

Presence of specific antibiotic (*tet*) resistance genes in infant faecal microbiota

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Abstract

The widespread use of antibiotics for medical and veterinary purposes has led to an increase of microbial resistance. The antibiotic resistance of pathogenic bacteria has been studied extensively. However, antibiotics are not only selective for pathogens: they also affect all members of the gut microbiota. These microorganisms may constitute a reservoir of genes carrying resistance to specific antibiotics. This study was designed to characterize the gut microbiota with regard to the presence of genes encoding tetracycline resistance proteins (*tet*) in the gut of healthy exclusively breast-fed infants and their mothers. For this purpose we determined the prevalence of genes encoding ribosomal protection proteins (*tet M*, *tet W*, *tet O*, *tet S*, *tet T* and *tet B*) by PCR and characterized the gut microbiota by FISH in stools of infants and their mothers. The gene *tet M* was found in all the breast-fed infants and their mothers. *tet O* was found in all of the mothers' samples, whilst only 35% of the infants harboured this gene. *tet W* was less frequently found (85% of the mothers and 13% of the infants). None of the other genes analysed was found in any sample. Our results suggest that genes carrying antibiotic resistance are common in the environment, as even healthy breast-fed infants with no direct or indirect previous exposure to antibiotics harbour these genes.

Introduction

Infectious diseases are the most common cause of morbidity in children and adults. The widespread use of antibiotics for the eradication of infectious agents has led to increasing microbial resistance. Research has mainly focused on the effect of antibiotics on the quality and quantity of pathogenic bacteria. However, antibiotics are not selective for pathogens: they also affect all members of the gut microbiota. Under antibiotic pressure, such microorganisms may constitute a reservoir of genes carrying resistance to specific antibiotics, especially in very highly populated ecosystems such as the human gut.

The normal gut microbiota can be viewed in two distinct ways. On one hand, the gut mucosal barrier is immature during its initial establishment, and postnatal intestinal colonization is necessary for the correct establishment and regulation of the epithelial barrier. On the other hand, colonizing bacteria constitute the major source of hospital- and community-acquired infections: most microbes use the mucosa as port of entry. Moreover, the normal intestinal

microbiota may act as an environmental reservoir of genes carrying resistance to antibiotics. Indeed, bacterial resistance has been shown to be common in all age groups, including children.

Tetracyclines have been used extensively in human and animal infections because of their broad spectrum and lack of major adverse side-effects. During the last decade tetracycline human use, especially paediatric use, has decreased, and it is not indicated for children below 8 years or pregnant women (American Academy of Pediatrics, 2003). However, tetracyclines are still widely used in some countries as growth promoters in animal feeds (Chopra & Roberts, 2001). Therefore, intestinal bacteria are extensively exposed to small amounts of these antibiotics. The occurrence of some genes carrying tetracycline resistance in commensal *Escherichia coli* strains isolated from infants and the persistence of these resistant strains in the gut have recently been assessed (Karami *et al.*, 2006). However, most of the members of the human intestinal microbiota cannot be cultured (Harmsen *et al.*, 1999; Suau *et al.*, 1999), making it

necessary to use culture-independent techniques to understand antibiotic resistance in this community.

Hence, we have used tetracycline resistance and healthy exclusively breast-fed infants as a model to evaluate antibiotic resistance in the absence of selective environmental pressure. We assessed faecal samples from breast-fed infants for the presence of *tet* antibiotic resistance genes. The study was designed to characterize the gut microbiota and determine the prevalence of genes encoding ribosomal protection proteins in stools of healthy exclusively breast-fed infants and their mothers through the use of culture-independent methodologies.

Materials and methods

Study subjects

Twenty-nine infants from a prospective follow-up study evaluating the effects of maternal diet on the risk of developing allergic disease in childhood (Hopppu *et al.*, 2000) were included in this study. The sole inclusion criterion for the study was that the infants were exclusively breast-fed and healthy, and free of any symptoms or signs of acute or chronic disease. In twenty cases, a faecal sample from the mother was available for analysis. No antimicrobial or probiotic preparations were in use before or after delivery.

Quantification of faecal microorganisms by FISH

Faecal specimens were collected and immediately cooled to 6–8 °C, and then transported to the hospital within 24 h to be frozen at –75 °C. Bacterial cells were harvested for FISH to be performed as described elsewhere (Langendijk *et al.*, 1995; Kirjavainen *et al.*, 2002). The probes for the FISH method were Bif164 (5'-CATCCGGCATTACCACCC), Bac303 (5'-CCAATGTGGGGGACCTT), His150 (5'-TTATGCGGTATTAATCT(C/T)CCTTT) and Lab158 (5'-GGTATAGCA(T/C) GTGTTTCCA), specific for bifidobacteria,

bacteroides, clostridia (perfringens/histolyticum subgroup) and lactobacilli/enterococci, respectively. All probes were of commercial synthesis origin and 5' labelled with fluorescent dye Cy3. Total counts were assessed using the nucleic acid stain DAPI (4',6-diamidino-2-phenylindole), as described by Porter & Feig (1980). Sample processing was completed as previously described (Kirjavainen *et al.*, 2002), and counting was conducted using a Leica Laaborlux D epifluorescence microscope. At least 15 random fields were counted on each slide and the average count was used for analysis.

Detection of *tet* resistance genes in faecal samples

DNA extraction

Faecal DNA was extracted using the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions for Gram-positive bacteria.

Oligonucleotides

The PCR primers shown in Table 1 were used to detect the presence of the corresponding genes coding for the various groups of ribosomal protection proteins. All oligonucleotides were purchased from Thermo Electron Corporation (Thermo Biosciences, Ulm, Germany).

PCR conditions

Amplification of the DNA was performed using a PCR iCycler apparatus (Bio-Rad, Espoo, Finland). The total volume of each PCR was 50 µL, employing 1 µL of DNA extract as a template. The reaction mixture was composed of 1 × ExTaq buffer (Takara Shuzo, Orsu, Japan), 0.2 µM of each primer, 50 µM of each dNTP (Amersham Biosciences, Helsinki, Finland), and 2.5 U *ExTaq* DNA polymerase

Table 1. PCR primers targetting the various classes of ribosomal protection proteins used in this study

Primer	Class targetted	Sequence	Amplicon size (bp)	Annealing temperature	Reference
ptet3 ptetB	TetW	AAGCGGCAGTCACTTCCTCC TCAAGTATCCCAGCGAAACC	1239	60	*
TetM-FW	TetM	ACAGAAAGCTTATTATATAAC	171	55	Aminov <i>et al.</i> (2001)
TetM-RV		TGGCGTGTCTATGATGTTAC			
TetO-FW	TetO	ACGGARAGTTTATTGTATAACC	171	60	Aminov <i>et al.</i> (2001)
TetO-RV		TGGCGTATCTATAATGTTGAC			
TetS-FW	TetS	GAAAGCTTACTATACAGTAGC	169	50	Aminov <i>et al.</i> (2001)
TetS-RV		AGGAGTATCTACAATATTAC			
TetT-FW	TetT	AAGGTTTATTATATAAAAAGTG	169	46	Aminov <i>et al.</i> (2001)
TetT-RV		AGGTGTATCTATGATATTAC			
TetB/P-FW	TetB/P	AAAACCTATTATATTATAGTG	169	46	Aminov <i>et al.</i> (2001)
TetB/P-RV		TGGAGTATCAATAATATTCAC			

*Primers ptet 3 and ptet B were a gift from Dr D. Moine.

Table 2. Bacterial levels per gram of faeces in the subjects included in this study

Microorganism	Infants (N = 29)	Mothers (N = 20)
	<i>Bifidobacterium</i>	6.5 × 10 ⁹ 1.8 × 10 ⁹ (2.1 × 10 ⁹ –1.3 × 10 ¹⁰)
	<i>Bacteroides</i>	4.0 × 10 ⁸ (2.1 × 10 ⁸ –9.2 × 10 ⁸)
	<i>Lactobacillus</i>	7.0 × 10 ⁸
	<i>Enterococcus</i>	(4.1 × 10 ⁸ –9.4 × 10 ⁸)
	<i>Clostridium</i>	1.8 × 10 ⁹ (1.1 × 10 ⁹ –1.9 × 10 ⁹)
Total	1.1 × 10 ¹⁰ (6.2 × 10 ⁹ –1.8 × 10 ¹⁰)	1.3 × 10 ¹⁰ (1.1 × 10 ¹⁰ –1.8 × 10 ¹⁰)

Results are expressed as the median interquartile range.

(Takara Shuzo). The thermal cycle program consisted of the following time and temperature profile: an initial cycle of 94 °C for 5 min, 30 cycles of 30 s at 94 °C, 30 s at the annealing temperature of the corresponding primer pair (indicated in Table 1) and 30 s at 72 °C, and a final extension step of 7 min at 72 °C. PCRs with universal primers were carried out as controls to check the suitability of the extracts for PCR (data not shown). Amplified products were subjected to gel electrophoresis in 1% agarose gels and were visualized by ethidium bromide staining.

Statistical analysis

The levels of bacteria in the gut microbiota were assessed using analysis of variance to compare groups. The data are expressed as medians with interquartile ranges. Correlation between *tet* genes was tested using Fisher's exact test. All statistical analyses were performed using STATVIEW computer software, version 4.5 (Abacus Concepts Inc., Berkeley, CA).

Results

Clinical characteristics

All the infants (16 males and 13 females) were exclusively breast-fed at the time of the analysis, and most of them (23 out of 29, 79%) were vaginally delivered. Their age ranged from 0.5 to 3 months, and their birth weight and length between 2890 and 4800 g, and 47 and 54 cm, respectively. The age of the mothers ranged from 22–36 years.

Detection of *tet* resistance genes in faecal samples

The presence of genes encoding tetracycline resistance proteins of the ribosomal protection family was tested by PCR using specific primers (Table 1) in faecal samples from mothers and infants. *tet M* was the most widely distributed gene, being present in all the subjects analysed. The gene *tet*

O was found in all the mothers' samples, whilst only 35% of the infants harboured this gene. *tet W* was the next most frequently found resistance gene, being present in 85% of the mothers and 13% of the infants. None of the other genes analysed (*tet S*, *tet T* and *tet B*) was found in any sample.

Evaluation of gut microbiota: FISH

The faecal levels of the various bacterial groups analysed are shown in Table 2. The total number of *Bifidobacterium* in faecal samples was higher in mothers positive for *tet W* [2.6 × 10⁹ (1.4 × 10⁹–3.8 × 10⁹)] than in those negative for the gene [6.4 × 10⁸ (1.8 × 10⁸–8.7 × 10⁸) (*P* = 0.02)]. In contrast to this, in infants with higher *Bifidobacterium* numbers no difference was detected. However, those positive for *tet W* tended to have lower numbers of clostridia in faeces [8.4 × 10⁷ (2.8 × 10⁷–9.1 × 10⁷)] than those negative for the gene [1.8 × 10⁸ (5.4 × 10⁷–4.3 × 10⁸) (*P* = 0.10)]. The same observation was made for *tet O*, i.e. clostridial levels were lower in the positive samples [5.9 × 10⁷ (2.8 × 10⁷–9.7 × 10⁷)] than in the negative ones [3.4 × 10⁸ (5.5 × 10⁷–4.5 × 10⁸) (*P* = 0.056)].

In infants, the presence of *tet W* and *tet O* showed a good correlation (*P* = 0.006), indicating that both genes tend to be present in the same individuals.

Discussion

It is important to characterize resistance to commonly used antibiotics, such as tetracyclines, in commensal intestinal microorganisms. The goal is to assess the potential for horizontal gene transfer to pathogenic microorganisms. However, the lack of culturability of most of the members of the human intestinal microbiota limits our understanding of antibiotic resistance in this community and makes it necessary to use culture-independent approaches.

Several mechanisms can confer resistance to tetracycline (Roberts, 2005), among them the presence of ribosomal protection proteins. Currently, 11 classes of genes coding for ribosomal protection proteins have been identified, with *tet M*, *tet W*, *tet Q* and *tet O* being the most widely distributed (Roberts, 2005). In certain bacteria some of these genes, such as *tet W*, *tet S*, *tet Q*, *tet M*, are harboured in conjugative transposons (Roberts, 1996; Lancaster *et al.*, 2004; Melville *et al.*, 2004), implying a high potential for genetic exchange, especially in environments with high bacterial concentrations such as the human intestine.

This study shows that *tet* resistance genes are normally present in the human gut microbiota of both healthy adults and breast-fed infants. We found that *tet M* was the most widely distributed gene, being present in all the subjects analysed, even in breast-fed infants having had no direct or indirect previous exposure to antibiotics. This gene has been found in a broad variety of microorganisms, including both

Gram-positive and Gram-negative bacteria (Roberts, 1994), explaining its occurrence in all the analysed samples. *tet O* is another of the most widely distributed genes carrying resistance to tetracycline (Roberts, 2005). This gene was also commonly found, being present in all the mothers analysed and in one-third of the infants. The gene *tet W* was the next most frequently found, in agreement with previous reports indicating the presence of *tet W* in gastrointestinal environments (Scott *et al.*, 2000; Aminov *et al.*, 2001). This gene has been found in two important human intestinal microorganisms, namely *Fusobacterium prausnitzii* and *Bifidobacterium longum* (Scott *et al.* 2000). None of the other genes analysed (*tet S*, *tet T* and *tet B*) was found in any sample. In this sense, it is interesting to note the lack of *tet S*, despite its occurrence in some important food-related bacteria such as lactococci and enterococci (Perreten *et al.*, 1997). Our results extend previous studies reporting that *tet M*, *tet O* and *tet W* are among the most widely distributed genes coding for tetracycline ribosomal protection proteins (Roberts, 2005), to exclusively breast-fed infants. It has also been reported that tetracycline-resistant bacteria constitute c. 11% of the total cultivable oral bacteria, *tet M* being the most commonly found, followed by *tet W* and *tet O* (Villedieu *et al.*, 2003), which is in agreement with our observations. The presence of some of these genes in broad-host-range conjugative transposons could explain their wide distribution (Villedieu *et al.*, 2003).

The occurrence of *tet O* and *tet W* was higher in mothers than in infants. This could be because of the lack of a fully diverse and established microbiota in infants, and may indicate age-related changes in microbiota driven by antibiotic pressure, pointing out at these genes as markers of exposure to tetracyclines. Interestingly, in infants *tet W* and *tet O* tend to be present in the same individuals. Although combinations of some of these genes in the same microorganisms have been reported, the combination *tet O/tet W* has only been reported in *Neisseria* (Villedieu *et al.*, 2003). Thus, the correlation observed in our samples appears to result from the presence of different microorganisms each harbouring one of the genes rather than from the presence of one microorganism harbouring both.

The total number of *Bifidobacterium* in faecal samples was higher in mothers positive for *tet W* than in those negative. This finding agrees with the fact that *tet W* is the only tetracycline ribosomal protection protein gene that has been reported in bifidobacteria. In contrast to this, in infants with higher *Bifidobacterium* numbers this difference was not detected. However, those positive for *tet W* or *tet O* had lower numbers of clostridia in faeces. This could be indicative of a correlation between high levels of bifidobacteria and low levels of other microorganisms such as clostridia, as has been previously suggested (Kalliomäki *et al.*, 2001).

Our results suggest that bacterial resistance genes are common in the environment, as healthy breast-fed infants who had no previous exposure to antibiotics or probiotics harbour those genes. In this sense, certain antibiotics can cross over from maternal plasma to breast-milk (Mathew, 2004). The effect of maternally transferred antibiotics on infant microbiota resistance should be evaluated. There is a need to characterize the species and strains carrying these resistance genes as well as the effects of antibiotic exposure in the development and composition of the human gut microbiota, as this bacterial community may act as a reservoir of genes carrying resistance to antibiotics.

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