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MINIREVIEW

Horizontal transfer of antibiotic resistance genes in clinical environments

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Abstract: A global medical crisis is unfolding as antibiotics lose effectiveness against a growing number of bacterial pathogens. Horizontal gene transfer (HGT) contributes significantly to the rapid spread of resistance, yet the transmission dynamics of genes that confer antibiotic resistance are poorly understood. Multiple mechanisms of HGT liberate genes from normal vertical inheritance. Conjugation by plasmids, transduction by bacteriophages, and natural transformation by extracellular DNA each allow genetic material to jump between strains and species. Thus, HGT adds an important dimension to infectious disease whereby an antibiotic resistance gene (ARG) can be the agent of an outbreak by transferring resistance to multiple unrelated pathogens. Here, we review the small number of cases where HGT has been detected in clinical environments. We discuss differences and synergies between the spread of plasmid-borne and chromosomal ARGs, with a special consideration of the difficulties of detecting transduction and transformation by routine genetic diagnostics. We highlight how 11 of the top 12 priority antibiotic-resistant pathogens are known or predicted to be naturally transformable, raising the possibility that this mechanism of HGT makes significant contributions to the spread of ARGs. HGT drives the evolution of untreatable "superbugs" by concentrating ARGs together in the same cell, thus HGT must be included in strategies to prevent the emergence of resistant organisms in hospitals and other clinical settings.

Key words: antibiotic resistance, horizontal gene transfer, conjugation, transduction, natural transformation.

Résumé : Une crise médicale mondiale est en train de se dérouler au fur et à mesure où les antibiotiques perdent de leur efficacité envers un nombre croissant d'agents pathogènes bactériens. Le transfert horizontal de gènes (THG) contribue significativement à la propagation rapide de la résistance, mais la dynamique de la transmission de gènes qui confèrent la résistance aux antibiotiques est mal comprise. Plusieurs mécanismes de THG libèrent les gènes de la transmission héréditaire verticale normale. La conjugaison par les plasmides, la transduction par les bactériophages et la transformation naturelle par l'ADN extracellulaire permettent toutes au matériel génétique de sauter d'une souche à l'autre et d'une espèce à l'autre. Ainsi, le THG ajoute une dimension importante aux maladies infectieuses selon laquelle un gène de résistance à un antibiotique (GRA) peut être à l'origine d'une éclosion en transférant la résistance vers plusieurs agents pathogènes non apparentés. Les auteurs passent ici en revue le faible nombre de cas où un THG a été détecté dans des environnements cliniques. Ils discutent des différences et des synergies établies entre la propagation de GRA chromosomiques ou codés dans des plasmides, en considérant plus particulièrement les difficultés de détecter la transduction et la transformation par les méthodes diagnostiques génétiques de routine. Ils soulignent comment 11 des 12 agents pathogènes prioritaires résistants aux antibiotiques sont connus ou présumés être naturellement transformables, soulevant la possibilité que ce mécanisme de THG apporte une contribution significative à la propagation de GRA. Le THG contrôle l'évolution de super-bactéries incurables en concentrant les GRA dans une même cellule; en conséquence, le THG doit être pris en compte dans les stratégies visant à prévenir l'émergence d'organismes résistants dans les hôpitaux et les autres milieux cliniques. [Traduit par la Rédaction]

Mots-clés : résistance aux antibiotiques, transfert horizontal de gènes, conjugaison, transduction, transformation naturelle.

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Antibiotic resistance and horizontal gene transfer

Antimicrobials that kill or inhibit infectious diseases are essential clinical tools, yet resistance continues to emerge, diversify, and spread rapidly. Globally, antimicrobial-resistant infections kill at least 700 000 people each year; within 30 years, resistant infections are predicted to kill 10 000 000 per year, greatly exceeding deaths from cancer (O'Neill 2014). This apocalypse of resistance is estimated to become the greatest challenge in healthcare by 2050. Antibiotics are a subset of antimicrobials that inhibit essential functions in bacteria. Antibiotics are natural products or derivatives of natural products and are used widely to treat and prevent bacterial infections in humans and other animals. Most antibiotic-resistant infections are thought to occur in hospitals, where they increase the risks associated with medical treatments and undermine the ability of hospitals to provide safe places to heal (Davies and Davies 2010; Kizny Gordon et al. 2017). Bacterial antibiotic resistance (AR) is already making routine surgeries and hospital visits increasingly risky. The epidemic is particularly problematic in long-term acute care facilities, where over 25% of healthcare-associated infections are caused by antibiotic-resistant bacteria (O'Neill 2014). Resistant bacterial populations spread when antibiotics exert selective pressures that favour resistance. Antibiotics can also eliminate susceptible microbial populations, reducing competition and expanding the resources available to resistant bacteria (Sommer and Dantas 2011; Conlan et al. 2014). Additionally, AR is spreading rapidly because once a resistance gene evolves in one bacterium, it can spread to other cells and other bacterial species (Huddleston 2014; Juhas 2015; Klümper et al. 2015).

To tackle the rising problem of AR, we must understand how bacteria acquire and transmit resistant genes in clinical settings. Horizontal gene transfer (HGT) allows microbial species to acquire new genetic material from outside their clonal lineage. Through HGT, microbes can sample and share a large gene pool, which may encode traits that are useful in their local environment (Sørensen et al. 2005; Pilla et al. 2017). For example, when bacteria are faced with strong selective pressures, such as the presence of antimicrobials, horizontal acquisition of antibiotic resistance genes (ARG) enables diversification of genomes and creates a potential for rapid fitness increases. Indeed, HGT can be faster than spontaneous mutations to provide genes necessary for survival (Charpentier et al. 2012). HGT also contributes to infections and outbreaks by transferring pathogenic traits such as virulence genes and the ability to form biofilms (Hiller et al. 2010). While the study of HGT has progressed for some environments, such as wastewater treatment plants, studies to track and quantify rates and drivers of HGT have not yet been conducted in clinical environments where pathogens are most problematic.

Table 1. 2017 WHO list of priority pathogens and their natural competence ability.

Pathogen (resistance)	Natural competence
Priority: Critical	
Acinetobacter baumannii (carbR)	Yes
Pseudomonas aeruginosa (carbR)	Yes
Enterobacteriaceae (carbR, ESBL), including *Escherichia coli*, *Klebsiella*	Predicted
Priority: High	
Enterobacteriaceae — *Salmonella* (flrqR)	Predicted
Staphylococcus aureus (vanR)	Yes
Helicobacter pylori (clarR)	Yes
Enterococcus faecium (vanR)	No
Neisseria gonorrhoeae (flrqR, cephR)	Yes
Campylobacter spp. (flrqR)	Yes
Priority: Medium	
Enterobacteriaceae — *Shigella* spp. (flrqR)	Predicted
Streptococcus pneumoniae (penR)	Yes
Haemophilus influenzae (ampR)	Yes

Note: carbR, carbapenem-resistant; flrqR, fluoroquinolone-resistant; vanR, vancomycin-resistant; clarR, clarithromycin-resistant; cephR, cephalosporin-resistant; penR, penicillin-non-susceptible; ampR, ampicillin-resistant.

The purpose of this review is to explore how the three primary mechanisms of HGT in bacteria (conjugation, transduction, and natural transformation) may contribute to the spread of AR in clinical environments. The role of plasmids in the transfer and global spread of many ARGs is well established, and recent studies outlined below have uncovered important examples of plasmid-mediated transfer of ARGs in hospitals and in patients. Conversely, the frequency of gene transfer by transduction or by natural transformation is poorly characterized. Although these two mechanisms are presumed to have less of a role than conjugation, transduction and natural transformation cannot be overlooked because these mechanisms of HGT can transfer both plasmids and chromosomally encoded ARGs.

Importantly, the World Health Organization published its first ever list of antibiotic resistant "priority pathogens" in 2017 (Tacconelli and Magrini 2017). The list is composed of 11 species and one group of bacteria considered to pose the greatest threat to human health because of the severity of the diseases they cause and the loss of effective antibiotics to treat them. Eight of these species can engage in DNA uptake through natural competence, while the three types of *Enterobacteriaceae* on the list are predicted to be naturally competent in nature (Table 1) (Cameron and Redfield 2006). This high potential for natural transformation underscores how enhanced research attention is required to characterize how the multiple mechanisms of HGT contribute to the spread of AR.

HGT is understudied in clinical environments

Quantifying the rate and direction of gene flow is a crucial knowledge gap that must be filled to effectively mitigate the spread of AR. Reservoirs of antibiotic-resistant organisms in hospitals have been well documented (Huddleston 2014; Kizny Gordon et al. 2017; Weingarten et al. 2018), as have transmission routes between these reservoirs (Paterson and Bonomo 2005; Hota et al. 2009; Breathnach et al. 2012; Weingarten et al. 2018), but the rates of horizontal transfer in clinical environments and the impacts of HGT on disease frequency remain unknown or speculative. Many aspects of clinical environments are unique and quite unlike natural environments where HGT is better characterized. Bacterial activities in clinical environments are influenced by variables such as the regular use of detergents and disinfectants, temporal aspects of human movement and contact networks, and specialized physical features designed to reduce and prevent transmission of infectious diseases. Moreover, ethical considerations prevent scientific experimentation with ARG transmission in healthcare settings, and strict privacy rules reduce the ease of conducting targeted studies to track HGT. Altogether, the unique features of clinical settings necessitate focused studies to determine the kinetics and frequency of HGT events.

Plasmids, bacteriophages, and extracellular DNA are the three primary drivers of HGT through the processes of conjugation, transduction, and natural transformation, respectively. Plasmids and bacteriophages are ubiquitous genetic features of bacteria. The capacity for natural transformation is more sporadically distributed, yet it predates diversification of the bacterial Gram-positive and Gram-negative clades (Johnston et al. 2014). Gene transfer by each of the three mechanisms is favoured between closely related organisms, but can occur between phylogenetically distant organisms (Wiedenbeck and Cohan 2011). The ubiquity of plasmids and bacteriophages and the broad phylogenetic distribution of natural transformation means that these ancient agents of HGT function in a vast array of environments and ecosystems. In other words, the transfer of ARGs will occur by the same mechanisms in clinical environments, on human hosts, in human communities, and in almost all natural environments.

Although very little data are available regarding sources, mechanisms, or frequencies of ARG transfer between clinical reservoirs (Andersson and Hughes 2017), there are multiple cases in which the same ARG has been detected simultaneously on hospital surfaces and in patients (Hota et al. 2009; Lowe et al. 2012; Conlan et al. 2014). Recently, the rate of $bla_{\text{OXA-48}}$ plasmid transfer between *Klebsiella pneumoniae* and *Escherichia coli* within hosts was estimated from hospital epidemiological data paired with mathematical modeling (Haverkate et al. 2015). These analyses provide estimates of genetic transfer rates that can be empirically tested and refined.

Community outbreaks of infectious diseases driven by HGT further illustrate the potential impacts of HGT events in clinical settings. For example, *Shigella* outbreaks emerged in the United Kingdom due to the transfer of a plasmid-encoded ARG (Baker et al. 2018). The dissemination of a plasmid encoding azithromycin resistance allowed previously low frequency pathogens to spread because traditional antibiotics were no longer effective. Ultimately, multiple outbreaks of different strains occurred as a result of independent acquisition of the same plasmid (Baker et al. 2018). This example of environmental *Shigella* is no different from the evolutionary processes and molecular mechanisms that facilitate HGT between potential pathogens in clinical environments. Unfortunately, infections with antibiotic-resistant bacteria in clinical settings are more likely to be fatal, particularly for immunocompromised patients who are already more likely to be exposed to clinical environments (Mathers et al. 2011; Snitkin et al. 2012; Tofteland et al. 2013; Conlan et al. 2014; Martin et al. 2017).

Conjugation mobilizes plasmid-borne ARGs in clinical environments

Plasmids are extrachromosomal genetic elements that replicate independently of chromosomes. Persistence of these selfish genetic elements is improved when they carry genes that are useful to the host cell, such as ARGs in the presence of antibiotics. Consequently, many different ARGs circulate on plasmids (Blair et al. 2015). Plasmids disseminate through bacterial populations primarily through the process of conjugation. Conjugation requires physical contact between two cells in the same environment, followed by the formation of a bridge that enables the transfer of a plasmid from a donor to a recipient cell (reviewed in Sørensen et al. 2005).

Plasmid-mediated resistance to β-lactam antibiotics provides prime examples of how HGT exacerbates AR challenges in hospitals. Extended-spectrum β-lactamases (ESBLs) and carbapenemase confer resistance by hydrolyzing β-lactam antibiotics, including penicillin, carbapenems, and cephalosporins (Paterson and Bonomo 2005). β-Lactam resistance genes are commonly located on plasmids and thus disseminate by inter- and intraspecies conjugation in the *Enterobacteriaceae*, *Pseudomonas*, and *Acinetobacter* (Valenzuela et al. 2007; Mathers et al. 2011; Balm et al. 2013; Tofteland et al. 2013; Conlan et al. 2014; Huang et al. 2015; Martin et al. 2017; Gorrie et al. 2018; Phan et al. 2018; Weingarten et al. 2018). On a global scale, the plasmid pCT spread the $bla_{\text{CTX-M-14}}$ to animals and humans across multiple continents (Cottell et al. 2011). The broad and rapid dissemination of ESBLs indicates that these genes function effectively in new host cells immediately after HGT. This facility of gene transfer has resulted in ESBL-

containing bacteria being detected on hospital surfaces (Kac et al. 2004; Lowe et al. 2012; Boyd et al. 2015; Weingarten et al. 2018) and in wastewater downstream of hospitals (Breathnach et al. 2012; Korzeniewska and Harnisz 2013; Egbule 2016; Ludden et al. 2017; Weingarten et al. 2018). As well, ESBL-encoding plasmids were inferred to have transferred between species within a hospital sink environment (Boyd et al. 2015; Weingarten et al. 2018), highlighting how microenvironments in hospitals can enable gene transfer among bacteria.

Whole-genome sequencing revealed that ESBL outbreaks can be caused by transmissible genetic elements moving between bacterial species. Several of the first documented cases of plasmid-mediated outbreaks occurred between 2007 and 2013 when plasmids encoding bla_{KPC} originated in *K. pneumoniae* and transmitted to at least five other species in three separate hospitals (Mathers et al. 2011; Tofteland et al. 2013; Conlan et al. 2014). In all cases, one or more carbapenemase variants were located in transposable elements which transferred horizontally (Mathers et al. 2011; Tofteland et al. 2013; Conlan et al. 2014). In one case, a *K. pneumoniae* isolate lost the *bla*-encoding plasmid, perhaps due to plasmid instability and metabolic cost, which was determined by the isolation of the same strain 2 years apart (Tofteland et al. 2013). Based on the detection of identical ARG-carrying plasmids in patients and on hospital surfaces, outbreaks were likely prolonged by plasmid exchange between reservoirs (Mathers et al. 2011; Tofteland et al. 2013). Based on genomic sequencing, carbapenem-resistant isolates were determined to have been acquired through nosocomial or outside transmission (Conlan et al. 2014). The fine-scale resolution achieved by DNA sequencing paired with clinical data has great implications for tracking outbreaks and improving infection control measures within hospitals. However, epidemiological links can be hard to confirm due to infrequent environmental sampling for ARGs.

The observation of HGT within a single patient is remarkable and has broad implications for the emergence of new infectious diseases. A single person was infected with multiple strains of carbapenem-resistant *Klebsiella* and *Escherichia*, and researchers were able to reconstruct the transmission events where conjugation transferred the bla_{KPC-3} resistance gene between bacterial species residing in the human host (Mulvey et al. 2016). The same bla_{KPC-3} carbapenemase gene was situated within three transposon variants that differed by a single nucleotide and a 100 bp deletion. One of these transposons jumped between two different plasmid incompatibility groups and across multiple strains of *K. pneumoniae* and *E. coli* (Mulvey et al. 2016). DNA sequencing revealed single nucleotide variants of bla_{KPC-3}, which enabled reconstruction of the transmission events that spread the transposon and plasmids between organisms in the infected individual over time (Mulvey et al. 2016).

Laboratory experiments have confirmed that plasmids isolated from AR outbreaks in hospitals can transfer by conjugation between bacterial species. For example, conjugation transferred plasmids with clinically relevant ESBL-encoding genes bla_{OXA-48}, bla_{NDM-1}, bla_{KPC-2}, and bla_{SHV-1} from *K. pneumoniae* hospital isolates to laboratory *E. coli* (Pitart et al. 2011; Tofteland et al. 2013; Zheng et al. 2016).

Plasmid transfer also impacts clinical resistance in Gram-positive bacteria, such as *Staphylococcus aureus*. Infections caused by methicillin-resistant *S. aureus* (MRSA), a persistent colonizer of hospitals and a frequent cause of hospital-acquired infections, are difficult to treat with conventional antibiotics (Grundmann et al. 2006). Vancomycin is a last-resort antibiotic for treating MRSA and other antibiotic-resistant infections. However, vancomycin-resistant *S. aureus* (VRSA) evolved from MRSA through horizontal acquisition of a plasmid from *Enterococcus faecalis* (Weigel et al. 2003; Gardete and Tomasz 2014). This event provides a key example of how HGT concentrates resistance genes in genomes, contributing to the emergence of so-called "superbugs" for which effective antibiotic treatments are lacking.

Conjugation can be stimulated by antibiotics, which is particularly relevant in clinical settings where antibiotics are used frequently. Exposure to minimum inhibitory concentrations of two antibiotics (kanamycin and streptomycin, or gentamycin and chloramphenicol) was found to stimulate conjugation between multiple Gram-negative species (Xia et al. 2008; Zhang et al. 2013). Antibiotics can act as chemical signals that modulate transcription of genes for virulence, DNA repair, and DNA transfer (Goh et al. 2002; Prudhomme et al. 2006; Xia et al. 2008; Lopatkin et al. 2016). Genes such as *oppA* and *rbsB*, which are involved in plasmid transfer and membrane transport systems, were found to be upregulated in *E. coli* after antibiotic treatment (Zhang et al. 2013). Indirectly, antibiotics can modify cell wall composition or stimulate expression of SOS response genes to activate conjugative phenotypes (Al-Masaudi et al. 1991; Goh et al. 2002; Beaber et al. 2004; Yim et al. 2007; Lopatkin et al. 2016). For example, vancomycin binds to peptidoglycan precursors, and it was found to stimulate plasmid transfer in *S. aureus*, perhaps by changing the cell wall composition (Al-Masaudi et al. 1991). Fluoroquinolone antibiotics cause DNA damage that can stimulate the SOS DNA repair response; after induction by antibiotic treatment, SOS genes have been shown to stimulate conjugation between *Vibrio cholerae* and *E. coli* (Beaber et al. 2004). This conjugative activity is possibly a selfish mechanism used by plasmids to escape crippled host cells. Conversely, an antibiotic-inducible conjugative phenotype was disputed when Lopatkin and colleagues tested many parameters involving conjugation in the presence of antibiotics and found that the physiological state of cells and energy availability had greater impacts on conjugation frequency than antibiotics did

(Lopatkin et al. 2016). Moreover, an antibiotic may simultaneously decrease the potential for conjugation by reducing the size of the sensitive recipient population (Lopatkin et al. 2016). The above conjugation experiments have been conducted in laboratories, but as multiple antibiotics are present in clinical environments, these phenomena could be occurring in clinical settings at currently undetected levels.

Even without antibiotic pressure in the environment, and despite the metabolic cost of plasmid replication, plasmids can be stably maintained in bacterial populations by dedicated partitioning systems and post-segregational killing of cells that lose plasmids (Sørensen et al. 2005; Sommer and Dantas 2011). The persistence of AR plasmids in the absence of antibiotics is problematic because it increases the potential for HGT despite improved antimicrobial stewardship efforts.

Transduction in clinical settings

Transduction is acknowledged as a potential contributor to the spread ARGs, especially between members of the same species (Dzidic and Bedeković 2003; Hens et al. 2006; Gillings 2017; and reviewed in Brown-Jaque et al. 2015). Transduction occurs when viral particles transfer bacterial genes. After infection with a bacteriophage, bacterial DNA is sometimes accidentally packaged in a bacteriophage capsid. A capsid containing bacterial DNA is fully capable of binding to a recipient cell and injecting the foreign DNA. If the transferred bacterial DNA is recombined into the genome of the recipient cell, transduction has occurred.

There is currently only indirect evidence that transduction occurs in hospitals. Bacteriophages isolated from hospital-acquired MRSA infections were found to readily transduce ARGs to sensitive strains in the laboratory (Stanczak-Mrozek et al. 2015). Similarly, tetracycline and penicillin resistance genes could be transduced between hospital isolates of *S. aureus* (Mašlaňová et al. 2016), consistent with the known ability of bacteriophages to transmit chromosomal ARGs from MRSA to recipient strains in the laboratory (Chlebowicz et al. 2014). Because bacteriophage-mediated transfer of AR can occur in laboratories, transduction could be a significant contributor to emergence and persistence of AR in clinically relevant *S. aureus*. In Gram-negative bacteria, transduction has been observed to transfer multiple ARGs, including ESBL genes, from *Pseudomonas* hospital isolates to other *Pseudomonas* strains in the laboratory (Blahová et al. 2000). Similarly, β-lactamase genes can be transduced between *Acinetobacter* strains in the laboratory (Krahn et al. 2016).

It is hypothesized that some ESBL-producing Gram-negative pathogens arose through transduction. The CTX-M family of ESBL genes circulating on plasmids is proposed to have originated in *Kluyvera*, a member of the *Enterobacteriaceae* that infrequently causes infections (Sarria et al. 2001). In *Kluyvera*, chromosomally encoded CTX-M genes and flanking sequences are highly similar (>95%) to plasmid-encoded CTX-M genes (Humeniuk et al. 2002; Olson et al. 2005; Zhao and Hu 2013). Transfer of a $bla_{\text{CTX-M-10}}$ gene from a *Kluyvera* chromosome to an ARG plasmid circulating in a hospital is thought to have been mediated by the flanking bacteriophage elements (Oliver et al. 2005). The horizontal transfer of ESBL genes from a minor pathogen like *Kluyvera* has resulted in widespread dissemination of CTX-M alleles among clinically relevant *E. coli* and *Klebsiella* (Oliver et al. 2005; Zhao and Hu 2013). Other cases of AR mobilization by transduction likely exist but have yet to be detected.

Recent studies found that over 70% of hospital fecal samples tested positive for bacteriophages containing ARGs (Quirós et al. 2013), and hospital wastewaters were contaminated with bacteriophages containing ARGs (Marti et al. 2014; Subirats et al. 2016). Antibiotics may drive transduction of ARGs because antibiotic-treated mice were found to have more bacteriophages carrying ARGs in their intestines compared with non-antibiotic-treated mice (Modi et al. 2013). Some bacteriophages can transport DNA fragments greater than 100 kb (Ochman et al. 2000), which is sufficient to transfer plasmids. Bacteriophages were able to transduce a 5667 bp plasmid containing tetracycline and aminoglycoside resistance genes between *S. aureus* strains in the laboratory (Groisman and Ochman 1993; Zeman et al. 2017). Bacteriophages were also able to transduce a 5620 bp plasmid containing a kanamycin resistance gene between *Serratia* and *Kluyvera* species, albeit at a lower rate than transduction of chromosomal resistance genes (Matilla and Salmond 2014). Chloramphenicol and tetracycline resistance genes on 27 kb plasmids were also transduced by bacteriophage between *Actinobacillus* (Willi et al. 1997). Transduction of plasmid-borne ARGs can be so efficient in *S. aureus* that it can surpass the transduction frequency of a chromosomal methicillin resistance gene (Chlebowicz et al. 2014). Thus, both chromosomal- and plasmid-borne ARGs can transfer by transduction, and antibiotics likely increase the rates of transfer.

Natural competence and transformation in clinical settings

Natural transformation occurs when naturally competent bacteria take up extracellular DNA and an imported gene(s) is recombined into the host genome (reviewed in Johnston et al. 2014). Several clinically relevant antibiotic-resistant pathogens are capable of DNA uptake and natural transformation, including *Acinetobacter*, *Haemophilus*, *Neisseria*, *Pseudomonas*, *Staphylococcus*, and *Streptococcus* (Johnston et al. 2014; Traglia et al. 2014). *Escherichia* and *Klebsiella* are leading causes of community-acquired and hospital-acquired antibiotic-resistant infections; although neither genus has demonstrated natural transformation in the laboratory, both are predicted to be naturally competent

in nature (Cameron and Redfield 2006; Palchevskiy and Finkel 2006). This prediction also applies to all other *Enterobacteriaceae* (Cameron and Redfield 2006), raising the possibility that natural transformation contributes to the spread of ARGs in many priority pathogens (Table 1).

The molecular mechanisms of natural competence and transformation have been studied and exploited in laboratory conditions for many decades, but the frequency of natural transformation events in nature is poorly understood. Frederick Griffith famously discovered that nonvirulent *Streptococcus pneumoniae* strains can be transformed into virulent pathogens inside infected mice (Griffith 1928). Similarly, *Helicobacter pylori* has been observed to acquire genes by natural competence and transformation in a colonized human and in mouse infection models (Kersulyte et al. 1999; Kennemann et al. 2011; Dorer et al. 2012), and resistance to the antibiotic metronidazole has been positively correlated with capacity for natural transformation in clinical isolates (Yeh et al. 2002). Despite evidence of natural transformation events during infection, currently there is no direct evidence that natural transformation contributes to ARG transmission between bacteria in clinical environments. However, this lack of evidence may be due to the difficulty of detecting uptake and recombination of exogenous DNA. Nordgård and colleagues attempted to identify the rate of ARG transformation in human gut microbiota but did not detect any transformants (Nordgård et al. 2012).

Antibiotics themselves can increase transformation rates in certain bacteria in laboratory conditions (reviewed in Charpentier et al. 2012), suggesting that the very presence of antibiotics may facilitate HGT and the dissemination of resistance genes. For example, when adding quinolones to *Streptococcus* cultures, competence genes were highly expressed and transformation rates were elevated (Prudhomme et al. 2006). Many bacterial species engage in natural competence when living in biofilms, and quorum sensing in biofilms can even stimulate competence in neighbouring species (Nadell et al. 2009; Seitz and Blokesch 2013). Biofilms present one of the greatest challenges for decontamination efforts in clinical settings, as demonstrated in Hota et al. (2009), thus the combination of biofilm persistence and the potential for exposure to high concentrations of antimicrobials may synergize to enhance natural competence and transformation.

Horizontal transfer of accessory resistance cassettes compared with core housekeeping genes

AR is achieved primarily through three alternate cellular pathways: enzymatic destruction or modification of an antibiotic, increased efflux or reduced influx, or modification of the drug target to prevent interference of essential cellular functions. These AR mechanisms can be encoded by accessory genes that are not required in normal cellular metabolism and physiology. In other words, many ARGs function as gene cassettes, which likely evolved to facilitate "plug-and-play" functionality after acquisition by an antibiotic-sensitive organism. AR can also be achieved through modification of core genes; for example, mutations in ribosomal genes can reduce antibiotic interference with essential translation functions, and modifications to the expression of pores in a cell envelope can reduce antibiotic entry into a cell.

The three primary mechanisms of HGT each have distinct features that favour the transfer of certain genetic loci. Conjugation is dedicated to the transfer of plasmids and, thus, plays a fundamental role in the dissemination of plasmid-borne ARGs. As discussed above, plasmids can also transfer through transduction, and natural competence can take up extracellular plasmids. Nevertheless, plasmid transfer by either of these mechanisms is likely to be rare compared with conjugation. Many clinically relevant forms of resistance are carried on plasmids owing to the shared benefits for both an ARG and its host plasmid: the ARG gains mobility through a bacterial community while the plasmid gains persistence by providing a selective benefit to host cells.

After a plasmid arrives in a new host cell, plasmid-borne transposable elements can further mobilize ARGs by copying them to a different plasmid or to the chromosome (Valenzuela et al. 2007; Bennett 2008; Skipper et al. 2013; Conlan et al. 2014; Mulvey et al. 2016; Ludden et al. 2017). Thus, transposons create another transmission dynamic in which an ARG in a transposon can transfer to different plasmids and so increase the potential of the ARG to transmit to additional bacterial recipients through conjugation. Some transposons can direct conjugation in the absence of plasmids, but this activity is not discussed here. Infectious disease surveillance efforts must recognise plasmids as the possible unit of transmission as opposed to classic clonal outbreaks of antibiotic-resistant species (Linares et al. 2006; Mathers et al. 2011; Tofteland et al. 2013; Conlan et al. 2014).

Resistance to some clinical antibiotics arises through mutation of a gene(s) that encodes a drug target, creating a resistance allele of an otherwise sensitive core gene. This type of mutation often removes a drug interaction without destroying the function of an essential gene. For example, single nucleotide point mutations in *gyrA*, which encodes essential DNA gyrase, confer resistance to fluoroquinolones and nalidixic acid (Hooper 2001). Because transduction and natural transformation can transfer any chromosomal or plasmid genes, resistance alleles of core chromosomal genes are predicted to transfer primarily through transduction or natural transformation. Horizontal transfer of alleles will occur most frequently between closely related individuals because allelic exchange relies on homologous recombination to

convert the genotype of a recipient cell. Indeed, both transduction and natural transformation have broad applications in molecular microbiology because of their efficiency at transferring ARGs. From the perspective of infectious disease testing and control, the horizontal transfer of an antibiotic resistance allele can be difficult to detect because all members of a species encode an allele (mutant or wild type) of the resistance gene.

Fluoroquinolone resistance presents an example of how multiple forms of HGT could synergize to produce AR. Fluoroquinolones such as ciprofloxacin and norfloxacin are clinically important antibiotics, but their success results in environmental pollution, as these antibiotics can be detected in wastewater downstream of hospitals (Szczepanowski et al. 2008; Ory et al. 2016). Resistance to fluoroquinolones is on the rise, particularly in *S. aureus*, *Pseudomonas*, *Acinetobacter*, *Clostridium*, and *Enterobacteriaceae* (Dalhoff 2012). Fluoroquinolones target DNA topoisomerases, and resistance requires amino acid changes to reduce antibiotic affinity for the topoisomerase called DNA gyrase. In most bacteria, including *Enterobacteriaceae*, clinical resistance to fluoroquinolones can only be achieved through the additive contributions of multiple genetic loci (Hooper 2001; Corkill et al. 2005; Kary et al. 2017; Osei Sekyere and Amoako 2017). Fluoroquinolones have a demonstrated ability to induce natural competence in *Streptococcus* (Prudhomme et al. 2006); thus, antibiotic exposure increases the potential for HGT and consolidation of chromosomal resistance alleles. Plasmid-borne resistance arises from the quinolone resistance genes, *qnr* (Jacoby et al. 2008). In *Acinetobacter*, *Klebsiella*, and *Escherichia*, *qnr* genes usually associate with β-lactam resistance on multidrug resistance plasmids (Paterson and Bonomo 2005; Lewis et al. 2010; Conlan et al. 2014; Sana et al. 2014; Ludden et al. 2017). As expected for plasmid-borne resistance genes, there have been several reports of quinolone resistance transferring horizontally by conjugation (Corkill et al. 2005; Sana et al. 2014; Osei Sekyere and Amoako 2017). In summary, the primary resistance alleles in gyrase as well as supporting resistance alleles in other topoisomerase genes are most likely to transfer by transduction or natural competence, and these mutations in the core genome can be enhanced by acquisition of plasmid-encoded accessory genes.

Detecting and anticipating HGT

Bacterial population density and cell proximity differentially impact the various mechanisms of HGT. Bacteria that occupy the same environment, such as human intestines or sink surfaces, are more likely to share DNA via direct cell-to-cell transfer processes such as conjugation (Andersson and Hughes 2017). Conversely, transducing bacteriophage particles provide protection for DNA and support its persistence. Thus, bacteriophages can transfer genes between bacteria separated by space and time. Yet even with their ability to persist, transducing particles derived from bacteriophages with narrow host ranges will have an increased likelihood of loss from a gene pool due to the low probability of encountering a suitable host.

For natural transformation to occur, cells do not need to make physical contact with one another nor do donor and recipient need to be simultaneously present. The timescale for natural transformation is shorter than transduction because environmental DNA will degrade faster than DNA protected in a bacteriophage particle. Because natural competence for DNA binding and uptake is an active process governed by a competent cell, natural transformation requires bacteria to express genes for DNA binding and uptake, DNA must be sufficiently intact and in the vicinity of a competent bacterium, and the DNA must recombine with the genome rather than be degraded (Palchevskiy and Finkel 2006; Johnston et al. 2014; Mell and Redfield 2014).

Bona fide transduction and natural transformation events in hospitals may have gone undetected so far because rates are very low and (or) because it is challenging to identify recombination events, particularly when core chromosomal genes are transferred. In other words, transduction and natural transformation outside controlled laboratory conditions are hard to measure because transferred DNA fragments are disguised among chromosomal regions that are conserved in all members of a species. Also challenging is that HGT results in a web of gene transmission events that can obscure phylogenetic relatedness between bacterial strains, which in turn can complicate tracking and surveillance efforts (Mathers et al. 2011; Conlan et al. 2014; Martin et al. 2017). Conversely, HGT can provide genetic features that can be easily tracked and identified in clinical outbreaks, such as plasmids. Plasmid conjugation transfers novel genes and, thus, is relatively easy to track by DNA sequencing and PCR (Snitkin et al. 2012; Boyd et al. 2015; Gorrie et al. 2018; Simner et al. 2018). Even classic restriction fragment length polymorphism analysis can resolve the presence and absence of plasmids, as in the case of the loss of the *Salmonella* virulence plasmid in a human infection (Alexander et al. 2015). Experiments using specific genetic markers as bait DNA might help identify cases of DNA mobility by transduction and transformation in clinical environments. Altogether, the ubiquity of HGT and the diversity of resistance genes highlight the importance of long-term systematic studies using metagenomics and other tools to track the mobility of ARGs (Andersson and Hughes 2017).

Conclusion

Horizontal transfer in clinical settings occurs primarily through conjugation, yet laboratory studies also implicate natural transformation and transduction as contributors to the spread of resistance genes. Condi-

tions for natural transformation and transduction do exist in clinical environments, but currently we have limited abilities to detect these gene transfer mechanisms outside of controlled laboratory experiments. The multiple examples of antibiotic-induced gene transfer underscore the need to understand how clinical treatments and activities could exacerbate HGT of ARGs. More studies are needed to quantify the rates and direction of HGT in clinical environments.

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