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CULTURE BASED EVALUATION OF MICROBIOTA IN CHILDREN WITH ACUTE APPENDICITIS

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Treatment strategies for acute uncomplicated appendicitis have evolved and now conservative antibacterial treatment is recommended over surgical treatment, especially for paediatric patients. The aim of this study was to evaluate microbiota in paediatric patients with acute uncomplicated and complicated appendicitis, and antibacterial susceptibility of the causative microorganisms. Bacteriological identification was conducted using the VITEK2 analyser. Antibacterial susceptibility tests were performed and the results were evaluated in accordance with the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) "Clinical breakpoints and dosing of antibiotics" (Version 7.0, January 2019). Serodiagnosis of Yersinia enterocolitica *was performed using indirect haemagglutination. The results revealed differences in microbiota in cases of acute complicated and acute uncomplicated appendicitis.* Pseudomonas aeruginosa *was identified more frequently in cases of acute complicated appendicitis. Mixed culture was prevalent in cases of both acute complicated and acute uncomplicated appendicitis. Very few positive extended spectrum beta-lactamase (ESBL)* Escherichia coli *cultures were identified. Most of strains of* Pseudomonas aeruginosa *were resistant to amoxicillin with clavulanic acid, ertapenem, ampicillin and cefotaxime. Some of* E. coli *isolates were resistant to ampicillin and to amoxicillin with clavulanic acid.*

Key words: *appendicitis, microbiota,* Pseudomonas aeruginosa, Escherichia coli*, extended spectrum beta-lactamase, antibacterial susceptibility.*

INTRODUCTION

Acute appendicitis (AA) is one of the most common paediatric abdominal pathologies. It requires surgery, and despite advances in diagnostics and treatment, a uniform understanding of the aetiology and pathogenesis of appendicitis is still missing (Naher *et al.,* 2013; Essenmacher *et al.,* 2018; Snyder *et al.,* 2018). The physiology of the appendix has also not been fully investigated. It is recognised that the vermiform appendix plays a role in the development and maturation of the immune system (Gebbers and Laissue, 2004; Rhee *et al.,* 2005). Involvement of microbes in the pathogenesis of appendicitis is not entirely understood; however, recent research suggests that the appendix functions as a microbiota reservoir in the gastrointestinal tract. The belief is that it ensures the repopulation of microbiota

during acute illnesses, in which the gastrointestinal tract is colonised by pathogens such acute gastroenteritis, and after antibacterial treatment (Guinane *et al.,* 2013).

Historically, the only effective treatment method of appendicitis and prevention of septic complications has been surgery, namely, an appendectomy, which has been practiced for over 130 years (Rogers *et al.,* 2016). This view has been challenged in recent years as conservative treatment using antibiotics has supplanted surgical interventions (Lamps, 2010). Clinical research has demonstrated the efficacy of antibacterial treatment, but nonetheless, 27% of patients each year still require surgery (Roberts, 1988).

One of the most important issues in paediatric surgery is distinguishing between acute uncomplicated appendicitis (AnA) and acute complicated appendicitis (AcA) upon commencing conservative treatment, as complicated cases indicate a delay in establishing diagnosis and require emergency surgery. These cases constitute more than 35% of all AA cases in Latvia. Our current research showed that the total number of AA cases treated surgically at the Children's Clinical University Hospital has not changed, but there has been an increased incidence of AcA (Kakars *et al.,* 2017). Increase use of conservative treatment necessitates the evaluation of algorithms for antibacterial treatment, as they can differ among clinical institutions (Salo *et al.,* 2017).

The aim of this study was to evaluate microbiota in paediatric patients with acute uncomplicated and complicated appendicitis, and antibacterial susceptibility of the causative microorganisms.

MATERIALS AND METHODS

This study included children between the ages of seven and 17 admitted to the Children's Clinical University Hospital due to acute abdominal pain with signs and symptoms suggesting the possibility of appendicitis. All patients were examined to confirm or exclude this diagnosis. Preoperative screening involved physical examination, complete blood count, abdominal ultrasound (US) and detection of serum values of C-reactive protein (CRP) and interleukin-6 (IL-6).

The paediatric surgery team supervising patients with appendicitis received a written consent form from the caregiver and assent from the patient if they were 13 years of age or older. The consent form provided information to the patient and the caregiver about the research objective and methodology used for examining the biological material.

Ethical approval: All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (Rīga Stradiņš University; reference number: 21/27.04.2017; and Children's Clinical University Hospital; reference number: SP-37/2018), and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from a parent of each individual participant included in the study. Clinical data collected prior to surgery included patients' age, sex, and current medical history.

Altogether, a total of 67 patients were eligible and recruited for this study. All of these patients had an appendectomy, and either laparoscopic or conventional laparotomy was performed in all of the patients. Intraoperatively, a microbiological culture swab from the peritoneal cavity was collected. After the removal of the appendix, an extra submucosal swab was taken to avoid bacterial dissemination from the following swabs. The appendix was dissected longitudinally and under sterile conditions, and swab samples were taken from the distal and proximal part of the appendiceal lumen. Each pair was placed in Amies medium for immediate transfer and subsequent bacterial culture (Schulin *et al.,* 2017). They were cultivated under aerobic and anaerobic conditions. Cultivation was performed on blood agar (Supplement, Oxid, UK; defibrinated sheep blood – E&O laboratories limited, Scotland), MacConkey (Oxid, UK) and tripticase soy (Oxid, UK) agar. Bacterial identification was performed using the VITEK2 analyser (Biomerieux, France).

Antibacterial susceptibility tests were performed and the results were evaluated in accordance with the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST), "Clinical breakpoints and dosing of antibiotics" (Version 7.0, January 2019) (Anonymous, 2019). Overnight cultures were suspended in physiological saline to 0.5 McFarland units (McFarland Densitometer DEN-1, Biosan, Latvia). The suspension was inoculated on Mueller-Hinton agar (Oxid, UK). Selected antibiotics were placed on the inoculated plates and included ceftazidime 10 μg, ampicillin 10 μg, cefotaxime 5 μg, meropenem 10 μg, imipenem 10 μg, amikacin 30 μg, gentamicin 10 μg, ciprofloxacin 5 μg, chloramphenicol 30 μg, ertapenem 10 μg, amoxicillin+clavulanic acid 30 μg, and piperacillin+tazobactam 36 μg (Liofilchem, Italy). The plates were incubated at +35 ± 1°C temperature for 18 ± 2 h. A double disk synergy test (DDST) was used to confirm extended spectrum beta-lactamase (ESBL). Disks containing cephalosporins (cefotaxime, ceftazidime) were applied to plates next to a disk with clavulanic acid (amoxicillin-clavulanic acid). A positive result was indicated if the inhibition zones around any of the cephalosporin disks were augmented in the direction of the disk containing clavulanic acid. Results were evaluated by measuring the zone of inhibition, and resistance was interpreted in accordance with the EUCAST breakpoints.

Haematoxylin-eosin staining was performed and the grade of inflammation was assessed, differentiating gangrenous and phlegmonous appendicitis. Serodiagnosis of *Yersinia enterocolitica* was performed using indirect (passive) haemagglutination.

For statistical analysis, Microsoft Excel 2016 and IBM SPSS Statistics 22 were employed. Results were expressed as median values and interquartile ranges (IQR). The com-

parison between groups was made using both the Mann–Whitney U-Test (two groups) and Kruskal–Wallis test (three groups) for non-parametric distribution, and the Fisher Exact Test was applied on normally distributed variables to determine correlations between them. A p-value of < 0.05 was considered statistically significant. The data was entered in SPSS and validated by an additional statistical analyst for accuracy.

RESULTS

A total of 67 children having arrived at the Emergency Department were included in this study (33 males, 34 females). Patient age ranged 7–17 years with a median age of 12 years. Depending on the intraoperative and bacteriological findings, two patient groups were established (Table 1). Patients with positive culture from samples of the peritoneal cavity were classified in the AcA group, which consisted of 34 patients (50.7%). Those patients with a negative culture were classified in the AnA group, which consisted of 33 patients (49.3%). There was no statistically significant difference between the results of the samples taken from the anatomical parts of the appendiceal lumen. *E. coli* was the prevalent representative of appendiceal intraluminal microbiota in both complicated and uncomplicated cases, totalling 51 patients (76.0%). *P. aeruginosa* was the prevalent microorganism of the extraluminal appendiceal microbiota (AcA/AuA:13/1). There were some differences in microbiota of the proximal and distal parts of the appendix between patients with acute complicated and acute uncomplicated appendicitis. In 22 of 34 AcA cases (55.0%) microbiota were identical, but the microbiota in distal and proximal parts differed for the remaining 12 cases (35.0%).

Bacteria were grown from samples of submucosa in both acute complicated and uncomplicated appendicitis. *E. coli* was the prevalent species, with *P. aeruginosa* being the second most commonly isolated microorganism (Table 1). Histological examination of surgically removed specimens showed a significant correlation between gangrenous appendicitis in AcA and phlegmonous appendicitis in AnA (Chi-square = 15.246, df = 1, $p < 0.001$). More than a half of the patients (61.3%) who had a drainage tube inserted were diagnosed with AcA ($p = 0.004$). A simple comparison suggested that AnA had a slightly shorter median postoperative hospital stay of five versus six days. *Yersinia enterocolitica* antibody detection preoperatively was negative in all cases. The remaining demographics and clinical characteristics of the patients are shown in Table 2.

Bacterial culture resulted in positive intraluminal samples with growth of one or several strains from each appendix. Table 2 shows the number of cases of the most common isolates for the AcA and AnA patient groups. Frequently, mixed strains were found at culture. The most common bacteria isolated from the appendix were *Escherichia coli* in 46 (37.4%), followed by *Pseudomonas aeruginosa* in 16 (13.0%), *Klebsiella pneumoniae* in nine (7.3%), *Bacterioides fragilis* in six (4.9%), and *Citrobacter braakii* in four

Table 1. Types of isolated bacteria, frequency and percentage in acute complicated appendicitis and acute uncomplicated appendicitis

	AcA		AnA		Total isolates No.	p-value
	No.	%	No.	%		
Escherichia coli	24	47.1	27	52.9	51	0.460#
Pseudomonas aeruginosa	13	92.9	1	7.1	14	0.098*
Klebsiella pneumoniae	3	50.0	3	50.0	6	0.480
Citrobacter braakii	0	0	3	100.0	4	0.053*
Bacterioides fragilis	1	20.0	4	80.0	5	0.321
Mixed cases	23	51.1	22	48.9	45	0.867
Total	60		63		123	

AcA, acute complicated appendicitis; AnA, acute uncomplicated appendicitis, # Pearson Chi-square test, * Fisher Exact test

Table 2. Characteristics and distribution of the study population

	AcA	AnA	Total	p-value
Children, n (%)	34 (50.7)	33 (49.3)	67	0.288
Age, median (IQR)	12 (9–15)	11 (10–15)	–	0.200
Laboratory values				
WBC count ($\times 10^9$/l),	16.7	14.5	–	< 0.001
CRP (g/l),	27.6	10.3	–	0.050
Duration of symptoms, h (IQR)	28 (16–51)	20 (12–26)	–	0.038
Alvarado score, points	8	7	–	< 0.001
Type of surgery, n (%)				
Laparotomy	7 (63.6)	4 (36.4)	11	0.349
Laparoscopy	26 (48.1)	28 (51.9)	54	
Histology, n (%)				
Phlegmonous	10 (29.4)	24 (70.6)	34	< 0.001
Gangrenous	23 (74.2)	8 (25.8)	31	
Drainage tube, n (%)	19 (70.4)	8 (29.6)	27	0.008
Length of hospital stay, days (IQR)	6 (5–9)	5 (4–6)	–	0.006

AcA, acute complicated appendicitis; AnA, acute uncomplicated appendicitis; IQR, interquartile range; WBC, white blood cells; CRP, C-reactive protein. Median values are presented with IQR (25%, 75%)

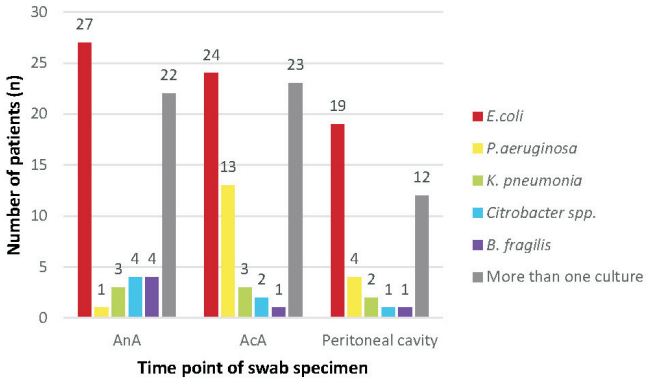

Fig 1. Types of organisms isolated.

samples (3.3%) (Table 2, Fig. 1). The 46 samples isolating *E. coli* had various antibacterial sensitivities: four (~8.7%) strains were resistant to ceftazidime; 17 (~37%) to ampicillin; three (~6.5%) to cefotaxime; one (~2.17%) to imipenem; four (~8.7%) to ciprofloxacin; three (~6.5%) to chloramphenicol; two (~4.34%) to ertapenem; nine (~19.5%) to

amoxicillin/clavulanic acid, and two (~4.34%) to piperacillin-tazobactam. All strains were susceptible to meropenem, amikacin and gentamicin. Additionally, two ESBL-producing strains of *E. coli* were also isolated.

P. aeruginosa was isolated from 15 samples. Antibacterial resistance was shown for four (26.7%) strains to ceftazidime; 11 (73.3%) to ampicillin; eight (53.3%) to cefotaxime; five (33.3%) to imipenem; five (33.3%) to chloramphenicol; 11 (73.3%) to ertapenem; 11 (73.3%) to amoxicillin/clavulanic acid and one (6.67%) to piperacillin-tazobactam. All strains were susceptible to meropenem, amikacin, gentamicin and ciprofloxacin. Other bacteria from the *Enterobacteriaceae* family were also isolated, such as *Citrobacter spp., Klebsiella spp.,* and others. *Citrobacter spp.* were resistant to amoxicillin/clavulanic acid, and *Klebsiella spp.* were resistant to ampicillin.

DISCUSSION

Research has shown that appendiceal luminal obstruction, by creating a closed loop, is the cause of appendicitis. Lymphoid hyperplasia in the follicles of the submucosa is the typical cause of luminal obstruction in children. Faecolith is mentioned as a precipitating obstructive factor (Bhangu *et al.,* 2015), while parasites (e.g. nematodes) and inflammatory constrictions are less common causes. Obstruction leads to increased bacterial proliferation, thus increasing the intraluminal pressure that subsequently impedes blood flow, which results in congestion and ischemia that promotes bacterial colonisation.

Although obstruction is the leading theory behind pathogenesis of appendicitis, it is not fully consistent with the data obtained in research and clinical practice. Therefore, bacteria are believed to also play a role in the pathogenesis. There is also a hypothesis highlighting the importance of genetic predisposition as the prevalence of appendicitis is higher among first-degree relatives. Finally, perforated and non-perforated appendicitis, which represent disease progression from an early to a late stage, are epidemiologically recognised as two distinct processes (Bhangu *et al.,* 2015; Abdussemee *et al.,* 2018; Essenmacher *et al.,* 2018). There is a dispute regarding whether the most common causative agents are *E. coli* and anaerobic *Clostridium perfringens* (Abdussemee *et al.,* 2018), as opposed to *Klebsiella spp.* and *Enterobacter spp.* (Parthiban and Harish 2017), or *Bacteroides fragilis, Pseudomonas aeruginosa* and *Peptostreptococcus spp*. (Roberts, 1988; Lamps, 2010; Guillet-Caruba *et al.,* 2011; Chen *et al.,* 2012).

The results of our study demonstrated that 53.0% of *P. aeruginosa* isolates were resistant to cefotaxime, and about a third against ceftazidime. Cefotaxime is included in the guidelines of the Children's Clinical University Hospital for treatment of intraperitoneal infections in paediatric patients (Anonymous, 2013). Although both antibiotics belong to third generation cephalosporins, ceftazidime has shown more efficacy in the treatment of infections with gram negative bacteria, especially *Pseudomonas spp.*, an important causative agent of nosocomial infections (Anonymous, 2019). Treatment typically involves the use of beta-lactam antibiotics; however, ceftazidime is the most important antibacterial agent, which functions as a cell wall synthesis inhibitor by binding to penicillin-binding protein 3 (PBP3) (Koss *et al.,* 2016). Nevertheless, ceftazidime-resistant strains have been discovered. Resistance mechanisms against ceftazidime in *P. aeruginosa* include the production of beta-lactamase encoded by genes acquired via horizontal gene transfer, or by increased production of a drug-induced, broad-spectrum, chromosomally encoded class C beta lactamase with an altered affinity (Fisher and Mobashery, 2014). The frequency of resistance against ceftazidime in isolates can fluctuate between 35% to 86% (Du *et al.,* 2010; Noha *et al.*, 2015).

In our study, *P. aeruginosa* was prevalent in samples obtained from patients with acute complicated appendicitis. All strains were sensitive to meropenem, which inhibits cell wall synthesis and is not affected by beta-lactamase (Baldwin *et al.,* 2008). Drusano *et al*. (2018) investigated the potential use of fosfomycin in the treatment of infections with *P. aeruginosa* and discovered that the bacteria rapidly developed resistance against fosfomycin. Therefore, they suggested switching treatment from monotherapy to combination therapy with fosfomycin and meropenem. A synergistic effect was observed with fosfomycin eradicating the meropenem-resistant mutants and meropenem working against fosfomycin-resistant strains. Thus, this combination was recommended as a treatment strategy for wider use in the future (Drusano *et al.,* 2018). Another combination displaying promising results in research settings is meropenem in conjunction with ceftazidime (Feng *et al.,* 2017). In the past decade, the following antibiotic combinations have been investigated as potential treatment options (Gracia *et al.* 2018).

Ceftazidime-Avibactam. Avibactam is a member of the class of azabicycloalkanes. Avibactam is a non-beta-lactam beta-lactamase inhibitor that is available in combination with ceftazidime (Avycaz). This combination was approved by the Food and Drug Administration (FDA) on 25 February 2015 for the treatment of complicated intra-abdominal infections in combination with metronidazole (Anonymous, 2019). This combination has shown efficacy of up to 90% against ceftazidime-resistant strains of *P. aeruginosa* (Mazuski *et al.*, 2016; Qin *et al.,* 2017). Combined treatment with ceftazidime-avibactam and colistin has shown promise in treating infections with XDR (extremely drug-resistant) *P. aeruginosa* (Xipell *et al,* 2017).

Ceftolozane-Tazobactam. Ceftolozane-tazobactam was approved by the FDA in 2014, shortly before ceftazidime-avibactam was approved for the same indications. It is highly effective in combinations with meropenem and levofloxacin (Solomkin *et al.,* 2015; Wagenlehner *et al.,* 2015; Gracia *et al.,* 2018).

In our study, amikacin demonstrated significant efficacy against isolates from samples. It is a broad-spectrum semi-

synthetic aminoglycoside antibiotic, derived from kanamycin with antimicrobial properties. Amikacin irreversibly binds to the bacterial 30S ribosomal subunit, specifically locking 16S rRNA and S12 protein within the 30S subunit. This leads to interference with the translational initiation complex and misreading of mRNA, thereby hampering protein synthesis and resulting in the bactericidal effect. This agent is usually used in short-term treatment of serious infections due to susceptible strains of Gram-negative bacteria (Anonymous, 2019). Data is scarce regarding amikacin-resistant *pseudomonas*. Research conducted by Loho *et al.* (2018) showed that only two of 20 *P. aeruginosa* isolates were resistant against amikacin. Its combination with doripenem is synergistic and improves treatment results.

The most commonly isolated microorganism from our patients' samples was *E. coli*, especially from those treated for acute uncomplicated appendicitis. This finding concurs with results obtained by other authors (Naher *et al.,* 2013; Bhangu *et al.,* 2015; Abdussemee *et al.,* 2018; Bazzaz *et al.,* 2018; Essenmacher *et al.,* 2018; Rickard *et al.,* 2018; Snyder *et al.,* 2018; Turel *et al.,* 2018). Our data revealed that strains of *E. coli* are sensitive to antibacterial agents such as amikacin, imipenem, and meropenem, which is in line with recent studies by other researchers (Bazzaz *et al.,* 2018). There were strains resistant against other antibacterial agents included in the treatment guidelines; three and four of 47 were resistant to cefotaxime and ceftazidime, respectively. Only two isolates were ESBL-positive. This complies with data from other studies that showed the prevalence of ESBL-producing *E. coli* in Latvia. 11% of *E. coli* present in animal microbiota produce ESBL (Terentjeva *et al.,* 2019), whereas in the adult population, ESBL are produced by only 1.6% of *E. coli* (Ny *et al.,* 2018).

CONCLUSIONS

In cases of acute complicated appendicitis, *P. aeruginosa* is the prevalent microorganism, whereas *E. coli* is the most commonly isolated microorganism in acute uncomplicated appendicitis. Antibiotic treatment strategies in cases of acute complicated appendicitis should include antibiotics with different mechanisms of action to achieve a synergistic effect and prevent the development of antibiotic resistance. Mixed culture results were prevalent in both acute complicated and acute uncomplicated appendicitis. The incidence of ESBL producing microorganisms was low in these acute appendicitis cases.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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MIKROBIOTAS BAKTERIOLOĢISKA IZVĒRTĒŠANA BĒRNIEM AKŪTA APENDICĪTA GADĪJUMĀ

Pēdējo gadu laikā ir mainījusies akūta nekomplicēta apendicīta ārstēšanas taktika, kuras ietvaros rekomendēta antibakteriālā terapija nevis ķirurģiska operācija, īpaši bērniem. Šī pētījuma mērķis bija izvērtēt mikrobiotu pediatriska vecuma pacientiem ar akūtu komplicētu un nekomplicētu apendicītu, kā arī izvērtēt etioloģisko ierosinātāju antibakteriālo jutību. Darbā tika izmantotas bakterioloģiskās metodes, mikroorganismu identifikācija veikta, izmantojot VITEK2 analizatoru. Antibakteriālās jutības testi tika veikti un rezultāti izvērtēti saskaņā ar Eiropas antimikrobiālās jutības testēšanas (EUCAST, Versija Nr. 7.0, 2019. g. janvāris) rekomendācijām. *Yersinia enterocolitica* serodiagnostikā tika izmantota netiešā hemaglutinācijas redakcija. Pētījuma rezultāti liecina, ka ir microbiota atšķirības akūta komplicēta un nekomplicēta apendicīta gadījumā. Akūta komplicēta apendicīta gadījumā prevalēja *Pseudomonas aeruginosa.* Akūtu apendicītu gadījumā pārsvarā izdalītas mikroorganismu asociācijas. No pētījumā iekļautajiem pacientiem tikai atsevišķos gadījumos tika izdalīti ESBL producētāji. Daļa *Pseudomonas aeruginosa* celmu bija rezistenti pret amoksicilīnu ar klavulānskābi, ertapenēmu, ampicilīnu un cefotaksīmu. Daļa izdalīto *E. coli* celmu bija rezistenti pret ampicilīnu un amoksicilīnu ar klavulānskābi.