identified as *H. influenzae*, which further complicates the role of the diagnostic laboratory in guiding antibiotic therapy for infections involving *H. influenzae*.

Objectives

To screen the *ftsI* gene sequences obtained from clinical isolates of non-typeable *Haemophilus influenzae* (NTHi), and normal flora isolates of *H. haemolyticus* for the presence of mosaic *ftsI* gene structures, and to evaluate the role inter-species recombination of the *ftsI* gene has on the formation of resistant *ftsI* genes and the subsequent dissemination of β-lactamase-negative ampicillin-resistance in *Haemophilus species*.

Methods

The *ftsI* genes of 100 *Haemophilus* isolates comprising genetically defined β-lactamase-negative ampicillin-susceptible (gBLNAS) and β-lactamase-negative ampicillin-resistant (gBLNAR) isolates of NTHi (n=50) and *H. haemolyticus* (n=50) were analysed in this study. Both the flanking regions and full-length ftsI gene sequences of all study isolates were screened for mosaic structures using *H. influenzae* Rd and *H. haemolyticus* ATCC 33390 as reference parental sequences, and bioinformatics was performed for recombination analysis using SimPlot..

Results

Of the 100 clinical isolates analysed 33% (33/100) harboured mosaic *ftsI* gene structures containing distinct ftsI gene fragments similar to both reference parental sequences. The inter-species recombination events were exclusively encountered in the *ftsI* gene of gBLNAR isolates of both NTHi (Figure 1) and *H. haemolyticus*, and were always associated with the formation of a mosaic fragment at the 3'-end of the *ftsI* gene. There was no evidence supporting the horizontal gene transfer (HGT) involving the entire *ftsI* gene among the clinical isolates *in vivo*.

THE ROLE OF INTER-SPECIES RECOMBINATION CONT'D

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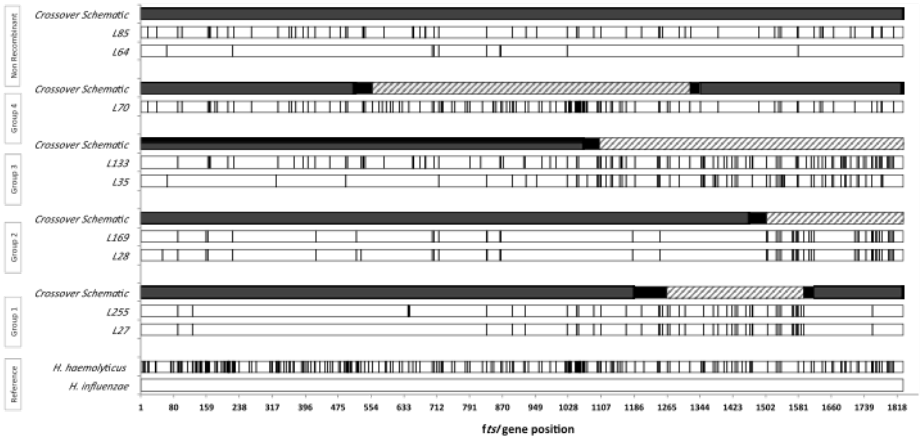

Figure 1. Mosaic ftsI *gene structures identified in clinical NTHi isolates.*
Schematic representation of the divergence in the ftsI *gene of NTHi study isolates compared to the* ftsI *reference sequence of* H. influenzae *Rd. Solid black lines, denote the position of the nucleotides that differ from the corresponding nucleotide in the reference* H. influenzae *sequence; Crossover schematics, show the proportion of the ftsI gene most similar to reference sequences; Solid grey shading, most similar to* H. influenzae Rd *sequence; Hashed grey shading, most similar to* H. haemolyticus *ATCC 33390; Solid black shading, crossover location.*

Conclusion

We provide evidence for the HGT and inter-species recombination of the *ftsI* gene among gBLNAR isolates of NTHi and *H. haemolyticus* in a clinical setting, highlighting the importance recombination of the *ftsI* gene has on the emergence of altered PBP3s and BLNAR-mediated resistance.

Further Information can be found in the recent publication:

Witherden EA, Bajanca-Lavado MP, Tristram SG, Nunes A (2014). The role of inter-species recombination of the *ftsI* gene on the dissemination of altered penicillin-binding protein 3 mediated resistance in *Haemophilus influenzae* and *Haemophilus haemolyticus*. J Antimicrobial Chemother; In Press. [doi: 10.1093/jac/dku022].

INCREASING EMERGENCE OF IMP-4 PRODUCING ENTEROBACTERIACEAE THROUGHOUT QUEENSLAND HOSPITALS

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Emergence of carbapenemase-producing *Enterobacteriaceae* (CPE) has resulted in increasing difficulties in the treatment of multidrug-resistant Gram-negative bacteria. The types of enzymes vary by region with OXA-48-production is the major cause of CPE in parts of Europe [1]. In the USA, the KPC-producers are still the predominant type of CPE [2]. Cases of NDM-producing *Enterobacteriaceae* have been occasionally reported in Australia, and usually in patients who had a travel history [3, 4]. In contrast, IMP-producing *Enterobacteriaceae* which has been reported as the predominant CPE in Australia has not been associated with international travel [5-7].

Carbapenemase encoding genes are usually located on large plasmids. Plasmids can be classified based on their incompatibility (Inc) groups, or replicon types. Plasmids of different replicon types are associated with the carriage and transmission of particular antibiotic resistance genes [8]. The majority of bla_{IMP} genes have been found on plasmids belonging to incompatibility groups L/M and A/C [5].

Although cases of IMP-producing *Enterobacteriaceae* have been reported occasionally from Melbourne or Sydney, currently, there has been no data from Queensland. Meropenem resistant *Enterobacteriaceae* were referred by hospitals and pathology laboratories to a state reference laboratory between 2009 and 2013. Here, we report the significant increase in CPE mainly due to IMP-4 producing *Enterobacteriaceae* over the past 2 years. We also report the features of plasmids carrying these carbapenemase encoding genes.

Enterobacteriaceae isolates with reduced susceptibility to meropenem by Vitek 2 (bioMerieux) were referred from hospitals in Queensland to a state reference laboratory from 2009 to 2013 for the confirmation of the presence of carbapenemase encoding genes. Only one isolate per patient was included unless the patients had multiple species; then all species were included. PCR and sequencing for various carbapenemase and β-lactamase encoding genes and aminoglycoside resistance genes were performed on all isolates using previously described methods [4, 9, 10]. Since *Enterobacter* spp especially *E. cloacae* were predominant, the relatedness of *E. cloacae* was determined by a semi-automated method, rep-PCR typing (Diversilab, bioMerieux). All isolates were characterised for the most commonly replicon types associated with carbapenemase encoding genes, i.e. L/M, H12 and FII using previously described methods [11]. A specific set of primers was designed to target H12 replicon in this study.

A total of 32 *Enterobacteriaceae* isolates were confirmed as IMP-4-producing *Enterobacteriaceae* by PCR and sequencing. The CPE isolates were referred from hospitals in wide geographical area of Queensland, including Townsville, Rockhampton, Caloundra, Toowoomba, Logan and Brisbane. There was no evidence of hospital outbreaks. The majority of CPE were isolated in 2013 (n=22). Seven CPE were isolated in 2012 and only three CPE were isolated prior to 2012. The IMP-producing *Enterobacteriaceae* comprised of *E. cloacae* (n=19), *E. aesburiae* (n=2), *K. pneumoniae* (n=3), *E. coli* (n=3), *E. hermannii* (n=1), *S. marcescens* (n=1), *C. freundii* (n=1), *C. koseri* (n=1) and *P. mirabilis* (n=1) (Table 1). All isolates showed resistance or reduced susceptibility to carbapenems, especially to ertapenem. All isolates showed the production of carbapenemase by MHT. All isolates showed MBL phenotypes using EDTA. The IMP-producers usually possessed bla_{TEM}. However, bla_{SHV}, bla_{CTX-M} and bla_{CMY} were less frequently found in IMP-producers (Table 1). All IMP-producers were susceptible to amikacin.

INCREASING EMERGENCE OF IMP-4 PRODUCING ENTEROBACTERIACEAE THROUGHOUT QUEENSLAND HOSPITALS CONT'D

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There were no predominant clones observed amongst *Enterobacter* spp. Several *E. cloacae* were clonally but not epidemiologically related. Two pairs of identical strains which were related both temporally and geographically suggesting person-to-person transmission. The majority of IMP-4-producers were positive for H12 replicon plasmids (66%). Although L/M replicon was less common amongst IMP-producers (22%), the L/M bla_{IMP}-carrying plasmids were successfully transferred by transformation. All the bla_{IMP-4} genes were located in integron class 1. The majority of isolates had an integron size of 1.2 kb. A gene encoding aminoglycoside resistance was located downstream to $bla_{IMP-4.}$

The emergence of CPE is not only occurring overseas, but also in Australia. IMP-mediated resistance was initially described in bla_{IMP-1} producing *Serratia marcenscens* in Japan [12] with bla_{IMP-4} first being reported in *A. baumannii* in Hong Kong in the 1990's [13]. bla_{IMP-4} was first detected in Melbourne and Sydney in 2002 and has since been the most commonly reported variant of bla_{IMP} in Australia [5-7].

The association of bla_{TEM} and *aac-6'-Ib* in IMP-producers have been reported previously [5]. In our study, we proved that these genes are located on bla_{IMP-4}-carrying plasmids. A previous study in Australia showed that the majority of bla_{IMP-4} was carried in A/C and L/M plasmids [5]. Only one isolate from China in H12 has been described [5]. Here, we have demonstrated the predominance of H12 plasmids carrying bla_{IMP-4} in Queensland. Unique to IMP-4-producers in our study, there were no other variants of bla_{IMP} found. In contrast studies from South Asian countries and China have described outbreaks due to other variants of bla_{IMP} [14-17]. The variant IMP-4 is also found in other states in Australia as the predominant type of MBL present [5-7]. The mechanism of spread of the IMP-4-producers in Queensland is unknown. Environmental contaminations, especially in burns units and intensive care units, have been attributed to the sources of outbreaks elsewhere in Australia [6, 18]. The source of acquisition of CPE was not studied here and remains subject to speculation, particularly in view of the low incidence and wide geographic distribution. In order to reduce transmission, the investigation of the reservoir of IMP-producers warrants urgent further study. The number of cases reported here is likely to be a gross estimation of the true burden by CPE. In conclusion, given the steady increase in prevalence of CPE, routine screening for isolates with reduced susceptibility to carbapenems should become part of standard laboratory practice. Further efforts are required to eliminate the spread of CPE in Australia.

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INCREASING EMERGENCE OF IMP-4 PRODUCING ENTEROBACTERIACEAE THROUGHOUT QUEENSLAND HOSPITALS CONT'D

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Table 1. Characteristics of IMP-4-producing *Enterobacteriaceae* from Queensland.

Species	Number of isolates	*bla*$_{IMP}$ variant	*bla*$_{TEM-1}$	*bla*$_{SHV}$	*bla*$_{CMY-2}$	*bla*$_{CTX-M}$	*aac-6-Ib*	Replicon types	
								H12	L/M
E. cloacae	19	*bla*$_{IMP-4}$	18	3	2	2	18	12	5
E. aesburiae	2	*bla*$_{IMP-4}$	2	2	0	0	2	2	0
E. coli	3	*bla*$_{IMP-4}$	2	0	0	0	3	1	0
E. hermannii	1	*bla*$_{IMP-4}$	1	1	0	0	1	1	0
K. pneumoniae	3	*bla*$_{IMP-4}$	3	3	0	0	3	1	2
C. freundii	1	*bla*$_{IMP-4}$	1	0	0	0	1	1	0
C. koseri	1	*bla*$_{IMP-4}$	1	0	0	0	1	1	0
S. marcescens	1	*bla*$_{IMP-4}$	1	0	0	0	1	1	0
P. mirabilis	1	*bla*$_{IMP-4}$	1	0	0	0	1	1	0
Total	32		30	9	2	2	31	21	7

GENOMICS OF PERSISTENT *STAPHYLOCOCCUS AUREUS* INFECTION

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Persistent *Staphylococcus aureus* infection is a well recognized, difficult to treat condition, however the molecular basis for persistence is not understood. We have previously investigated a pair of clinical *S. aureus* isolates obtained from a patient before and after a protracted persistent infection. We showed that the day-115 isolate had a reduced growth rate, resistance to innate immune factors, and a chronically active stringent response. Here, we have undertaken a more detailed investigation by phenotypic and/or genomic characterization of 47 blood stream isolates obtained from 23 positive blood culture specimens and one spinal aspirate over the 115 day infection.

All isolates underwent detailed antibiotic susceptibility testing, growth characterization, and measurement of delta hemolysin expression (an indication of quorum sensing activity) and 15 isolates were sequenced using the Illumina MiSeq platform. The genome sequences of the first (JKD6210) and last (JKD6229) clinical isolates were fully assembled, annotated, and validated using Optical Mapping.

There were 29 hVISA/VISA and 16 VSSA isolates. All the hVISA/VISA isolates had a growth defect, and most isolates produced delta hemolysin. The hVISA/VSSA phenotype emerged at day 45. Interestingly there were mixed populations of VSSA and hVISA in some blood cultures. Comparative genomic analysis by read mapping and SNP detection identified unique transient mutations and selected conserved mutations associated with antimicrobial resistance or innate immune evasion. Read-mapping also revealed substantial and varied chromosome duplications up to 98kb in antibiotic resistant sub-clones of *S. aureus*. Such chromosome flux has not been previously described in *S. aureus*, and was experimentally confirmed by genome closure and optical mapping. Intriguingly, these chromosome flux seem to coincide with the reduction in vancomycin susceptibility.

This study highlights several adaptive strategies employed by *S. aureus* to persist in the face of antimicrobial therapy within the milieu of the host to cause persistent infection and the power of thorough genomic comparisons to uncover clinically significant mutations in addition to SNPs.

Abbreviations: hVISA, hetero- vancomycin-intermediate *Staphylococcus aureus*; VISA, vancomycin-intermediate *Staphylococcus aureus*; VSSA, vancomycin susceptible *Staphyloccus aureus*

2014 - 2015 MEETING CALENDAR

2014

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10-13 May 2014, Barcelona, Spain
Website: http://escmid.org/dates_events/

114th American Society for Microbiology Annual Meeting
17-20 May, Boston, USA
Website: http://www.asm.org

7th European Meeting on viral zoonoses
24-27 May 2014, St Raphael, France
Website: http://www.euroviralzoon.com

Microbiology in the new Genomcis Era – Genomics 2014
24-27 June, Paris, France
Website: www.genomes-2014

Molecular typing methods for pathogens
30 June - 4 July, Lyon, France
Website: http://escmid.org/dates_events/

Australian Society for Microbiology, Annual meeting
6-9 July, Melbourne
Website: www.the asm.org.au

20th IEA World Congress of Epidemiology
Aug 17-21, Anchorage, Alaska
Website: http://ieaweb.org/

International Union of Microbiological Societies (IUMS) 2014 Congress
27 July to 1 August, Montreal, Canada
website: www.montrealiums2014.org/

16th International Symposium on Staphylococci and Staphylococcal infections
26-29 August, Chicago, USA
Website: http://isssi2014.com/

54th ICAAC
6-9 Sept, Washington D.C. USA
Website: http://www.asm.org

ID Week 2014: IDSA
8-12 Oct, Philadelphia, USA
Website: www.idsociety.org

15th Asia Pacific congress of Clinical Microbiology and Infection.
26-29 Nov, Kuala Lumpur
Website: http:/www.apccmi2014.org/

2015

Antimicrobials 2015
26 - 28 Febraury 2015, Brisbane
Website: http://antimicrobials.com

25th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID 2015)
25 - 28 April 2015, Copenhagen, Denmark
Website: http://escmid.org/dates_events/

55th ICAAC
18-21 Sept, San Diego, USA
Website: http://www.asm.org