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Short Communication

Amoxicillin–clavulanic acid resistance in fecal *Enterobacteriaceae* from patients with cystic fibrosis and healthy siblings

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Abstract

Background: The present study set out to detect and identify amoxicillin–clavulanic acid (AMC)-resistant *Enterobacteriaceae* in fecal samples of two patients with cystic fibrosis (CF) and their respective siblings.
Methods: Fecal *Enterobacteriaceae* were enumerated onto EMB agar containing amoxicillin (AMX). A total of 173 CF isolates and 41 sibling isolates were grouped into seven Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) clusters and identified through 16S rRNA and *rpo*B sequence analysis.
Results: The fecal microbiota of patients with CF revealed a higher prevalence of AMX resistant *Enterobacteriaceae* compared to that of their healthy siblings. Whereas all selected isolates of healthy siblings were assigned to *Escherichia coli*, isolates of patients with CF belonged to *Klebsiella oxytoca* (58.4%), *E. coli* (28.3%), *Klebsiella variicola* (7.5%) or *Citrobacter* sp. (5.8%). *A*ll tested CF isolates showed a high resistance rate to AMX, and a lower level of resistance to the combination with clavulanic acid. In contrast, all tested sibling isolates were susceptible for both AMX and AMC.
Conclusion: The higher abundance of AMX resistance in the investigated patients with CF suggests that frequent AMC administration may be one of the major contributing factors in the proliferation of *Enterobacteriaceae* and the development of resistant strains in the gastrointestinal tract.

Keywords: Cystic fibrosis; Gut microbiota; Amoxicillin–clavulanic acid; Antibiotic resistance; *Enterobacteriaceae*

Chronic endobronchial infections in patients with cystic fibrosis (CF) are currently managed with frequent treatment with high doses of multiple antimicrobial agents. Whereas the effect of recurrent antibiotic therapies on CF-lung microbiota has been extensively studied [1,2], their influence on the digestive microbiota in this patient population is poorly documented [3–5]. In studies of populations without chronic clinical diseases, however, it has been shown that antibiotics may trigger a substantial reduction of metabolically important bacterial groups [6,7] while other groups such as the *Enterobacteriaceae* often proliferate upon antimicrobial administration [8,9]. Furthermore, the selective pressure of prolonged antibiotic treatment in patients with CF may promote exchange of resistance genes triggering proliferation of antimicrobial resistant strains [10]. Antibiotic therapy may also decrease colonization resistance eliciting overgrowth with already-present yeasts and/or opportunistic pathogenic bacteria such as *Clostridium difficile* [11]. Depending on the severity of the dysbiosis, these microorganisms may enter the bloodstream and cause systemic infection. Evidence that even short periods of antibiotic treatment can cause long-term persistence of resistant bacteria in the gut exists [12].

Amoxicillin (AMX), a broad-spectrum β-lactam penicillin, is recommended by the European guidelines as the first-choice antibiotic for treating mild respiratory and other common infections in non-hospitalized patients [13]. Despite these

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guidelines, in the last decade a significant increase is observed in the use of AMX in combination with the β-lactamase inhibitor clavulanic acid for treating lower respiratory tract infections. Amoxicillin–clavulanic acid (AMC) serves to treat more severe lower respiratory tract infections such as pulmonary exacerbations with *Staphylococcus aureus* and/or *Haemophilus influenzae* in young patients with CF [14]. Frequent use of these antibiotics increases the concern for emerging development and spread of antibiotic resistance genes. Within the *Enterobacteriaceae*, a bacterial family which is typically associated with abdominal infections, resistant isolates have been frequently isolated from intestinal microbiota following AMX [15,16] and AMC [17] administration, and may consequently pose a significant therapeutic challenge. From a clinical point of view, it is thus relevant to gain more insight into the impact of frequent antibiotic treatment courses on the development of antimicrobial resistant strains in the gut of patients with CF. In the present study, the prevalence and identity of AMC resistant fecal *Enterobacteriaceae* were compared between two patients with CF and their respective healthy siblings.

Stool samples were collected from 2 patients with CF and their corresponding healthy sibling (Table 1). A detailed description of the materials and methods is available as Supplementary content.

The prevalence of AMX resistant *Enterobacteriaceae* was assessed by determining the number of colony forming units per gram fecal sample (CFU/g) from eosin methylene blue (EMB) agar plates containing 0, 8 and 128 ppm AMX. Samples of the two patients with CF consistently yielded higher counts on EMB agar without AMX (6.77 ± 1.06 mean $\log_{10}$ CFU/g) compared to healthy siblings (5.80 ± 0.81 mean $\log_{10}$ CFU/g). On plates containing 8 ppm AMX, no decrease in CFU was observed for fecal samples of patients with CF (6.91 ± 0.03 mean $\log_{10}$ CFU/g), while for fecal samples of healthy siblings the counts drastically declined (2.06 ± 0.84 mean $\log_{10}$ CFU/g). On plates containing 128 ppm AMX, the EMB agar counts for samples of patients with CF (6.91 ± 0.03 mean $\log_{10}$ CFU/g) were still within the same range as counts on plates containing 0 or 8 ppm AMX. For samples of healthy siblings, counts on plates containing 128 ppm AMX decreased to an average of 1.58 ± 3.36 mean $\log_{10}$ CFU/g.

Following enumeration, a total of 173 patient isolates and 41 sibling isolates were collected. No isolates were obtained from sample P1s3. Despite the inclusion of an antifungal agent in the medium, only yeast colonies were recovered from this sample. Matrix-Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF MS) was used to dereplicate

Table 1
Overview of clinical characteristics of volunteers, isolates, MALDI-TOF MS results, sequencing results and MICs.

Volunteer	Date of birth	Sampling date	Sample code	Antibiotic history [a]		No. of isolates	MALDI-TOF MS clusters	Final identification	Source (μg/ml AMX) [b]	MIC range	
				Antibiotic agent, route and doses	Administration period					AMX [c]	AMC [c]
Patient 1	12/03/2005	17/02/2008	P1-s1	Augmentin PO 3 × 4 ml	15/10/2007–01/05/2008	80	IV	*Klebsiella oxytoca*	4	–	2/1
									128	256	–
									256	>256	2/1
						18	VII	*E. coli*	4	>256	32/16
									128	>256	32/16
		4/08/2008	P1-s2	Augmentin PO 3 × 225 mg	10/07/2008–01/08/2008	21	IV	*Klebsiella oxytoca*,	128	>256	16/8–32/16
						13	V	*Klebsiella variicola*	128	>256	8/4–16/8
						10	VI	*Citrobacter* spp.	16	>256	32/16
									128	>256	32/16
						13	VII	*E. coli*	16	>256	8/4
									128	>256	4/2–32/16
		2/05/2009	P1-s3	Augmentin PO 3 × 5 ml Duracef PO 3 × 5 ml Tobramycin INH 2 × 1 amp Ciproxin PO 3 × 150 mg Colistine b INH 2 × 2 million units	3/02/2009–10/02/2009 10/02/2009–23/04/2009 13/02/2009–12/03/2009 23/04/2009–23/07/2009 23/04/2009–23/07/2009	0 [d]	–	–	–	–	–
Sibling 1	20/09/2001	17/02/2008	S1-s1	No > 1.5 years	N/A	20	III	*E. coli*	1	4	2/1
		4/08/2008	S1-s2	No	N/A	4	III	*E. coli*	8	8	4/2
		2/05/2009	S1-s3	No	N/A	15	I	*E. coli*	–	–	–
Patient 2	2/07/1998	1/12/2007	P2-s1	Augmentin PO 4 × 250 mg	27/11/2007–4/12/2007	3	I	*E. coli*	256	>256	8/4
						15	II		256	>256	8/4
									128	>256	–
Sibling 2	7/05/1992	2/12/2007	S2-s1	No > 3 years	N/A	2	I	*E. coli*	1	8	2/1

[a] PO: per oral; INH: inhaled, N/A: not applicable.
[b] Concentration of AMX (μg/ml) in EMB agar plates from which the isolates were picked.
[c] AMX: amoxicillin, AMC: amoxicillin–clavulanic acid.
[d] Only yeasts could be recovered for this sample.