BREAK POINT

2015 - ISSUE 12

WELCOME TO ANOTHER EDITION OF BREAKPOINT

The New Year and January holidays have presented themselves and passed in a flash, and once again the Annual ASA meeting is upon the readership of "Breakpoint". In the first issue of the year, Sally Partridge, on behalf of her co-authors, provides an update of the work supported by an ASA research grant (2013), entitled "Sequencing antibiotic resistance plasmids from the *Enterobacteriaceae".* An update from John Turnidge on the more pertinent musings of the CLSI and EUCAST follows. The committee trusts that 2015 will be a fruitful conference year for gazing into the future of antimicrobials and mircoorganisms alike. As always suggestions towards improving the Newsletter are very welcome.

Sharon Chen
ASA Breakpoint Editor

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IN THE NEWS

Enhanced Gram-negative activity of novel antibiotic ceftolazone-tazobactam

A blessings in the guise of another new cephalosporin may be upon us? - In 2014, the US Food and Drug Administration approved **ceftolozane-tazobactam**, a novel intravenous cephalosporin-beta-lactamase inhibitor combination. The combination has broad-spectrum *in vitro* activity against aerobic and aerobic Gram-negative rods, **including *Pseudomonas aeruginosa* and most extended-spectrum-beta-lactamase-producing Gram negative organisms**. In as yet unpublished clinical trials, clinical cure rates with ceftolozane/tazobactam alone or in combination with metronidazole were similar to those with comparator antibiotics for complicated urinary tract and intra-abdominal infections (1,2).

IN THE NEWS CONT'D

Multistate outbreak of listeriosis associated with caramel apples !

A multistate outbreak of listeriosis associated with commercially produced prepackaged caramel apples was reported in the United States in December 2014 [7]. Updated case counts, a list of states in which cases have been reported, and guidance from the United States Centers for Disease Control and Prevention (CDC) can be found on the CDC's website)

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3. Centers for Disease Control and Prevention. Multistate outbreak of listeriosis linked to commercially produced, prepackaged caramel apples. http://www.cdc.gov/listeria/outbreaks/caramel-apples-12-14/index.html (Accessed on December 24, 2014).

SEQUENCING ANTIBIOTIC RESISTANCE PLASMIDS FROM THE *ENTEROBACTERIACEAE*

Sally Partridge[1], Jan Bell[2] and Jonathan Iredell[1]
Westmead Millennium Institute[1] and SA Pathology[2]

Introduction

Multi-resistant *Enterobacteriaceae*, such *Klebsiella pneumoniae* and *Escherichia coli*, producing extended-spectrum β-lactamases and/or carbapenemases in combination with resistance to other antibiotics are an increasing global health problem. Much of this problematic multi-resistance is due to different combinations of resistance genes carried on large conjugative plasmids that can spread between isolates, strains and, in some cases, different species[1]. Resistance genes are often found as part of large complex multi-resistance regions (MRR) consisting of mosaic assemblies of resistance genes and associated insertion sequences (IS), integrons (In) and transposons (Tn) that appear to belong to a limited set[2]. These MRR are inserted in a plasmid 'backbone' encoding proteins required for plasmid replication and conjugation and plasmid stability/partitioning systems and 'addiction' systems that ensure plasmids are maintained in bacterial populations.

Plasmid backbones were originally classified based on incompatibility, i.e. the inability of two similar plasmids to coexist in one bacterial cell, by experimentally introducing two plasmids into the same cell and determining whether one was lost. Subsequently hybridisation methods[3] and more recently PCR-based replicon (PBRT)[4,5] and degenerate-primer MOB typing (DPMT)[6] methods have been developed. The number of plasmids present in an isolate and their sizes can be examined by S1 nuclease digestion followed by pulsed-field gel electrophoresis (S1/PFGE)[7], coupled with hybridisation to determine which plasmid carries which resistance genes. Plasmids can also be compared by methods such as digesting with restriction enzymes. For some Inc groups, typing systems based on the idea of multi-locus sequence typing used for bacterial isolates have been developed e.g. for IncI1 plasmid pMLST five target genes are amplified, sequenced and compared with a database[8].

Understanding associations between resistance genes, MRR and different plasmid backbones and the factors determining the success of certain plasmids is vital in controlling the spread of multi-resistance. Sequencing of complete plasmids obviously provides the most information and 'next generation' technology is now making this economically feasible, although assembly of MRR sequences, which typically have multiple long repeats, from short-read data can be challenging. The aim of our ASA Research Grant proposal was to completely sequence selected plasmids carrying important resistance genes. Preliminary data was presented at the ASA meeting in Melbourne 2014.

Plasmid sequencing and assembly.

We originally intended to use the Roche 454 system to sequence plasmids, as we had done previously, but the more accurate Illumina sequencing method became more accessible. Although Illumina sequencing produces shorter read lengths than 454, reads from the latter method are still too short to cover the long repeats (e.g. IS) typically found in MRR, so 454 may not have any advantage over Illumina for these types of sequences. To simplify assembly, in most cases we used a transconjugant carrying a single plasmid to prepare the DNA template for sequencing and we found that using a combination of different assembly programs gave the best results. As expected Illumina reads were assembled into contigs of similar lengths to those we previously obtained for other plasmids by 454. Annotation with the Attacca annotation system, developed with Dr Guy Tsafnat (Centre for Health Informatics, Macquarie University, Sydney)[9] indicated that contigs often ended at the boundaries of mobile elements or other repeated elements. Attacca annotation of sequences available in GenBank was used to guide design of PCRs to join contigs. RAST[10], BLAST searches and the sequences of well-characterised plasmids were used to annotate plasmid backbones.

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Assembly problems caused by the "shufflon" in IncI1 plasmids

Four of the plasmids sequenced in this work are classified as IncI1. IncI1 (and IncI2) plasmids typically carry a "shufflon" multiple inversion system that generates variation in the C-terminus of the PilV tip adhesin of the thin pilus, which is required for mating in liquid media and may have a role in specificity of conjugation[11]. The complete IncI1 shufflon in the archetypal plasmid R64 consists of seven 19 bp inverted repeats that flank and separate four segments (A, B, C, D). Rci, a shufflon-specific recombinase encoded by the rci gene adjacent to the shufflon, catalyses recombination between the repeats, resulting in inversion, rearrangement and/or deletion of segments[12]. Segments A-C each contain two oppositely-oriented partial open reading frames (orf) and segment D has a single orf. The arrangement of shufflon segments dictates which of these seven possible orfs will be joined in frame with the 5' end of the adjacent pilV gene.

For all four IncI1 plasmids described below, sequence assembly generated several contigs corresponding to the shufflon. Depending on the assembly programme used, these either ended precisely at the boundaries of the shufflon repeats or included one complete shufflon segment flanked by fragments of two others. As the former gave no information about the arrangement and the latter often contradicted one another, these contigs could not be assembled. Sequencing of PCR amplicons obtained with primers in the rci and pilV genes flanking the shufflon revealed mixed bases beyond the outermost shufflon repeat for all four plasmids, suggesting active rearrangement and that populations of plasmid molecules harbouring different arrangements of the shufflon are present in DNA preparations used for sequencing.

Many papers about complete sequencing of IncI1 plasmids do not report how the shufflon was assembled, possibly because some assembly programs produce a plausible arrangement. This may mean that closely-related plasmids are reported as having different shufflon arrangements, but our experience suggests that it is more likely that a number of different arrangements for each plasmid will be present. Some shufflons in sequenced plasmids are reported as missing one or more segments and although this has been verified by cloning and hybridisation for some plasmids, in some cases it may be an artefact of assembly. Examination of the sequences of the above IncI1 plasmid sequences plus those available in GenBank suggests that shufflon segment sequences are highly conserved, with variation mainly due to shufflon rearrangement. Variations at a few different positions appear common and may be useful for epidemiological purpose. Some do alter the protein sequence, but any effect on PilV function is not yet known.

Following discussions between our PhD student Kaitlin Tagg and Dr. Michael Brouwer (Netherlands) at the International Society for Plasmid Biology (ISPB) conference (Palm Cove, Queensland, 27th Oct-1st Nov 2014), a joint manuscript on assembly of shufflons from next-generation sequencing data has been submitted to the journal "Plasmid" for a special issue associated with the meeting. This work will also form the basis for future experiments on shufflon function, as part of Kaitlin's PhD project.

An interesting IncI1 plasmid carrying the *bla*$_{CMY-2}$ plasmid *ampC* gene

The most common plasmid-borne ampC (class C β-lactamase) genes found in *E. coli* are *bla*$_{CMY-2}$-like genes, mainly carried by IncA/C and IncI1 plasmids. *bla*$_{CMY-2}$ has been captured from the *Citrobacter freundii* chromosome by the insertion sequence ISEcp1, which is able to pick up genes adjacent to its right-hand end by using uses its own left-hand inverted repeat (IR$_L$) with a sequence other than its own right-hand IR (IR$_R$) during transposition. Direct repeats of 5 bp generated

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during transposition define the ends of the 'transposition unit' (TU) and ISEcp1 also provides a promoter for expression of adjacent genes[13].

Our surveys of local Sydney clinical isolates identified bla_{CMY-2} as the most common *ampC* gene in *E. coli*, and these isolates often also carried IncI1 plasmid, but no IncA/C plasmids were identified (unpublished). Transconjugants carrying bla_{CMY-2} on an IncI1 plasmid were obtained from several isolates and pMLST identified some as belonging to ST2, a type commonly associated with bla_{CMY-2}. As no ST2 plasmid sequence was available, one such plasmid (pJIE512b) was initially selected for sequencing.

As we reported in Antimicrobial Agents and Chemotherapy[14] and in an oral presentation at the ISPB meeting (Kaitlin Tagg), sequencing and assembly of pJIE512b gave a 92,339 bp plasmid with similar overall organisation to other IncI1 plasmids. A 4,831 bp insert containing 161 bp of the right end of ISEcp1, truncated by IS1294, and 2,823 bp region of the *C. freundii* chromosome encompassing bla_{CMY-2} was followed by a 159 bp fragment of IncA/C backbone. As IS1294 can also capture adjacent regions, this suggested that this element had inserted into ISEcp1 adjacent to bla_{CMY-2} in an IncA/C plasmid and subsequently transferred itself and the adjacent region, including bla_{CMY-2} and a piece of IncA/C backbone, into an IncI1 plasmid. A similar bla_{CMY-2} context was identified in other IncI1 plasmids, but with a variant of IS1294 inserted in a different position and a longer IncA/C fragment, suggesting two independent IS1294-mediated mobilisations of bla_{CMY-2} from an IncA/C to an IncI1 plasmid. IncA/C plasmids are considered "broad host-range", found in many species of *Enterobacteriaceae*, while IncI1 plasmids may be better adapted to *E. coli* and *Salmonella spp.*, suggesting a direction for the "flow" of resistance genes.

The complete sequence of a plasmid carrying the second type of insertion and belonging to IncI1 ST2 appeared in GenBank during this work (pR7AC; accession no. KF434766). Comparison of pJIE512b and pR7AC revealed substantial differences across the backbones, in addition to different insertions carrying bla_{CMY-2} in different positions. This diversity in plasmids belonging to the same sequence type highlights the need to develop better plasmid typing methods. Sequencing of additional IncI1 plasmids, including those carrying bla_{CMY-2}, and analysis of available IncI1 plasmids is planned to start to address this.

IncI1 plasmids carrying the globally-dominant $bla_{CTX-M-15}$ ESBL gene

We found that bla_{CTX-M} genes, particularly the widespread $bla_{CTX-M-15}$, are dominant in western Sydney[15,16], mostly associated with IncF and IncI plasmids. In F plasmids, $bla_{CTX-M-15}$ has generally been found in a 2.971-kb ISEcp1-$bla_{CTX-M-15}$-orf477Δ TU inserted in a truncated Tn2 located within larger MRR[17]. Sequencing of three different IncI1 plasmids carrying $bla_{CTX-M-15}$ revealed that ISEcp1-$bla_{CTX-M-15}$ is associated with different Tn2-derivatives in backbones that are organised similarly to other IncI1 plasmids, but with insertions/deletions and sequence differences

In pJIE113 the typical 2.971-kb TU interrupts a complete Tn2 inserted directly in the plasmid backbone. This context may be the progenitor of the version with the truncated Tn2 seen in IncF plasmids or ISEcp1-$bla_{CTX-M-15}$ move between the two plasmid types by homologous recombination in the flanking Tn2 segments. In the second plasmid, pJIE139, the ISEcp1-$bla_{CTX-M-15}$ TU lies within a novel Tn2/3 hybrid transposon with an internal deletion, which again suggests movement of ISEcp1-$bla_{CTX-M-15}$ by homologous recombination. In the third plasmid, pJIE174, the TU is truncated by two copies of IS26 and is found within a larger region bounded by the ends of Tn2 that also includes a gentamicin resistance gene *(aac(3)-*

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IId). bla$_{CTX-M-15}$ and the associated ISEcp1-derived promoter may be able to move from this structure by the actions of IS26, independently of both ISEcp1 and Tn2.

pJIE113 is almost identical to plasmids isolated from the 2011 *E. coli* O104:H4 outbreak in Europe[18], with only a few nucleotide differences, and appears to be the earliest characterised version of this plasmid. pJIE174 is almost identical to pESBL-12 from the Netherlands[19], again illustrating how successful plasmids spread globally. In contrast, both the backbone and the Tn2/3 ISEcp1-*bla*$_{CTX-M-15}$ insertion of pJIE139 are significantly different from other sequenced IncI1 plasmids and it may be a more local or less successful plasmid, or similar plasmids may be identified in the future.

This work was presented as a poster at the ISPB conference by Dr Andrew Ginn and is under review for the "Plasmid" Special Issue.

IncF plasmids carrying *bla*$_{CTX-M-15}$ from *E. coli* ST131 and ST405.

Preliminary characterisation of plasmids carrying *bla*$_{CTX-M-15}$ from Sydney suggested that they carry related MRR with simple insertions and/or deletions that are easy to explain, but differences in the backbones[17]. We selected eight IncF plasmids, most carrying two or three IncF-type replication systems, from *E. coli* ST131 and ST405 isolates for complete sequencing. Assembly of these plasmids from Illumina data was difficult despite already having mapped the MRR[17] and in some cases there appear to be different backbone arrangements due to recombination between IS elements present in multiple copies.

As expected from preliminary characterisation, pJIE100 was almost identical, except for a deletion, to pC15-1a from Canada, the first IncF plasmid carrying *bla*$_{CTX-M-15}$ to have been sequenced[20]. The backbones of the other three plasmids from ST131 isolates , known to all carry the same MRR, are generally closely related to each other and to other plasmids from ST131 isolates from the UK[21,22]. Two of these plasmids have a novel ~5 kb IS26-mediated deletion of genes involved in phosphate acquisition, and potentially virulence, compared with the third. Most available IncF plasmids have the longer version of this region, but in one a larger region is deleted.

Three of the plasmids from *E. coli* ST405 were known to have related MRR and were found to have similar backbones, with some, but not all modules, in common with the plasmids from ST131 isolate and differences in the conjugation region. PCR and Sanger sequencing suggest three possible arrangements for one plasmid, mediated by IS26 and potentially disrupting a region encoding iron uptake functions likely to be important for virulence. The fourth plasmid from ST405 was significantly different from all of the other plasmids and carried an additional resistance region. PCR and sequencing suggests that different arrangements of this plasmid are present, due to recombination between different copies of IS1 or different copies of IS26.

This work was presented as a talk at the ISPB conference by Dr Carola Venturini and a manuscript is in preparation.

Plasmids carrying *bla*$_{NDM}$ from *K. pneumoniae* and *K. oxytoca* are closely related

We have characterised four isolates carrying *bla*$_{NDM-1}$ isolated in Australia[23]. One plasmid carrying *bla*$_{NDM-1}$ (pJIE2121), from a *K. pneumoniae* isolated in 2011 from a patient recently returned from India, was typed as IncFII$_Y$, denoting a type of plasmid apparently associated with *Yersinia spp.*[24]. No sequence for this type of plasmid with *bla*$_{NDM}$ was available at the time so

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pJIE2121 was sequenced. pJIE2121 is very closely related, apart from a deletion, to pKOX-NDM-1 from a 2010 *K. oxytoca* isolate from Taiwan[25], and to pRJF866 from a *K. pneumoniae* isolate from China (KF732966), both of which became available during this study. These plasmids also carry the *rmtC* gene encoding a 16S rRNA methyltransferase that confers high-level resistance to all clinically-important aminoglycoside antibiotics and again illustrates spread of successful plasmids between countries. This work could be included as part of a larger study on plasmids carrying bla_{NDM} genes.

Plasmids from *E. coli* and *K. pneumoniae* carrying bla_{KPC-2}

We have characterised one *E. coli* and seven *K. pneumoniae* from Australia carrying a bla_{KPC} gene[26]. Plasmids from the *E. coli* (ST131) and a *K. pneumoniae* (ST1048) isolated from the same patient were initially selected for sequencing, as the other isolates all belong to clonal complex (CC) 258 and each appeared to have ~4 plasmids closely related to those found in other CC258 isolates, for which many sequences are now available.

The *E. coli* isolate, JIE2543, carries two plasmids (pJIE543-1 and pJIE2543-2), each of which was obtained in a separate transconjugant. The *K. pneumoniae* isolate, JIE2540, also carries two plasmids, but only one, pJIE2540-2, could be transferred to *E. coli* by conjugation. Plasmid DNA from this transconjugant plus a complete genomic DNA preparation from JIE2540 were sequenced, to enable assembly of the second plasmid, pJIE2540-1, by subtraction. pJIE2543-1 is closely related to pKpQIL-like plasmids that often carry bla_{KPC} in *K. pneumoniae* CC258 isolates. Only one other complete sequence of a pKpQIL-like plasmid from *E. coli* is available and it has a 14.5 kb ISKpn14-mediated deletion in the backbone compared with pKpQIL and pJIE2543-1[27]. pJIE2540-1 is also closely related to pKpQIL and pJIE2540-1 except for a different, 13.2 kb ISKpn14-mediated deletion that includes part of the resistance region. pJIE2543-2 is closely related to pUTI89, a plasmid from a UTI *E. coli* isolate that carries virulence genes but no resistance genes, while pJIE2540-2 belongs to the IncA/C group and carries most of the resistance genes found in pJIE2540. Final assembly and analysis of both plasmids is underway.

Summary

Sequencing and analysis of IncI1 plasmids revealed assembly issues with the shufflon region, movement of an important resistance gene from a broad host-range to a narrow host-range plasmid and the probable involvement of homologous recombination in movement of $bla_{CTX-M-15.}$ Sequencing of IncF plasmids highlights the complexity of this group and $IncFII_K$ pKpQIL-like plasmids normally associated with *K. pneumoniae* CC258 were identified in a different sequence type and in *E. coli*. The high level of conservation between plasmids isolated in Australia and those reported overseas illustrates the global spread of plasmids. This work has led to publications and conference presentations and some of the data obtained will provide the basis for future studies.

Acknowledgements

We would like to thank the ASA for awarding us the Research Grant that enabled this project.

SEQUENCING ANTIBIOTIC RESISTANCE PLASMIDS FROM THE *ENTEROBACTERIACEAE* CONT'D

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CLSI AND EUCAST NEWS

John Turnidge
University of Adelaide

As January 2015 rolls around, CLSI and EUCAST both release their updated documents on MIC breakpoints - their M100 and EUCAST Breakpoint Tables, respectively. Both do us the courtesy of listing the changes and updates to their documents! Highlights from these are:

CLSI M100-S25

- MIC and zone diameter breakpoints for cefazolin when used to as a surrogate test for predicting susceptibility or urine isolates to oral cephalosporins, in Australia's case that is cephalexin, cefaclor and cefuroxime-axetil. These breakpoints are different from those to predict susceptibility to (parenteral) cefazolin
- MIC and zone diameter breakpoints for azithromycin and Salmonella Typhi. Note that these are for the Typhi serotype only, as there are insufficient data to know about whether these breakpoints would apply to other serotypes
- A pefloxacin screening disc diffusion test for reduced fluoroquinolone susceptibility in *Salmonella species*. Based on multiple studies, this reagent has the highest sensitivity and specificity for predicting the presence of the broad array of acquired resistance genes
- Epidemiological cutoff value (ECOFF) for vancomycin and *Propionibacterium acnes*. As the full suite of data required to establish a breakpoint for this bug-drug combination are not available, it was agreed that there was a need to have at least an ECOFF available for clinical use – with appropriate caveats appended to reports. This is the first deliberate use of an ECOFF in any CLSI document
- Promotion of the CarbaNP test over the Modified Hodge test for phenotypic confirmation of acquired carbapenemases.

EUCAST Breakpoint Tables V 5.0

- Ceftobiprole MIC breakpoints for Enterobacteriaceae, *Staphylococcus aureus* and *Streptococcus pneumoniae*
- Revised amikacin zone diameter breakpoints for Enterobacteriaceae
- Revised telavancin breakpoints for *Staphylococcus* spp. after accepting the change to the use of polysorbate-80 in the MIC test
- MIC breakpoints for *Mycobacterium tuberculosis* and two new antimycobacterial agents, delamanid and bedaquiline

Other CLSI Activities

CLSI subcommittees have been active on a range of fronts. The Antifungal Susceptibility Testing Subcommittee is currently creating a new document that will provide the methodology for establishing ECOFFs for moulds and the less common yeast species, where there are no or insufficient PK-PD and clinical data to establish formal clinical breakpoints. This will be accompanied by a supplement which will be more regularly updated with the actual ECOFFs. The challenge will be how to use these values in the clinical laboratory, and how they might be used in a report to clinicians.
A similar document in ECOFFs is also being prepared by the Veterinary AST subcommittee, but for an entirely different purpose, and this is for use of ECOFFs in food animal resistance surveillance programs.
The Antimicrobial Susceptibility Testing Subcommittee is working on a revision of it's M23 document which describes

CLSI AND EUCAST NEWS CONT'D

the type of data and the analytical techniques used to establish breakpoints and quality control ranges. The Veterinary equivalent, M37, is also undergoing revision. Both will be substantial updates. It is recommended that interested parties take time to read these documents when they are releases, because they provide great insights into modern breakpoint setting, which is not a simple process.

Other EUCAST Activities

EUCAST has recently started a Veterinary Subcommittee, VetCAST, whose charter is to work of methods and breakpoints for bacteria of importance in animal health. Also published on their website is an interesting article for Eurosurveillance on the 'market penetration' of EUCAST across Europe.

2015 - 2016 MEETING CALENDAR

2015

ASA 16th ANNUAL MEETNG
February 26-28, Brisbane, Queensland
Website: www.asainc.net.au

Pathology Update 2015
Feb 27 – Mar 1, Melbourne, Victoria
Website: www.rcpa.edu.au

New Perspectives in Infection Control
12-14 March, Kayseri, Turkey
Website: www.escmid.org/

ASID annual meeting
Mar 18-21, Auckland, New Zealand
Website: www.asid.net.au

The 2015 TB Summit
March 24-26, London, UK
Website: https://www.regonline.co.uk/

7th international Congress of the Asia Pacific Society for Infection Control
26-29 March 2015, Taipei, Taiwan
website: www.apsic2015.org

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

19th Congress of the International Society for Human and Animal Mycology
4-8 May 2015, Melbourne, Australia
Website: www.isham2015.com.au

115th Annual General Meeting, American Society for Microbiology
May 30 – June 5, New Orleans, USA
Website: http://www.asm.org

International Conference in Prevention and Infection Control
16-19 June, Geneva, Switzerland
Website: http://www.icpic2015.com

Australian Society for Microbiology Annual Meeting
12 -15 July, Canberra, ACT
Website: www.theasm.org.au

9th International Conference on Emerging Infectious Diseases
24-26 Aug, Atlanta, USA
Website: www.iceid.org/

***Clostridium difficile*: practical aspects of diagnosis and comparative genomics**
2-4 Sept, Maribor, Slovenia
Website: www.escmid.org/

STI and AIDS World Congress 2015-02-06
14-16 Sept, Brisbane, Australia
Website: www.worldsti2015.com/

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

ID week 2015
7-11 Oct, San Diego, CA
Website: www.idweekinternationa.com/

9th International Transplant Infectious Diseases Conference
13-15 Oct, Cancun, Mexico
Website: www.tts.org/

2016

26th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID 2015)
9-12 April 2016, Instanbul, Turkey
Website: http://escmid.org/dates_events/

16th Asia Pacific Conference on Clinical Microbiology and Infection (APCCMI)
30 Nov- 3 Dec, Melbourne, Australia
Website: http://www.asainc.net.au

21st International AIDS Conference
17- 20 July, Durban, SA
Website: www.aids2016.org/

In 2016, the ASM general meeting and ICAAC will be co-located in Boston, June 2016.