On the road again – the antibiotic resistance superhighway

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Antibiotic resistance (AMR) in Gram-negative bacteria, has expanded massively in recent years and is now of global public health concern. AMR determinants in many cases have originated from environmental bacteria. Horizontal gene transfer (HGT) has facilitated the dissemination of these genes to organisms of different species and genera, including those of clinical relevance. HGT occurs by three broad mechanisms, namely conjugation, transformation and transduction. Key elements of HGT include insertion sequences, transposons, integrons and other mobile genetic elements (MGEs) such as plasmids.

This Chapter will discuss the role HGT and MGEs play in the dissemination of AMR genes. Of particular epidemiological significance has been the emergence of extended-spectrum β-lactamases (ESBLs) and carbapenemases. Key examples ($bla_{\text{CTX-M}}$, bla_{KPC}, bla_{NDM}) will be used to illustrate genetic context, the evolutionary processes involved and highlight different patterns of dissemination which have occurred.

Keywords: Antibiotic resistance; mobile genetic elements; horizontal gene transfer

1. Introduction

HGT describes the transfer of DNA sequences between living cells followed by integration into and stable maintenance within the new host. There are three main mechanisms by which HGT between prokaryotes occurs; transformation, transduction and conjugation. That HGT between organisms occurs has long been known and utilised in molecular biological research, but the extent to which HGT has shaped bacterial evolution is more controversial, as it challenges conventional views of gradual, step-wise evolutionary change [1, 2]. HGT events are often difficult to prove conclusively, although several lines of evidence suggest that HGT has had a significant impact on prokaryotic evolution. Divergent phylogenies, regions of altered codon usage or GC % and the presence of gene/protein sequences, where the closest homologues are from different species or genera, all suggest the influence of HGT [1, 3]. The presence of complete operons, genomic islands or plasmids with significant regions of synteny, found in diverse genetic backgrounds, strongly suggests that HGT events have occurred [2, 3]. The increased availability of genome sequences of bacteria present in different ecological niches, facilitated by the next-generation sequencing revolution, is revealing the extent to which HGT has probably shaped bacterial evolution. Bacteria can be seen as having access to a "communal" gene pool, allowing the population as a whole to remain more responsive to changing ecological circumstances [2]. This is exemplified by the recent evidence of dissemination of AMR determinants, and the MGEs associated with them, between a diverse range of environmental, commensal and pathogenic bacteria, most likely driven by the wide spread use and abuse of antimicrobials in clinical practice and beyond. This mini-review will focus on examples in Gram-negative bacilli (GNB), in which the expansion in the preceding decades of AMR phenotypes poses a significant public health, clinical and scientific challenge [4-6].

2. Mechanisms of HGT and associated MGEs

Conjugation is often supposed to be the most important mechanism of HGT between bacteria and has been crucial in the evolution of AMR phenotypes in pathogenic bacteria. It is mediated by conjugative plasmids and integrative and conjugative elements (ICEs). Conjugation requires direct contact between donor bacterial cells, containing the plasmid or ICE, and recipient cells [7, 8]. In GNB, this often depends on a type IV secretion system (T4SS), which facilitates the formation of a conjugative pilus, produced by the donor cells. Single stranded DNA from the MGE is produced after nicking at the origin of transfer by a relaxase enzyme and is transferred from the donor cell to the recipient via the pilus. Complementary strands are then synthesised in both cells so that the donor and recipient both harbour the MGE (Figure 1).

Plasmids are extrachromosomal MGEs which replicate autonomously [8]. Self-conjugative plasmids produce their own conjugative machinery, while mobilisable plasmids are only capable of conjugation in the presence of a conjugative helper plasmid [7]. Some plasmids are not capable of conjugative transfer, although they may occasionally be horizontally transferred by transformation or transduction. Plasmids with closely related replicons cannot usually be maintained alongside each other in a cell and are thus said to be incompatible [9]. This is the basis of plasmid incompatibility (Inc) typing. ICEs incorporate both phage and conjugative plasmid related genes [10]. ICEs normally replicate integrated into the host chromosome but are capable of excision and replication, forming an extra-

chromosomal circular intermediate which can be transferred by conjugation. Plasmids and ICEs frequently harbour a large number of genes which have phenotypically important functions, including resistance to antimicrobials, disinfectants or heavy metals [8, 11].

Transduction describes the transfer of DNA by phages (viruses which infect bacteria, see Figure 1) [12]. This can occur by generalised transduction, where bacterial DNA is mispackaged into the phage capsid or "specialized transduction," where host genes near to the phage insertion site on the bacterial chromosome are packaged in the phage capsid along with the phage genome [13]. Phages play a significant role in the transfer of bacterial virulence functions, including toxins. However, transduction of AMR genes may be more frequent than previously thought [14]. Transduction does not require close cell-to-cell interactions like conjugation and the transducing DNA is protected from destruction by the phage capsid. A major limitation of transduction may be the narrow host ranges of most phages, but it has been shown that phages can transduct DNA into bacteria they cannot replicate in [12].

Transformation describes the uptake by bacteria of free DNA into the cytoplasm, which may then be integrated into the new host (see Figure 1) [15]. In order for transformation to take place, the organism must be "competent," that is, able to actively uptake and regulate the extracellular DNA. A number of bacterial species are naturally competent but many more may become so, under certain physiological conditions. Once the DNA has been taken into the cytoplasm it may be degraded or integrated onto the chromosome of the new host by recombinases. Integration is most often by homologous recombination, where DNA is integrated at sites with a significant degree of sequence homology [15]. Transformed plasmids may be maintained extrachromosomally. A limitation of this form of HGT is that in the environment DNA is vulnerable to degradation.

Frequently, AMR genes and other horizontally acquired genes are found in "hot spots" for the insertion of accessory genes, located within plasmids, ICEs or other "genomic islands". These regions often contain many other genetic elements such as insertion sequences (ISs), IS common regions (IS*CR*s), transposons (Tns) and integrons. Integrons are gene capture and expression systems which are likely to have been important in the evolution of these regions. Integrons code for an integrase which mediates recombination between specific sites within the integron and circular genetic elements called gene cassettes, usually comprising of a single open reading frame (ORF) and a recombination site. Integrons can contain arrays of multiple cassettes. Class 1 integrons are the most widespread type associated with AMR in clinically relevant bacteria. Associated gene cassettes coding for resistance to many antimicrobial classes including amingoclycosides, β-lactams, chloramphenicol and trimethoprim are commonly found in these elements, while the 3 prime conserved region also contains determinants of resistance to sulphonamide and quaternary ammonium compounds [16].

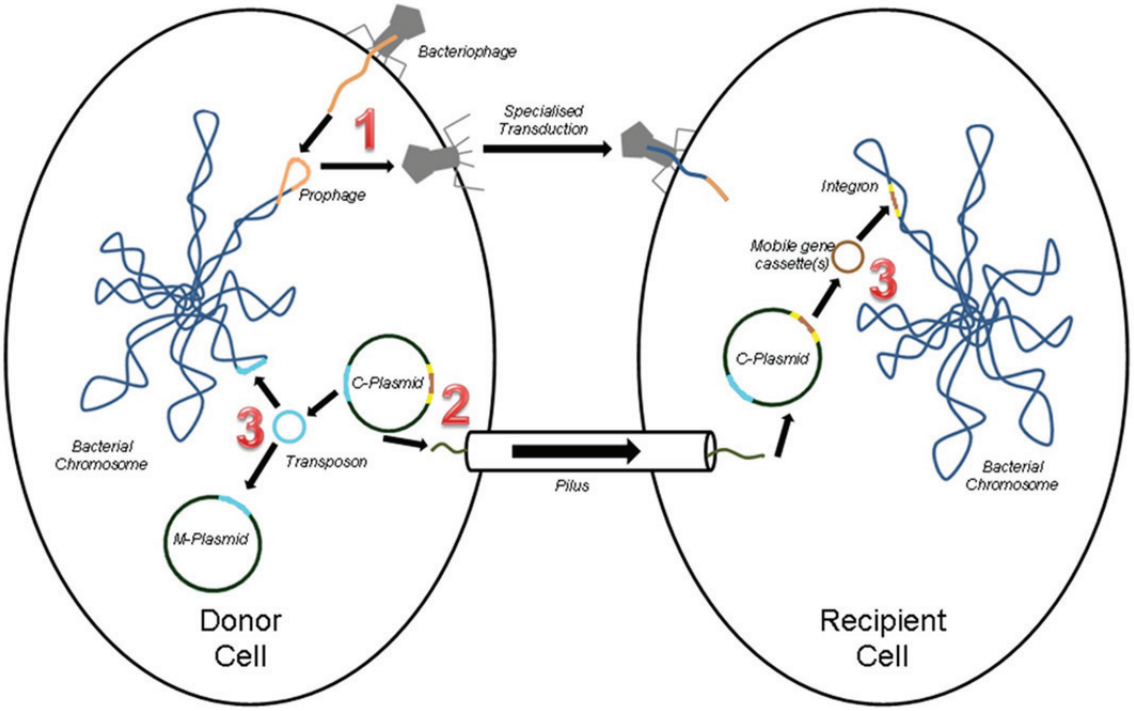

Fig. 1 Summary of HGT processes and mechanisms of genetic plasticity involving MGEs. (1) Specialised transduction involving bacteriophage. (2) Transmission of conjugative plasmid (C-Plasmid). (3) Recombination events involving MGEs (transposons, integrons and gene cassettes). C-plasmid, conjugative plasmid. M-plasmid, mobilisable.

Insertion sequences (IS) are short genetic elements which usually contain one or two ORFs coding for a transposase. Excision from the original location at the IS boundaries and insertion at the new genetic location is catalysed by the transposase(s). A few elements, such as IS*Ecp1*, IS*911* and IS*CR* elements, can also transpose flanking DNA sequences

[17]. Two similar ISs near to one another can form a composite Tn, where both ISs and any intervening host sequence can then be mobilised by a single transposition event [18]. Tns are larger genetic elements which are structurally and functionally diverse. They usually harbour genes not related to their own transposition which have important phenotypic properties, such as AMR genes [18]. The simplest Tns function similarly to ISs and have a limited number of ORFs coding for transposases and sometimes other proteins thought to be involved in initiating and regulating transposition between genetic locations.

3. The role of HGT in dissemination of AMR

The following section will describe some specific examples of key AMR determinants associated with multi-drug resistant or extensively-drug resistant isolates of GNB. The epidemiology and clinical importance of producers of the CTX-M family of ESBLs and the carbapenemases KPC (*Klebsiella pneumoniae* carbapenemases) and NDM (New Delhi metallo-β-lactamases) will be discussed briefly. There are no strict, widely accepted definitions for ESBLs. For our purposes, ESBLs are enzymes capable of leading to clinically relevant levels of resistance to extended-spectrum cephalosporins and monobactams, which are inhibited by β-lactam inhibitors such as tazobactam and clavulanate [19]. Carbapenemases, likewise, are capable of leading to clinically relevant carbapenem resistance. Different carbapenemase enzymes have varying activity against different classes of β-lactams [20]. β-lactam antimicrobials, which include penicillins, cephalosporins, carbapenems and monobactams, are widely used in clinical practice because of their efficacy, spectra of activity and tolerability.

In most cases, the strains carrying β-lactamases carry multiple additional resistance determinants, becoming resistant to many of the alternative antimicrobial agents, limiting therapeutic options. The genes coding for this multitude of resistance determinants are found in association with MGEs, including ISs, Tns and plasmids [5, 11, 20]. This has important implications, since evolutionary selection pressure for these strains (and indeed the main mobile elements harbouring resistance determinants) can be driven by a range of different antimicrobial agents [21]. Genetic contexts associated with $bla_{\text{CTX-M}}$, bla_{KPC} and bla_{NDM} will also be discussed. It should be noted that the terminology with regards to genetic contexts is often not defined and can lead to confusion. The term "genetic context" is used in this chapter as a general term to refer to the strain background, MGE(s) and DNA sequences immediately flanking the gene(s) referred to. To refer to only the sequences immediately flanking the gene(s), the term "immediate genetic context" will be used. Where a specific bacterial species, strain or mobile element is referred to these will be named in the text.

Certain bacterial clones have emerged which have proven to be particularly successful at causing human infections and at disseminating nosocomially and, in some cases, in the community. These so called "high risk clones" possess adaptive traits that increase their pathogenicity and survival, frequently including AMR determinants [22]. The spread of some AMR determinants and the MGEs which harbour them has been greatly facilitated by becoming established in high risk clones. At the same time, the spread of high risk clones is greatly enabled by the selective pressure of antibiotic usage in the clinical setting and animal husbandry [23]. The study of specific clonal lineages is useful as it facilitates the understanding of global epidemiology.

3.1 Extended-spectrum β-lactamases

ESBLs, in their widest designation, are among the most globally widespread mechanisms of antibiotic resistance. The first ESBLs, TEM and SHV, were Class A enzymes originating from *K. pneumoniae*. ESBLs are plasmid-mediated, and found in a large number of species within, but not restricted, to *Enterobacteriaceae*. The burden of ESBL producers is multiple. The mortality rates from bacteraemia of patients infected with ESBL-producing *Enterobacteriaceae* isolates are significantly higher than in those with non-ESBL producers. Moreover, in patients with other types of infections, a tendency towards higher rates of mortality in the ESBL group was noticed. Additionally, patients infected with ESBL-producing strains had significantly higher length of stay in the hospital than those with non-ESBL producers and thus higher infection-related hospital costs. Also, treatment failures and, thus, prolonged and varied antibiotic prescriptions were higher in patients infected with ESBL producers [24]. TEM & SHV enzymes are found at varying rates in different geographical locations. It is however the CTX-M lineage of ESBLs which are now the globally prevalent enzymes in *Enterobacteriaceae*, particularly *E. coli* and *K. pneumoniae* [6]. Unprecedented allelic variation within each ESBL lineage has resulted from point mutations in the sequence of these encoding genes and in some cases, genetic fusion events. Such mutations led to changes in the genetic and phenotypic make-up of the original TEM & SHV progenitors, consequently affecting substrate profile and increasing hydrolytic activity. At the time of publication, 170 CTX-M-type variants have been described (http://www.lahey.org/studies/), spread across 5 phylogenetic groups; group 1, 2, 8, 9 and 25.

The global dissemination of these bla_{ESBL}s can be attributed to the association of the encoding genes with MGEs, which allow gene transfer between genetic constructs, and their recruitment onto conjugative plasmids which facilitate the dissemination of bla_{ESBL} genes throughout bacterial populations. The spread of $bla_{\text{CTX-M}}$ genes, in particular, may be defined as allodemic; that is 'not as a result of dissemination of a particular clone, but of the spread of both multiple specific clones and/or mobile elements' [25]. Their proliferation has a globally pandemic scale, overtaking the

previously dominant TEM and SHV enzymes since the turn of the century [26]. CTX-M-type ESBLs arguably pose a significantly greater public health conundrum than their predecessor ESBLs due to their stronger hydrolytic activity against a wider range of β-lactam antibiotics, the alarming rate at which they have achieved global dissemination, and in keeping with this, the breakout of these enzymes from nosocomial infections into community cases [27]. CTX-M-producing *E.coli* and *K. pneumoniae* are the predominant causes of community acquired ESBL-positive urinary tract infections (UTIs) and pneumonia, respectively. *bla*$_{\text{CTX-M-15}}$, the behemoth of all ESBLs and a member of CTX-M group 1, is carried by the worldwide *E. coli* clone ST131 [36].

CTX-M-type ESBLs are not dissimilar to SHV-type, with regards to their promotion from chromosomal locations onto mobile vectors, which in turn facilitated their dissemination and emergence as a major public health concern. Mobilisation of *bla*$_{\text{SHV}}$ from the chromosome of *Klebsiella* spp., onto conjugative plasmids has occurred through two separate IS26-dependent events which led to the formation of large and small *bla*$_{\text{SHV}}$ transposons [28]. *bla*$_{\text{SHV-5}}$, a SHV enzyme responsible for resistant nosocomial outbreaks in Mexico, is encoded in a large transposon. Large SHV transposons are associated with numerous promoter variants, leading to an enhanced expression of the *bla*$_{\text{SHV}}$ genes [29]. *bla*$_{\text{CTX-M}}$ ESBLs are unique both in the unprecedented rate from which they emerged from environmental hosts and established a global epidemiological foothold, but also in the detail with which we have been able to map the genetic evolution and mechanisms of this emergence. Identification of chromosomal homologs have shown that *bla*$_{\text{CTX-M}}$ genes originate from species of *Kluyvera*, a genus of predominantly environmental species found in water, soil and sewage [30, 31]. More specifically, CTX-M group 1 and 2 alleles were mobilised from the chromosome of *Kluyvera ascorbata*, with genes from CTX-M groups 8 and 9 being originally recruited from *Kluyvera georgiana*. The origin of CTX-M group 25 has not yet been described. Plasmidic *bla*$_{\text{CTX-M}}$ homologs of the chromosomal *bla*$_{\text{KLUC-1}}$ in *Kluyvera cryocrescens* have also been described. Given the high percentage of identity between the plasmidic *bla*$_{\text{CTX-Ms}}$ and their chromosomal ancestors (100% identity is seen in many examples) it is accepted that these mobilisation events occurred comparatively recently, in contrast to the relatively ancient mobilisation of other plasmidic class A β-lactamases. Phylogenetic analysis by Barlow *et al*., (2008) has shown that ancestral *bla*$_{\text{CTX-Ms}}$ from *Kluyvera* spp. have been mobilised onto conjugative plasmids ~10 times more frequently than other ESBL genes [32].

Two particular mobile elements, IS*Ecp*1 and IS*CR1*, played a central role in 'picking up' *bla*$_{\text{CTX-M}}$s and introducing them to transmissible plasmids (See Figure 2). Other mobile elements such as IS*26* and IS*903* are also implicated in certain reports [33]. These mobile elements, and the destination plasmids, continue to be the driving force behind fluid movement of *bla*$_{\text{CTX-M}}$ genes between genetic contexts and through bacterial populations. IS*Ecp*1 is an insertion element that has no close identity to other IS elements, though it is part of the IS*1380* family, and achieves movement of downstream ORFs by a one-ended transposition process. The spacer regions between IS*Ecp*1 and *bla*$_{\text{CTX-M}}$ reading frames, and whether IS*Ecp1* is present in a whole or truncated form, influences the plasticity of this gene cassette and may influence the expression of the genes within [34].

bla$_{\text{CTX-M-14}}$, a member of CTX-M group 9, is the predominantly detected ESBL both in east Asia and the Iberian region of Europe. CTX-M-14 differs from CTX-M-9 by just a single amino acid substitution alanine-231-valine. Given the sequence identity between *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-9}}$, there is surprising variation in the immediate genetic context of these genes. In general terms, *bla*$_{\text{CTX-M}}$ genes of CTX-M group 1 (which includes *bla*$_{\text{CTX-M-15}}$), 2 and 9 (which includes CTX-M-9 and CTX-M-14) are most commonly associated with the upstream IS*Ecp*1 insertion elements. Often, IS*903*-like elements can be found downstream, which is certainly the case for *bla*$_{\text{CTX-M-14}}$ [33]. Conversely, *bla*$_{\text{CTX-M-9}}$ can be found downstream of an IS*CR1* element, and thus associated with class 1 integrons. In many of the published contexts, *bla*$_{\text{CTX-M-2}}$ is flanked by class 1 integrons with an ISCR1 immediately downstream probably having been pivotal in the formation of this genetic context. This association with class 1 integrons means that these *bla*$_{\text{CTX-M}}$ genes share a context with multiple other AMR determinants because of the integrons ability to incorporate and express mobile gene cassettes (see Figure 2).

The consequence of *bla*$_{\text{CTX-M}}$s association with IS*Ecp*1, IS*CR1*, and other mobile genetic elements, and the plasticity afforded to them by such relationships, is the dispersal of resistance genes onto conjugative plasmids across all major plasmid incompatibility types routinely carried by *Enterobacteriaceae*. This includes both narrow host range (IncF, IncHI2, IncI) and broad host range (IncA/C, IncN, IncL/M) plasmids, maximising the species into which these genes can be accommodated. IncH12 plasmids were largely, though not exclusively, responsible for the spread of *bla*$_{\text{CTX-M-9}}$ and *bla*$_{\text{CTX-M-2}}$ amongst pathogenic *E. coli* and *Salmonella enterica* strains. Recruitment of AMR determinants onto IncH12 plasmids has been a recent event, with no evidence of resistance gene cassettes or integrons upon these plasmids when they were first described in 1969. IncK plasmids have been proven to drive the epidemic spread of *bla*$_{\text{CTX-M-14}}$ epidemically across Europe within zoonotic, community and clinical isolates [35]. *bla*$_{\text{CTX-M-14}}$ genes have also been found upon IncA/C, IncF (primarily in Asia) and IncH12 plasmids. IncF-type plasmids have been responsible for the introduction of *bla*$_{\text{CTX-M-15}}$ into the *E. coli* ST131 clone and the resulting global dissemination of the gene.

It is in fact *bla*$_{\text{CTX-M-15}}$ that has arguably benefited from such genetic plasticity more than any other *bla*$_{\text{CTX-M}}$. IS*Ecp*1 is found upstream of *bla*$_{\text{CTX-M-15}}$, though in some cases this may be truncated and in association with IS*25* within a Tn3 transposon (the target site for IS*Ecp*1-$_{\text{CTX-M-15}}$ integration, see Figure 2). Within this genetic context, and upon IncF plasmids, *bla*$_{\text{CTX-M-15}}$ has established an ubiquitous, almost synergistic relationship with the *E. coli* ST131 (O25:H4). ST131 is a virulent extra-intestinal pathogenic *E. coli* (ExPEC) clone associated with clinical cases of bacteremia (17-

38%) and UTIs (6-22%) [36]. IncF plasmids hosted by ST131 have been shown to carry other resistance genes, in addition to $bla_{CTX\text{-}M\text{-}15}$, as for example, bla_{KPC} [37]. Two plasmids, pEK499 (IncFII/FIA fusion) and pEK516 (IncFII), from an epidemic ST131 strain isolated from the UK, were fully sequenced and found to carry 10 and 7 additional antimicrobial resistance determinants respectively [38]. This multi-resistant phenotype, and significantly virulent strain background, has provided *E. coli* ST131 the tools with which to become a globally disseminated pathogenic clone.

3.2 *Klebsiella pneumoniae* carbapenemases

KPCs are molecular class A carbapenemases almost invariably encoded on transferable plasmids. Currently, 24 distinct variants have been assigned (http://www.lahey.org/studies/other.asp#table1). They have a wide spectrum of hydrolysis, including penicillins, cephalosporins, cephamycins and carbapenems and their activity is weakly inhibited by clavulanic acid and tazobactam [39]. KPC was first reported in a *K. pneumoniae* isolate from the USA in 1996. By the mid-2000s, bacteria carrying bla_{KPC} had spread to several other countries, including Israel, Greece and Colombia. Hospital outbreaks due to *K. pneumoniae* harbouring mainly $bla_{KPC\text{-}2}$ and $_{\text{-}3}$ have since been reported worldwide and KPC-producers are now endemic in North-eastern USA, Argentina, Brazil, Colombia, Eastern China, Greece, Israel, Poland and Puerto Rico [40].

KPCs are especially prevalent in *K. pneumoniae* and, to a lesser extent, other *Enterobacteriaceae* where they play a pivotal role in β-lactam resistance phenotypes. Alongside NDM and OXA-48, KPCs are the main cause of carbapenem resistance among *Enterobacteriaceae* worldwide (Nordmann & Poirel, 2014) and are rarely reported in non-*Enterobacteriaceae*, e.g. *Acinetobacter* spp., *Pseudomonas* spp. and *Aeromonas* spp. [41, 42]. KPC-producing *Enterobacteriaceae* infections occur mostly in hospitalised patients, with community associated infections mainly in patients with previous health-care exposure [39, 41]. Although, KPC-producers are mainly nosocomial pathogens, they have also been found in rivers and sewage water [42].

KPC-producers usually display cross-resistance to fluoroquinolones, aminoglycosides and other important antimicrobial agents due to the presence of multiple other genetic determinants and remain susceptible to colistin, tigecycline and gentamicin [40, 41]. These antibiotics all have limitations in terms of efficacy and side effect profile. Furthermore, resistance has been emerging among *K. pneumoniae* isolates which is a worrisome finding, given the current lack of alternative treatment options [20, 23].

bla_{KPC} genes have disseminated rapidly across species and geographic regions not only due to clonal spread but also due to HGT. Carbapenem resistance in *K. pneumoniae* has escalated globally since 2007 and it is mainly due to the production of KPC enzymes. These have been identified in *K. pneumoniae* isolates of >100 STs. Nevertheless, the majority of isolates reported worldwide belong to ST258 and closely related STs (ST11, ST340 and ST512) [20]. In fact, >77% of outbreaks in the USA and 90% of all KPC-producing *K. pneumoniae* infections in Israel are caused by ST258 [40]. In Poland and Greece, ST258 isolates carry $bla_{KPC\text{-}2}$ and in Israel and Italy, ST258 and ST512 carry $bla_{KPC\text{-}3}$ on the closely related pKpQIL and pKpQIL-IT plasmids, respectively [43]. In China, the dominant clone is ST11 [41].

As mentioned before, *E. coli* ST131 is a successful clone due to its genetic armoury of virulence and AMR determinants. More recently, isolates of sublineage ST131-*fimH30* producing KPC were isolated in the USA between 2009 and 2012 [37]. The encoding genes were present in pKpQIL-like IncFIIk plasmids showing that the subclone-plasmid association was very persistent overtime and raising awareness for the risk of a successful combination for the future spread of bla_{KPC} in *E. coli* [37]. This report is especially worrisome since *E. coli* is a community pathogen and the spread of bla_{KPC} from *K. pneumoniae* to *E. coli* via plasmids and/or transposons is a potential health threat [37]. In the UK, and specifically in Manchester, dissemination of bla_{KPC} has been through horizontal dissemination of IncFIIK plasmids among *K. pneumoniae* and other *Enterobacteriaceae* isolates, as opposed to propagation of particular bacterial clones. These outbreak causing isolates exhibited an antibiotic susceptibility profile distinct from ST258 (susceptibility to fluoroquinolones and aminoglycosides) [41].

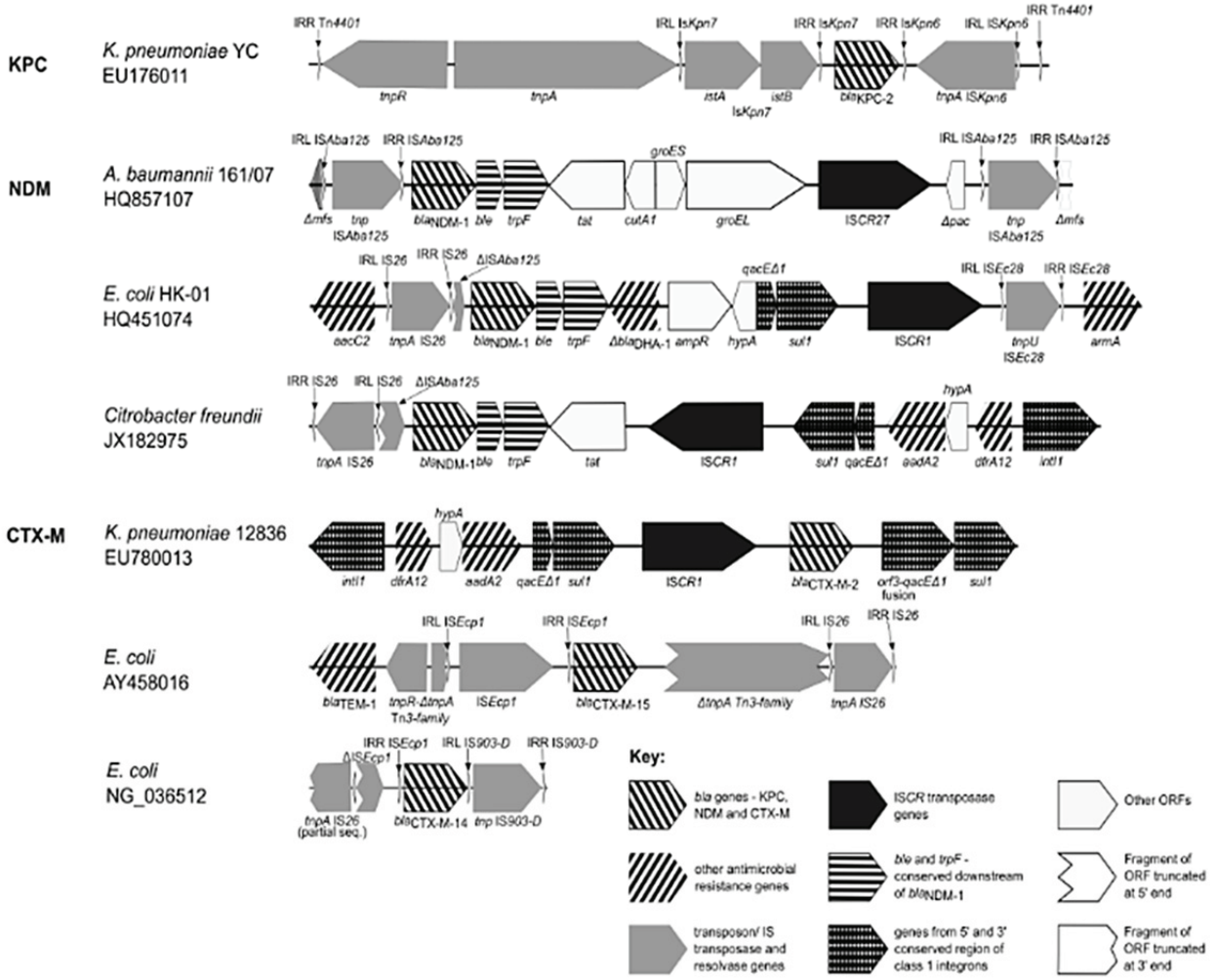

Fig. 1 Gene maps showing representative immediate genetic contexts of bla_{KPC}, bla_{NDM} and bla_{CTX-M}. Complete ORFs are shown as rectangles, with arrow heads indicating direction of translation. Shading or patterns indicate groups of genes according to the key. Truncated remnants of ORFs previously identified in other genetic contexts are indicated by jagged edges to rectangles/ arrow heads (see key). Small triangles indicated inverted repeats of ISs/ Tns. Labels excluding those described in the main text: IRR – inverted repeat right of IS/ Tn; IRL – inverted repeat left of IS/ Tn; *tnp*/ *tnpA*/ *tnpU* – transposase genes from ISs/ Tns; *tnpR* – transposon resolvase genes; *insA*/ *insB* – transposase genes of IS*Kpn7*; *Δmfs* – truncated major facilitator superfamily gene; *trpF* – phosphoribosyl anthranilate isomerase gene; *tat* – twin-arginine translocation pathway signal sequence domain gene; periplasmic divalent cation tolerance gene; *groES* – co-chaperonin gene; *groEL* – chaperonin gene; *qacEΔ1* – quaternary ammonium compound resistance gene (truncated); *sulI* – sulphonamide resistance dihydropteroate synthase gene; *aadA2* – aminoglycoside resistance 3"-adenylyltransferase gene; *dfrA12* – trimethoprim resistance dihydrofolate reductase gene. Summary of HGT processes and mechanisms of genetic plasticity involving MGEs.

Plasmids encoding bla_{KPC} on a Tn*4401* transposon are predominantly responsible for the dissemination of these genes among *Enterobacteriaceae*. Tn*4401* is a 10kb Tn*3*-based transposon bracketed by two 39-bp imperfect IRs and containing bla_{KPC}, with IS*Kpn6* downstream and IS*KPn7* upstream. The genes *tnpA* (Tn*3* transposase gene) and *tnpR* (Tn*3* resolvase gene) are located upstream of IS*KPn7* (see Figure 2). Tn*4401* is commonly flanked by a 5bp target site duplication, which is a signature of a transposition event [40, 44]. This transposon is highly conserved, with 6 isoforms (*a*-*f*) described, from which *a* and *b* are the most common The bla_{KPC} promoter region in the distinct isoforms varies, influencing the gene expression level, nevertheless, only slight variations on carbapenems MICs were noticed [43].

Although Tn*4401* is capable of transposition without a specific target site, it often integrates into Tn*1331*, creating a hybrid transposon structure that was observed in different backgrounds, remarkably, IncN, IncI2 and IncFIA plasmids. Tn*1331* carries AMR genes coding for aminoglycosides modifying enzymes and other β-lactamases. So far, bla_{KPC} has always been present in Tn*4401* in *K. pneumoniae* ST258. Additionally, it has been found associated with other mobile elements in non-ST258 lineages and other bacterial species [40]. In China, for example, bla_{KPC-2} was found in related but more complex chimeric elements. Plasmids of several distinct incompatibility groups have been found to carry bla_{KPC}, with IncFII plasmids being the most common [40]. As mentioned, often, other AMR genes are concomitantly present in these plasmids, including aminoglycoside, quinolone, trimethoprim, sulphonamide and tetracycline resistance

determinants. As a result, exposure to any of these agents can provide selective pressure in favour of the survival and spread strains harbouring these plasmids. *bla*$_{KPC}$ genes have only rarely been identified in chromosomal locations.

3.3 New-Dehli metallo-β-lactamases

NDM enzymes are metallo-β-lactamases (MBLs), so, unlike the other enzymes discussed here, rely on zinc ions at their active site for hydrolysis of the β-lactam ring. They are only distantly related to other known MBLs but have hydrolytic profiles similar to other clinically important enzymes, such as VIM-1 and VIM-2. This includes hydrolysis of penicillins, narrow and extended-spectrum cephalosporins as well as carbapenems, but not monobactams [45]. MBLs are not inhibited by β-lactamase inhibitors such as clavulanate but are inhibited by chelating agents such as EDTA and dipcolinic acid, which is exploited for phenotypic detection of Metallo-β-lactamase production in the laboratory.

NDM-1 was first described in a *K. pneumoniae* isolate from the urine of a patient returning to Sweden from India in 2008 [45], whilst the oldest known NDM producing bacterial isolates are from 2005 [46]. Not long afterwards, it was found to be one of the most frequently isolated carbapenemases among carbapenemase-producing *Enterobacteriaceae* isolated in the United Kingdom and to be present at relatively high prevalence in clinical isolates of *Enterobacteriaceae* in India, Pakistan and Bangladesh [47]. Several factors resulted in there being considerable political, media and scientific interest in NDM-producing bacteria. Firstly, most NDM-producing *Enterobacteriaceae* were associated with consistently high levels of resistance to carbapenems and were resistant to most other clinically useful antibiotics except tigecycline, colistin and fosfomycin. The consistently high rates of carbapenem resistance in *Enterobacteriaceae* harbouring NDM-1 contrast with findings for other important carbapenemases like KPC-2, VIM-1 and OXA-48 [20]. This may in part be related to the strong promoter associated with the *bla*$_{NDM}$ genes, but may also be a reflection of the strain backgrounds these genes are found in. Secondly, *bla*$_{NDM}$ genes also appear to have disseminated rapidly amongst clinically relevant pathogens, given how recently the earliest identifiable NDM-producing bacteria were isolated. Thirdly, NDM-producers detected in Europe and North America were predominantly found in travellers returning from the Indian subcontinent. There has been significant controversy, especially in India, because of this association with travel, the naming of the enzyme after New Delhi and the conclusions of one of the key early publications which raised concerns about the growing Indian medical tourism industry [47]. As a result NDM-producers have been studied intensively, apparently early in their dissemination into clinical bacteria.

In clinical isolates *bla*$_{NDM}$ genes are found most often in *K. pneumoniae* and *E. coli*. They are also found in many other species of *Enterobacteriaceae*, in *Acinetobacte*r spp. including the important nosocomial pathogen *Acinetobacter baumannii* and less frequently in a bewildering range of other Gram-negative species including *Pseudomonas* spp., *Stenotrophomonas maltophilia*, *Aeromonas* spp. and *Vibrio cholerae* [47-49]. Interestingly, whilst some clinical isolates have been associated with hospital acquired infections, clinical isolates have also been found in patients without apparent recent healthcare exposure, especially in the Indian subcontinent. Furthermore in some studies, particularly from the Indian subcontinent and China, NDM-producing bacteria of many different species have been readily detectable as human and animal gut colonisers and in the environment [48, 49]. NDM-producing Gram-negative bacteria have now been detected in many countries around the world, and in all continents except Antarctica. In most geographic locations, however, cases of colonisation and infection are sporadic and often associated with travel to higher prevalence regions.

Despite being associated with extensively-drug resistant phenotypes, the clinical importance of NDM-producing bacteria remains somewhat uncertain. Much of the time, NDM is found in species or strains which are of limited or uncertain pathogenic potential. Unlike for CTX-M and KPC-producing *Enterobacteriaceae*, there are no studies which conclusively show that NDM-producing bacteria are associated with higher mortality than non-NDM producers. One of the few studies comparing outcomes between NDM-producers and non-producers using an observational case-control design actually showed a higher mortality with the non-producers [50]. It is likely that the high mortality associated with some other AMR determinants is dependent on the establishment of multiple mechanisms of antimicrobial resistance in high risk clones. Although NDM has not yet become associated with a dominant pathogenic strain, it has been observed in strains with known pathogenic potential (ST131 and ST101 *E. coli*, ST15 and ST11 *K. pneumoniae* and ST1 *A. baumannii*), and an increasing number of outbreaks of infection with such strains, sometimes associated with significant mortality have been described [46, 51-53].

There is quite significant variation in the immediate genetic contexts found associated with *bla*$_{NDM}$ genes [49, 54-56]. It is believed that *bla*$_{NDM}$ genes were established in *Acinetobacter* spp. prior to dissemination amongst other GNB. In many *Acinetobacter* spp. *bla*$_{NDM}$ is found in a composite transposon, Tn*125*, formed by two copies of IS*Aba125*, as represented by the *A. baumannii* context shown in Figure 2 [56]. There is evidence that within *A. baumannii*, Tn*125* has been responsible for mobilisation to new genetic contexts, usually chromosomal, as the Tn is found at different insertion sites flanked by 3bp direct repeat, indicating transposition events. There is much greater variety in immediate *bla*$_{NDM}$ contexts in other species, with most of those which have been characterised so far being from *Enterobacteriaceae* isolates [49, 55]. In some *Enterobacteriaceae* isolates, sequences contain an intact IS*Aba125*, but in many others, IS*Aba125* is truncated by other ISs [55]. However, in all known examples a fragment of IS*Aba125* is preserved which contains part of the *bla*$_{NDM}$ promoter sequence. This promoter has been shown to induce strong expression of *bla*$_{NDM}$ genes and *ble*, a bleomycin resistance gene immediately downstream of *bla*$_{NDM}$ [57].

In most contexts, in organisms other than *Acinetobacter* spp., *ble* and *trpF* are still found downstream of bla_{NDM} but the rest of the contents of Tn*125* are often absent [54, 57]. A diverse range of ISs, with certain elements like IS*26* being more frequent, flank bla_{NDM} and its immediate genetic contexts, in several cases forming composite transposons which have potentially been responsible for moving the context to new genetic locations and replicons. A relatively common finding downstream of *trpF* is a truncated bla_{DHA-1} gene and its transcriptional regulator gene, *ampR*, followed by conserved region of a complex class 1 integron, including an IS*CR1* element. Some contexts have features which suggest that transposition of flanking DNA by IS*CR1* may have played a role in their formation. In general, immediate bla_{NDM} contexts contain multiple AMR genes and complete or partial class 1 integrons [5, 11]. Notably, several plasmids in *Enterobacteriaceae* harbour the genes *armA* or *rmtC*, coding for a ribosomal RNA methylase, resulting in high level resistance to most aminoglycosides currently used in clinical practice. The same plasmids often also harbour ESBLs such as $bla_{CTX-M-15}$ and AmpC type enzymes such as bla_{CMY-4} or bla_{CMY-6}, resulting in resistance to monobactams, the one major class of β-lactams not significantly inactivated by NDM-1. In many cases bla_{NDM} genes are found on plasmids of differing size and incompatibility types. The most commonly identified include IncA/C, IncF, IncN, IncL/M and IncH, although in many cases the incompatibility type could not be identified [11, 47, 48]. As expected, most of these plasmids harbour multiple other AMR determinants and in some cases heavy metal resistance genes. Most of the *Acinetobacter* spp. isolates for which plasmids have been characterised contain closely related conjugative plasmids of an unknown incompatibility type related to pNDM-BJ01 [58]. Several features of these plasmids suggest that they originate from an *Acinetobacter* spp. background. However, a small number of immediate genetic contexts characterised from *Enterobacteriaceae* contain remnants of the backbone of these plasmids within different plasmid Inc types. A single isolate of *Enterobacter aerogenes* has been described which contains a pNDM-BJ01-like plasmid [59] and, *in vitro*, these plasmids can be conjugatively transferred to *Enterobacteriaceae* as well as *Acinetobacter* spp. recipients [58]. Many isolates have also been described in which bla_{NDM} genes are apparently located on the chromosome [47, 48, 56] but for the most part these contexts have not been extensively characterised.

Although it is a common finding that AMR genes are associated with a number of genetic backgrounds and have variability in their genetic contexts, many of the most successful ones are predominantly associated in clinical practice with a limited number of successful strains and/ or MGE responsible for their acquisition [6, 20, 41] as illustrated by $bla_{CTX-M-15}$ within *E. coli* ST131 and bla_{KPC} within *K. pneumoniae* ST258. This has not yet occurred with bla_{NDM} genes and so the genetic variation associated with these genes is somewhat greater than has commonly been observed with other successful AMR genes. It is a moot point as to whether there is something unique about bla_{NDM} genes and their evolution, which has resulted in the great variety of genetic backgrounds the gene has been observed in, or whether we have fortuitously observed the dissemination of this resistance mechanism in real time, as it occurs. The dissemination of bla_{NDM} has been studied perhaps more intensively and with more non-clinical isolates than other resistance determinants because of the publicity it received soon after its discovery and the resulting political, media and scientific interest aroused.

4. Concluding remarks

AMR is a major public health issue due to its great impact on human health and animal welfare worldwide. Bacteria have increasingly evolved from being resistant to a narrow spectrum of antibiotics to becoming extremely-drug or even pan-drug resistant, meaning that treatment options for common infections in some settings are becoming ineffective. Additionally, AMR has a negative impact on healthcare expenditure. Evidence indicates that resistant bacteria benefit from selective environments where antibiotic exposure is higher, due to the genetic armoury they possess which gives them advantageous phenotypic traits. The association of genetic determinants of resistance with successful MGE and/or successful clonal lineages, such as *E. coli* ST131 and *K. pneumoniae* ST258, undoubtedly plays a major role in the spread of these traits. Why bla_{NDM} genes have spread so successfully despite relatively weak associations with any particular group of MGE or high risk clone, and what the consequences will be if this gene and its contexts do establish such associations, remain important questions.

There are still major gaps in information on the epidemiology of AMR bacteria and the factors which drive the dissemination of AMR amongst important pathogens. Worldwide surveillance is not standardised or coordinated and in some geographic regions is either weak or virtually non-existent. The role that HGT plays in the dissemination of AMR determinants has important implications for long term ambitions to control the global spread of AMR pathogens. Studies of bla_{CTX-M} and bla_{NDM} genes and their genetic contexts suggest that in fact environmental bacteria have played an important role as primary sources of these resistance determinants and potentially as reservoirs of MGEs capable of conferring multi-drug resistant phenotypes in bacteria which acquire these elements. In this way it is expected that species which readily acquire foreign DNA by HGT will remain rapidly responsive to changes in antimicrobial usage and able to acquire "new" resistance determinants from a vast and ancient evolutionary pool. In tackling these challenges it is clear that better surveillance of AMR pathogens is required globally, that the development pipeline of antimicrobial drugs needs to be strengthened and that there needs to be a focus on antimicrobial stewardship and infection control in both hospital and community medicine. In addition the importance of HGT and the potential for this to occur from non-pathogenic bacteria colonising humans and animals, and in environmental settings, suggest that other

factors may need to be addressed to mitigate the further dissemination of AMR. These could include use of antimicrobials in veterinary medicine, agriculture and aquaculture, contamination of the environment with antimicrobials from human and industrial waste and provision in developing countries of clean water supplies for washing, drinking and irrigation of crops. Whatever the eventual solutions it is clear that a rapid and co-ordinated political, scientific and healthcare response is required to address this issue of growing importance.

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