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Mini-Review

Long-term impacts of antibiotic exposure on the human intestinal microbiota

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Although it is known that antibiotics have short-term impacts on the human microbiome, recent evidence demonstrates that the impacts of some antibiotics remain for extended periods of time. In addition, antibiotic-resistant strains can persist in the human host environment in the absence of selective pressure. Both molecular- and cultivation-based approaches have revealed ecological disturbances in the microbiota after antibiotic administration, in particular for specific members of the bacterial community that are susceptible or alternatively resistant to the antibiotic in question. A disturbing consequence of antibiotic treatment has been the long-term persistence of antibiotic resistance genes, for example in the human gut. These data warrant use of prudence in the administration of antibiotics that could aggravate the growing battle with emerging antibiotic-resistant pathogenic strains.

Introduction

Antibiotic therapy can affect not only the target pathogen but also commensal inhabitants of the human host. The extent of the impact on non-target microbial populations depends on the particular antibiotic used, its mode of action and the degree of resistance in the community. Sometimes an imbalance in the commensal gut microbiota due to antibiotic administration can result in intestinal problems, such as antibiotic-associated diarrhoea (AAD) (McFarland, 1998). An additional concern is the increase in antibiotic resistance and the potential spread of resistance genes to pathogenic bacteria. Recently, it has been shown that even short-term antibiotic administration can lead to stabilization of resistant bacterial populations in the human intestine that persist for years (Jakobsson *et al.*, 2010; Jernberg *et al.*, 2007; Löfmark *et al.*, 2006). Although the consequences of long-term persistence of antibiotic resistance in the human gut are currently unknown, there are high risks that this could lead to increased prevalence of antibiotic resistance, reduce the possibility of successful future antibiotic treatments and subsequently lead to higher treatment costs. The short-term consequences of antibiotic administration have previously been reviewed and have primarily dealt with culture-based analyses. This mini-review will focus on the long-term consequences of antibiotics on the composition, ecology and resistance of the human gut microbiota and will highlight some recent studies based on molecular methods.

The normal human gut microbiota ecosystem

Practically all surfaces of the human body exposed to the environment are normally inhabited by micro-organisms. The intestine constitutes an especially rich and diverse microbial habitat. Approximately 800–1000 different bacterial species and >7000 different strains inhabit the gastrointestinal tract (Bäckhed *et al.*, 2005). These bacteria act together in many physiological processes and also interact with human cells, including those of the immune system. The diversity of the gut microbiota is relatively simple in infants but becomes more complex with increasing age, reaching a high degree of complexity in adults (Fanaro *et al.*, 2003). Lifestyle factors (Dicksved *et al.*, 2007) and diet (Flint *et al.*, 2007; Ley *et al.*, 2005, 2006) can also affect the diversity and composition of the

gut microbiota. Interestingly, the relative proportions of the two most dominant bacterial phyla i.e. *Bacteroidetes* and *Firmicutes*, were found to be correlated with obesity in mice and humans, respectively (Ley *et al.*, 2005, 2006), but a study by Duncan *et al.* (2008) did not see this same correlation in obese and lean humans. Molecular analyses have also revealed that the composition of the human intestinal microbiota is host-specific (Dicksved *et al.*, 2007; Jernberg *et al.*, 2007) and relatively stable over time (Jernberg *et al.*, 2007; Zoetendal *et al.*, 1998). Recent metagenome sequencing data of 124 individuals suggest the existence of a common core human gut microbiome (Qin *et al.*, 2010), but this core may exist more at the level of shared functional genes rather than shared taxa (Turnbaugh *et al.*, 2007, 2009).

Impact of antibiotics on normal gut microbiota composition

The ecological balance between the human host and associated micro-organisms described above can be disturbed by several factors, most dramatically by administration of antimicrobial agents. This perturbation mainly manifests as decreased colonization resistance of members of the commensal microbiota, which leads to varying states of disease as well as emergence of antibiotic-resistant strains (Fig. 1) (de la Cochetière *et al.*, 2005; Sjölund *et al.*, 2003). Short-term changes in the quantity and composition of the bacteria comprising the normal human flora as a response to antibiotic exposure have been extensively recorded (for example Sullivan *et al.*, 2001). However, only a few recent studies have investigated the long-term impacts of antibiotic administration, including development of resistance (Jakobsson *et al.*, 2007; Jernberg *et al.*, 2007; Lindgren *et al.*, 2009; Löfmark *et al.*, 2006; Nyberg *et al.*, 2007; Sjölund *et al.*, 2003).

Most studies to date that have addressed the impact of antibiotics on the intestinal microbiota have been performed using culture-based techniques. Disadvantages of culturing are that despite the use of specific selective media and anaerobic incubation conditions, there remains a substantial part of the microbiota, approximately 80 % (Eckburg *et al.*, 2005), that has not yet been cultured. On the other hand, several species representing the main groups of clinically important opportunistic bacteria can be routinely recovered on culture media, including members of the genera *Bacteroides*, *Streptococcus*, *Enterococcus* and *Staphylococcus* and the family *Enterobacteriaceae*. Culturing is still valuable because the pure cultures that are generated can be further analysed with respect to their physiology and antimicrobial susceptibility (Table 1). Furthermore, isolates can be subtyped to strain or clonal levels enabling specific strains or clones to be monitored over time or according to treatment (Jernberg *et al.*, 2007; Sjölund *et al.*, 2003).

The limitations of culture-based techniques can be largely circumvented by using molecular approaches (Brugère *et al.*, 2009). For example, community fingerprinting approaches based on 16S rRNA gene amplification and characterization, such as terminal-restriction fragment length polymorphism (T-RFLP), denaturing/temperature gradient gel electrophoresis (DGGE/TGGE) and 16S rRNA gene sequencing are useful for tracking temporal changes or perturbations in response to antibiotics (Fig. 2). More recently, second generation sequencing approaches (Hamady

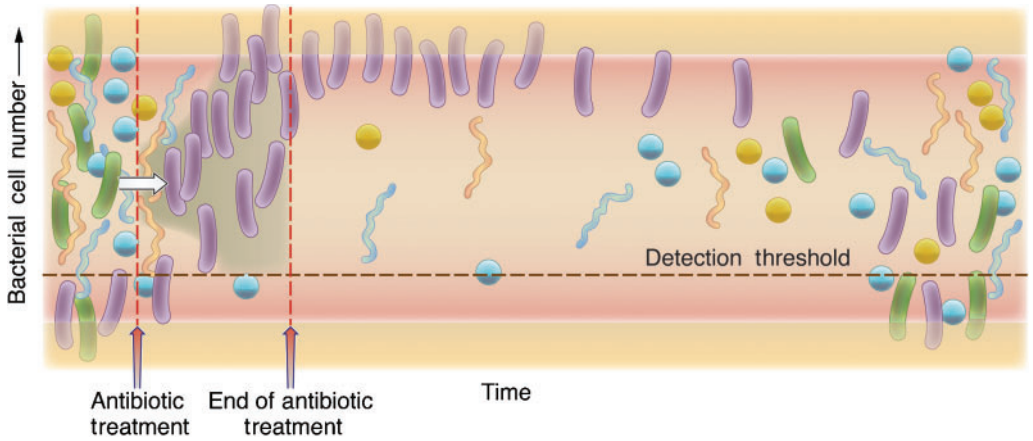

Fig. 1. Representation of the impact of antibiotic administration on the bacterial community of the colon. After the onset of treatment, an increase in resistant bacteria (purple rods) can be seen. This increase is due to either a susceptible bacterium (green rods) becoming resistant or resistant bacteria, already present in low levels, increasing in number due to their ability to survive the selective pressure provided by the antibiotic. The acquired resistance is often due to horizontal gene transfer or mutation events (white arrow). As a consequence of treatment, a temporary decrease in diversity can also be seen. Some bacteria may be protected from antibiotic exposure in the mucin layer (yellow shading) or in grooves in between the villi formed by host epithelial cells that line the intestinal channel (not shown). The figure is not drawn to scale and the timescale is relative.

Table 1. Impact of selected groups of antibiotics on the normal intestinal microbiota assessed by cultivation and MIC values

↓↓, Strong suppression; ↓, moderate suppression; ↑, increase in number; ↑↓, positive and negative effects seen in different studies. NC, No change detected; +, resistant strains detected. The table is adapted from the paper by Sullivan *et al.* (2001).

Antibiotic	Impact on:			Emergence of resistant strains in:	
	Anaerobes	Aerobic Gram positive cocci	Enterobacteria	Enterococci	Enterobacteria
Amoxicillin/clavulanic acid	NC	↑	↑	NC	NC
Ciprofloxacin (high conc. in faeces)	NC	NC	↓↓	NC	+
Clarithromycin/metronidazole	↓	↑	↓	+	+
Cephalosporins (high conc. in faeces)	NC	↑	↓↓	NC	+
Clindamycin	↓↓	↑	↑	+	+
Vancomycin	↓	↑↓	NC	+	+

& Knight, 2009), such as 454 pyrosequencing using specific bar codes to identify samples (Andersson *et al.*, 2008), have provided more in depth information about the impact of antibiotics on specific phylogenetic groups of the gut microbiota (Dethlefsen *et al.*, 2008).

Different antimicrobial agents can influence the normal gut microbiota in different ways. The extent of the antibiotic-induced alterations in the microbiota depends on several factors: i) the spectrum of the agent, ii) dosage and duration of treatment, iii) route of administration and iv) the pharmacokinetic and pharmacodynamic properties of the agent. For example, secretion of an antibiotic by intestinal mucosa, bile or salivary glands may subsequently interfere with the normal microbiota at different sites (Sullivan *et al.*, 2001). Other side effects of some antibiotics on the human host include disturbance of the metabolism and absorption of vitamins (Levy, 2000), alteration of susceptibility to infections (Levy, 2000) and overgrowth of yeast (Sullivan *et al.*, 2001) and/or *Clostridium difficile* (Edlund & Nord, 1993; Sullivan *et al.*, 2001). This review focuses on the impact of antibiotics from a bacterial perspective.

Since there is considerable subject-to-subject variability in the composition of the gut microbiota among humans (Turnbaugh *et al.*, 2009) the investigation of the impacts of antibiotics is currently best assessed on an individual basis. For example, in three healthy individuals treated with ciprofloxacin, certain abundant taxa showed inter-individual variation in response to the antibiotic. The overall effect of the antibiotic on many taxa was also stronger in two of the three individuals (Dethlefsen *et al.*, 2008). Grouping of microbial community data from several individuals can result in loss of statistical significance or false-negative results. Measuring individual divergences from baseline levels after treatment can overcome the problem of subject-to-subject variability (Engelbrektson *et al.*, 2006). However, variations in the microbiota due to other external factors, such as diet or stress, can complicate the task of untangling the specific impacts of antibiotic treatments. Therefore, more studies investigating the extent of long-term natural fluctuations in gut microbial

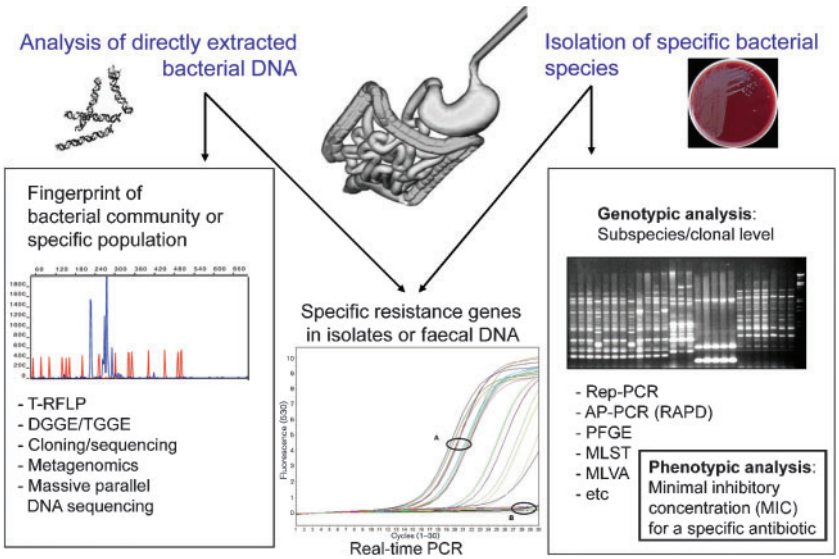

Fig. 2. Examples of experimental approaches that are currently used to assess the impact of antibiotic administration on the human microbiota. Rep-PCR, PCR of repetitive clonal elements; AP-PCR, arbitrarily-primed PCR; MLVA, multiple loci variable number tandem repeat analysis.

communities are needed to establish knowledge about the extent of baseline fluctuations over time.

Several antibiotics are specifically active against anaerobic bacteria that dominate in the human intestinal microbiota. They play an important role in maintaining a healthy gut, such as producing extensive amounts of volatile fatty acids. Therefore, treatment with antibiotics that select against important groups of anaerobic bacteria can have substantial consequences for the resultant functional stability of the microbiota. One example is clindamycin, a relatively broad-spectrum antibiotic that primarily targets anaerobic bacteria. Clindamycin is excreted in bile and concentrations can be high in faeces. Clindamycin has been shown to have a large negative impact on the intestinal microbiota as seen by reduced resistance to colonization by pathogens, leading to a high risk for pseudomembranous colitis due to *C. difficile* overgrowth (Bartlett, 2002). *C. difficile* is commonly isolated in low numbers from healthy individuals, but may increase in number as a consequence of antibiotic-induced disturbances, in particular following suppression of the normal beneficial members of the anaerobic microbiota. Gastritis and diarrhoea are other recorded clindamycin-induced effects on the intestinal flora and disturbances of normal bowel function can lead to symptoms such as bloating and intestinal pain (Levy, 2000; Sullivan *et al.*, 2001). Clindamycin has been shown, in short-term studies, to cause disturbances in the composition of the gut microbiota as well as to select for resistance. These studies have mainly been based on data from isolates and have indicated a normalization of the flora a few weeks following withdrawal of the treatment (Sullivan *et al.*, 2001). However, by using molecular approaches focused on the genus *Bacteroides*, we found long-term shifts in the composition of the intestinal microbiota of individual subjects after a short-term administration of clindamycin (Jernberg *et al.*, 2007) (Fig. 3). T-RFLP was used to assess changes in diversity using both general bacterial primers and bacteroides-specific primers. When general bacterial 16S rRNA gene primers were used, primarily short-term impacts were observed and normalization of the intestinal flora could be seen within 3 months. However, using bacteroides-group-specific primers, we found that specific populations in the community were significantly affected and some disturbances persisted even 2 years after treatment. The same sample material was analysed by culturing and although the total number of species of the genus *Bacteroides* returned to pre-treatment levels 21 days after administration, after an initial decline, the species composition was still significantly altered after 18 months (Löfmark *et al.*, 2006). The long-term impact of clindamycin on members of the family *Enterobacteriaceae* was also investigated in the same sample set (Nyberg *et al.*, 2007). Though intrinsically resistant to clindamycin, the level of antibiotic resistance increased, especially ampicillin resistance, and was high even 9 months after administration.

Several studies have looked at the impact of amoxicillin, or amoxicillin in combination with clavulanic acid, on the gut microbiota. The impact of amoxicillin with or without clavulanic acid on the normal microbiota has previously been shown to be mild to moderate when analysed by culture-based methods. However, an increase of resistant enterobacteria and a decrease in aerobic Gram-positive cocci in response to amoxicillin have been recorded (Edlund & Nord, 1993; Sullivan *et al.*, 2001). Molecular approaches have also been used to assess the impact of amoxicillin–clavulanic acid treatment on the gut microbiota. For example, major alterations in DGGE banding patterns were found after treatment, reflecting a shift in the bacterial constitution due to antibiotic administration (Donskey *et al.*, 2003). Barc *et al.* (2004) used a mouse model harbouring a human faecal flora to study the impact of amoxicillin–clavulanic acid. Specific members of the microbiota were quantified using fluorescent *in situ* hybridization (FISH) combined with flow cytometry. Antibiotic treatment had a strong effect on the number of specific groups detected among the aerobic and anaerobic species. For example, the FISH probes for *Clostridium coccoides* and *Eubacterium rectale* showed that these groups were highly affected and decreased in number after the antibiotic administration, whereas the quantity of *Enterobacteriaceae* and *Bacteriodes/Prevotella* groups increased. However, the total numbers of anaerobic micro-organisms were stable throughout the administration, and 7 days after treatment the levels of the different groups targeted by FISH normalized to pre-administration levels. In another study, de la Cochetière *et al.* (2005) used TGGE with general bacterial primers to investigate the impact of a 5 day course of amoxicillin on healthy volunteers. Five of six subjects had TGGE profiles that returned to their near initial compositions 60 days after administration, supporting the findings from cultivation-based studies showing normalization of the flora within a few weeks or months. Shifts in the gut microbiota of piglets were also observed after administration of amoxicillin, with a decline in diversity and reduction in some 'benefical' gut community members, including a butyrate-producing *Roseburia faecalis*-related population (Janczyk *et al.*, 2007) and this effect persisted at least 5 weeks after a single intramuscular administration. Young and co-workers investigated the short-term impact of amoxicillin–clavulanic acid in a patient suffering from AAD (Young & Schmidt, 2004). By creating a 16S rRNA gene clone library using general bacterial primers, it was shown that the major bacterial groups were partially restored 14 days after antibiotic treatment, except for the *Bifidobacterium* group that had been one of the major groups before treatment but could not be detected during the treatment or 14 days after.

We recently found that the gut microbiota was dramatically perturbed after taking a treatment regimen commonly used for *Helicobacter pylori* infections, consisting of clarithromycin, metronidazole and omeprazole (Jakobsson *et al.*, 2010). Using 454 pyrosequencing we found that some members of the faecal microbiota were affected for extremely long periods of time, i.e. up to 4 years post-treatment. In addition, we also looked at the impact of antibiotic administration on the throat microbiota and found that the throat bacterial

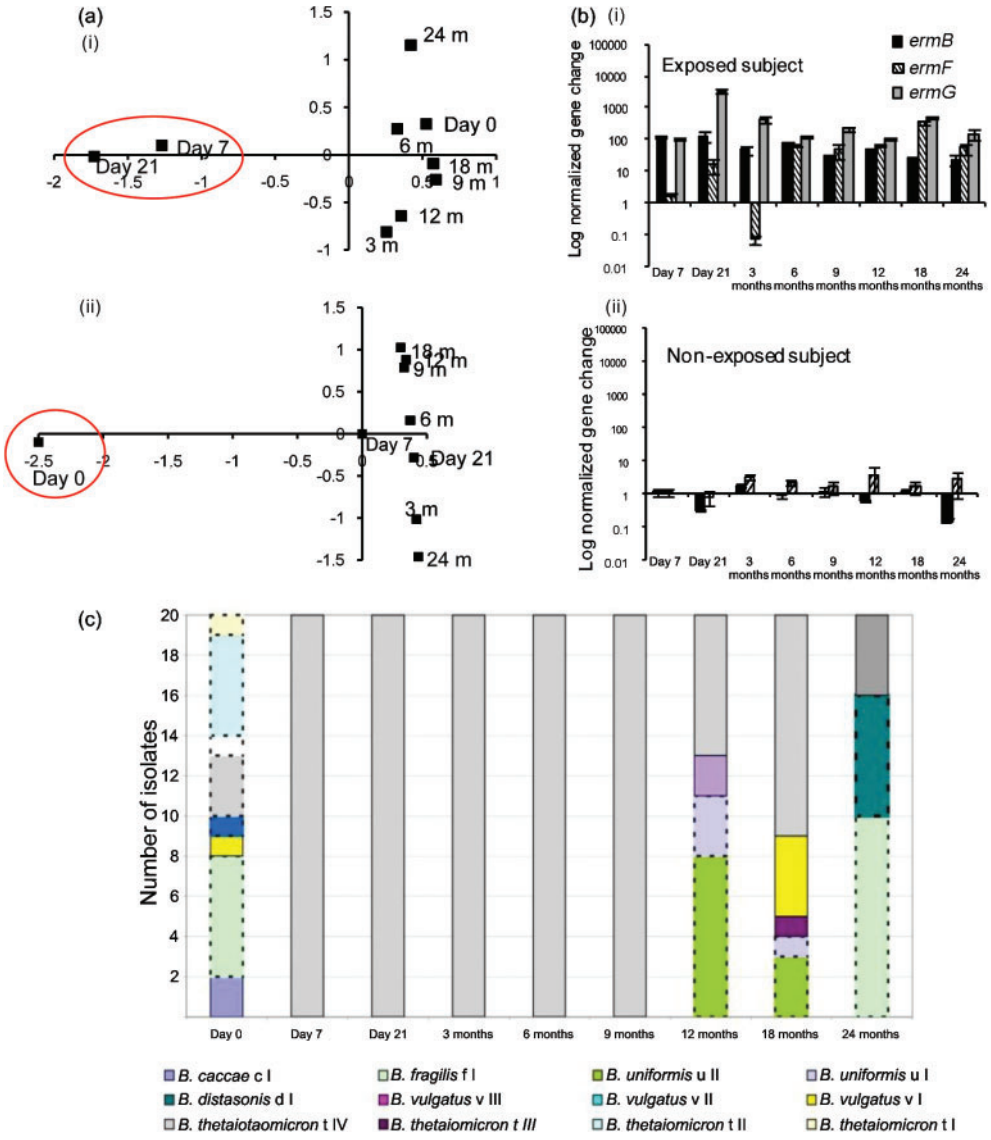

Fig. 3. Long-term impacts of a 7 day clindamycin administration on the human faecal microbiome. Faecal samples were collected before antibiotic administration (day 0), on the last day of antibiotic administration (day 7), 2 weeks after cessation of treatment (day 21), subsequently every 3 months for 1 year post-administration, and 18 months and 2 years post-administration. (a) Correspondence analysis (COA) plots of T-RFLP fingerprints over time from one subject exposed to clindamycin, showing shifts in the dominant members of the bacterial community (i) and the *Bacteroides* community (ii). (b) Normalized relative gene abundance of three specific *erm* genes compared with day 0. Representatives of one clindamycin-exposed subject (i) and one non-exposed subject (ii) are shown. (c) *Bacteroides* isolates from one clindamycin-exposed subject that were clonally typed using Rep-PCR. Bars with hatched lines represent clones susceptible to clindamycin and bars with solid lines represent resistant clones. This figure is adapted from a previous paper (Jernberg *et al.*, 2007).

community was more resilient than the faecal bacterial community (Jakobsson *et al.*, 2010). The actinobacteria were particularly negatively impacted in both the throat and gut samples immediately after antibiotic treatment, presumably due to clarithromycin that is known to target this group. Adamsson *et al.* (1999) also previously found that this antibiotic treatment regimen led to quantitative and qualitative alterations in the faecal microbiota and suggested that amoxicillin might be better from an ecological perspective than clarithromycin for eradication of *H. pylori* because it resulted in less emergence of resistant strains.

Impact of antimicrobial agents on the spread and stabilization of resistant bacteria and resistance genes

The spread of resistant bacteria and resistance genes depends on different factors but the major pressure is

antibiotic usage. Additional factors include the ability of resistant strains to colonize the gut, their relative fitness, mutation rates and efficiencies of horizontal transfer of resistance genes. Under the selective pressure of an antibiotic, a bacterium that acquires a resistance gene is often conferred with a benefit. When this selective pressure is no longer present, the resistant strain could have a lower fitness compared with its susceptible counterpart. However, this less competitive clone might compensate for this loss of fitness by acquiring compensatory mutations. The review by Andersson & Hughes (2010) discusses the fitness costs and mechanisms by which the bacterium can reduce these costs.

A few studies have investigated the impact of antibiotics on long-term persistence of antibiotic resistance. The prevalence of erythromycin-resistant enterococci was investigated in subjects treated with clarithromycin (Sjölund *et al.*, 2003). Using PFGE, it was shown that three of five subjects carried highly resistant enterococci clones 1 year post-administration and that these clones carried the *ermB* gene, conferring resistance to macrolides such as clarithromycin. In one patient, a specific resistant clone was detected 3 years after treatment in the absence of antibiotic pressure. In another study by Sjölund *et al.* (2005) macrolide-resistant *Staphylococcus epidermidis* was detected up to 4 years after patients had taken clarithromycin.

In our clindamycin study, we found significant increases in the levels of specific *erm* genes: *ermF*, *ermG* and *ermB* were detected in DNA extracted from the faecal samples by real-time PCR and these genes could still be detected 2 years after antibiotic administration (Fig. 3). Similarly, we observed long-term persistence of clindamycin-resistant *Bacteroides* clones following clindamycin treatment (Fig. 3) (Jernberg *et al.*, 2007). It was confirmed that most of the clones had acquired specific *erm* resistance genes. The initial cost of acquired resistance was high judging by slower growth in a culture of a resistant clone in competition with a susceptible clone (Löfmark *et al.*, 2008). However after 2 weeks, no growth disadvantage was detected and the subsequent resistant isolates that were collected retained their fitness up to 18 months after antibiotic treatment. Karami *et al.* (2008) also found no fitness burden in ampicillin-resistant *Escherichia coli* isolates compared with susceptible isolates from children up to 1 year old. These findings have important clinical implications, for example by providing extended opportunities for transmission of resistance genes to other species.

Few additional studies have looked at the long-term selection and stabilization of resistance genes within bacterial populations. Recently, the pharyngeal carriage of streptococci and the proportion of macrolide-resistant isolates was studied in healthy volunteers after exposure to either of the two macrolides azithromycin or clarithromycin over 180 days (Malhotra-Kumar *et al.*, 2007). An increase in resistant strains was seen for both groups compared with a placebo group immediately after treatment. The proportion of resistant isolates was higher after azithromycin treatment than after clarithromycin use, while clarithromycin selected for *ermB* genes, which could not be seen with the azithromycin treatment. The results implicate that macrolide use is the major driving force of macrolide resistance.

There is a high level of transfer of resistance genes within the intestine, as shown by several studies, but the picture is far from complete (Lester *et al.*, 2006; Salyers *et al.*, 2004; Scott, 2002; Shoemaker *et al.*, 2001). The intestine is apparently an ideal location for efficient transmission of resistance genes. This moist, warm environment with nutrients in abundance is comprised of high numbers of bacterial cells that are potential targets for resistance development and that also constitutes a large gene pool for resistance determinants (Fig. 1). After initial selection of resistance genes in the commensal microbiota, they may then potentially be transferred to pathogens. This is exemplified by a study that demonstrated transfer of a plasmid carrying a β-lactamase gene from a resistant *E. coli* strain to an initially susceptible strain in a child treated with ampicillin (Karami *et al.*, 2007). The authors concluded that antibiotics may not only select for resistant bacteria but also consequently increase the number of transfer events from the increased pool of resistant cells. Sommer *et al.* (2009) characterized the functional resistance reservoir in two unrelated healthy subjects who had not been exposed to antibiotics for at least 1 year. The microbiota was analysed using a metagenomic approach in addition to culturing. Sequencing of clones conferring resistance to 13 different antibiotics revealed 95 unique inserts that were evolutionarily distant from known resistance genes. This diverse gene pool of resistance genes in the commensal microbiota of healthy individuals could also potentially lead to the emergence of new resistant pathogenic strains.

Some bacteria are only transient inhabitants of the intestine and are resistant to colonization, such as many that originate from ingested foods. However, they can still play a key role in the introduction of resistance determinants that have the potential to be transferred to the commensal microbiota in the intestine during passage (Andremont, 2003; Salyers *et al.*, 2004). Another factor that might be contributing to the emerging resistance problem is the use of antibiotics or analogous compounds in agriculture. The use of these compounds in agricultural settings may lead to a more constant selective pressure for resistance to develop and could potentially contribute to a larger global resistance reservoir with potential introduction, for example via opportunistic pathogens, into the clinical environment (Aubry-Damon *et al.*, 2004; Heuer & Smalla, 2007).

Concluding remarks

Increasing antimicrobial resistance is a growing threat to human health and is mainly a consequence of excessive use of antimicrobial agents in clinical medicine. In addition to focusing on clinically relevant pathogens when monitoring

levels and risks for emergence of antimicrobial resistance, it is important to also consider the role of the enormously diverse human commensal microbiota. It is generally acknowledged that the use of antibiotics causes selection for and enrichment of antimicrobial resistance, but it has also been believed until recently that the commensal microbiota is normalized a few weeks following withdrawal of the treatment. As discussed above, increasing evidence suggests that this is not the case and that specific members of the microbiota may be positively or negatively affected for extended periods of time. With the onset of improved sequencing methods and other molecular approaches, increasing information is becoming available about how the biodiversity (richness and evenness) of the human microbiome is affected at different phylogenetic levels, from the community level to species, strains and individual clones. At the same time, it is possible to monitor the acquisition and persistence of resistance genes in the community. Together this information should help to provide knowledge of the natural dynamics of the normal microbiota and help us to understand the long-term consequences of antimicrobial treatment. This information is of great importance for the implementation of rational administration guidelines for antibiotic therapies.

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