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Effects of penicillin V on the faecal microbiota in patients with pharyngotonsillitis—an observational study

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Background: The intestinal microbiota functions as a reservoir of antibiotic resistance.

Objectives: To evaluate penicillin V (phenoxymethylpenicillin) effects on the faecal microbiota with focus on beta-lactam resistance.

Methods: We included 31 primary care patients with group A streptococcal pharyngotonsillitis treated with penicillin V for 5 (800 mg×4) or 10 days (1000 mg×3). Twenty-nine patients contributed with three faecal swab samples each. The faecal specimens were collected at the start of penicillin V treatment, after the last dose and at follow-up 7–9 days after completed treatment. Samples were inoculated semiquantitatively on selective screening agar plates to study beta-lactam resistance, species shifts among Enterobacterales and enterococci, and colonization with *Candida* spp. and *Clostridioides difficile*. Representative colonies were identified using MALDI-TOF. Results were analysed by non-parametric statistical methods.

Results: An increase in the proportion of patients colonized with ampicillin-resistant Enterobacterales, from 52% to 86% ($P=0.007$), and Enterobacterales with decreased susceptibility to third-generation cephalosporins, from 32% to 52% ($P=0.034$), was observed between the first and second samples. This increase was no longer significant at follow-up. New colonization with ampicillin-resistant Enterobacterales species and non-Enterobacterales Gram-negative species was observed, and persisted at follow-up.

Conclusions: Following treatment with penicillin V, we observed decreased susceptibility to ampicillin and third-generation cephalosporins, and prolonged colonization with non-*Escherichia coli* Gram-negative species. These findings challenge the perception that penicillin V has limited ecological effect on the intestinal microbiota, and emphasizes the importance of avoiding even narrow-spectrum antimicrobials when possible.

Introduction

Increasing antimicrobial resistance is a growing threat to human health and a consequence of the widespread use of antimicrobial agents.[1] Antimicrobial resistance is a leading cause of death in the world, estimated at 4.95 million deaths during 2019.[2] The main option for handling antibiotic resistance is to reduce antibiotic pressure by antimicrobial stewardship programmes[3] and changed behaviour.[4] In optimizing the use of available antibiotics, the ecological impact of the drug should be considered.[5] In addition to focusing on target pathogens when monitoring antimicrobial resistance, it is important to recognize the commensal microbiota of the human host. The distal gut harbours a rich commensal microbiota that serves as a breeding ground

for the transfer of resistance genes and selection of resistant microorganisms, hence acting as a reservoir of antibiotic resistance.[6] The impact on the microbiota depends on the antibiotics' mode of action and the pharmacokinetic profile,[7,8] the duration of treatment[9] and the local resistance epidemiology.[10–12]

Earlier studies of the effects of penicillin V (phenoxymethylpenicillin) on the intestinal microbiota found no, or very limited, alterations in the intestinal microbiota.[13–16] These studies were performed 20–40 years ago, in small populations and with dosage regimens lower than those presently in use. Current treatment guidelines recommend a more frequent intake and larger daily exposure of penicillin V,[17–19] resulting in greater time above MIC. The earlier studies did not specifically explore alterations in antimicrobial resistance. The aim of the present study was to evaluate the effects of modern dosage penicillin V on the faecal microbiota, with focus on emergence of beta-lactam resistance.

Patients and methods

Ethics

This research was conducted in accordance with the Declaration of Helsinki and national and institutional standards. The study was approved by the Regional Ethical Review board in Lund, Sweden, 25 June 2015 (reference number 2015/396). All participants were informed of the study, both verbally and in writing, and provided written consent before participation. In the case of children, both the child and the guardian/guardians provided consent before participation. The trial was registered in the EU Clinical Trials Register (EudraCT 2015-001752-30).

Study design

The present observational study constitutes a sub-study of a recent randomized, non-inferiority trial of penicillin V treatment for group A streptococcal pharyngotonsillitis. In the main study, penicillin V 800 mg four times daily for 5 days was compared with penicillin V 1000 mg three times daily for 10 days.[20] The present sub-study recruited participants from six primary healthcare centres in Sweden between September 2015 and February 2018.

Inclusion and exclusion criteria

Patients aged from 10 years seeking primary care for sore throat and having 3–4 Centor criteria (fever ≥38.5°C, tender cervical lymph nodes, tonsillar exudates and lack of cough),[21] and a positive rapid antigen detection test for group A streptococci, were asked to participate. Patients who had received antibiotics the previous month were excluded.

Data collection/sampling

Because the main study randomized patients to either 5 days' treatment (daily dose 3.2 g) or 10 days' treatment (daily dose 3.0 g), the study population consisted of patients with differing duration at risk for selective pressure promoting emergence of resistance. The participants noted each dose of penicillin V in a diary. All participants received materials for faecal swab samples with instructions for sampling, and prepaid envelopes for delivery to the Public Health Agency of Sweden where the samples were stored at −70°C pending analyses. Faecal samples, swabs from toilet paper, were taken at home using 1 mL Eswabs in a transport medium (Copan Diagnostics Inc., Mantua, Italy). Each patient contributed three faecal samples. Sample 1 was collected before or within 18 h of the first dose of penicillin V. If patients were unable to produce a sample within 18 h a swab sample was collected rectally. Sample 2 was taken directly after the last dose of penicillin V, and sample 3 was taken 7–9 days after treatment completion. Adverse events were recorded by the physician in the case report form at a follow-up visit 7–9 days after completed treatment. Regional research nurses made follow-up telephone calls 1 and 3 months after completed penicillin V treatment, to monitor potential adverse events and complications from treatment. In the event of complications details were collected from medical record retrospectively.

Microbiological methods

Each sample was inoculated on the following agar media: two sets of CHROMagar MH Orientation for the detection of various Enterobacterales and of specific resistances; one each of CHROMagar C3GR for detection of Enterobacterales with reduced susceptibility to third-generation cephalosporins; CHROMagar C. difficile for detection of *Clostridioides difficile*; CHROMagar Candida for detection of *Candida* spp. (all CHROMagar media from CHROMagar Company, Paris, France); and Enterococcus faecium ChromoSelect Agar Base (Merck, Germany) for detection and differentiation of enterococci. To detect ampicillin resistance among Enterobacterales two ampicillin discs (10 µg) were applied to one of the CHROMagar MH Orientation plates, at a distance of 3–4 cm. In addition, a linezolid disc (30 µg) was placed between the ampicillin discs for suppression of Gram-positive growth. Ampicillin resistance was applied as a marker for beta-lactam resistance in Enterobacterales. To analyse potential alterations in the *Bacteroides* population, the samples were inoculated on Brucella agar with kanamycin and vancomycin (BKV). The Substrate unit at Karolinska University Hospital prepared all agar media. The aerobic agar plates were incubated at 35 ± 1°C for 18–24 h, except for the CHROMagar Candida plates, which were incubated for 48 h. The anaerobic agar plates were incubated under anaerobic conditions at 35 ± 1°C for 48 h. After incubation, different colony types were counted and semiquantitatively scored as zero (0), 1, 2 or 3, where 0 corresponded to no growth, 1 to mild growth, 2 to moderate growth and 3 to rich growth, according to instructions and in relation to growth on a non-selective control plate. When analysing shifts in species (*Escherichia coli* towards non-*E. coli* Enterobacterales, *Enterococcus faecalis* towards non-*E. faecalis* enterococci, and *Candida albicans* towards non-*C. albicans* yeasts, respectively) 0–3 scores implied an approximate fraction of the latter of 0%, 25%, 50% and >75% of the total colony counts, respectively. Representative colony types, including non-Enterobacterales Gram-negatives from the different agar media, were isolated in pure culture and identified by MALDI-TOF (Biotyper, Bruker Corporation, the Netherlands).

Ecological alterations in the faecal microbiota were measured as emergence of resistance against beta-lactams, shift from *E. coli* to non-*E. coli* Enterobacterales, overgrowth of and species shift among enterococci, new colonization with *Candida* spp. and emergence of *C. difficile*. Colonization with Enterobacterales having reduced susceptibility to third-generation cephalosporins was defined as colonies isolated from the selective agar medium CHROMagar C3GR. Similarly, the density of colonies isolated within the presumed inhibition zone of ampicillin discs was scored semiquantitatively as 0, 1, 2 or 3. A shift in species within these genera to more intrinsically resistant species is generally considered a sign of ecological disturbance in the intestinal microbiota.[22] Reproducibility of the culture procedure was ascertained by conducting analyses in duplicates of the samples from the first five patients.

Statistical methods

The results of the culture analyses were presented descriptively and analysed by non-parametric tests. The null hypothesis was that the distribution of resistance among faecal Enterobacterales would be the same before and after penicillin V treatment, both between the different treatment regimens, and between samplings, regardless of treatment duration. Analyses were performed regarding the presence of

Enterobacterales with ampicillin resistance and decreased susceptibility to third-generation cephalosporins, respectively. New colonization with ampicillin-resistant Enterobacterales and non-Enterobacterales Gram-negative species was also analysed. A chi-squared test was used for comparison between the penicillin V treatment groups, 5 and 10 days. Differences within each patient were dichotomized, whether colonized with target isolates (semiquantitative scores 1–3) or not colonized (score 0) and analysed as paired data comparing samples 1 and 2, and samples 1 and 3, respectively, using McNemar test. Analyses of the number of resistant Enterobacterales and non-Enterobacterales Gram-negative species, between samples 1 and 2, and between samples 1 and 3, were performed using the paired Wilcoxon signed rank test. No adjustments for multiple testing were made. Statistical analysis was performed using IBM SPSS statistics version 28, and differences were considered significant at $P<0.05$ (two-tailed).

Results

Study population

We included 31 patients, none of whom had received antibiotics during the month prior to entering the study. Two patients lacked a second faecal sample. Twenty-nine patients had a complete set of three faecal samples and were included in all analyses. One of these had been treated with antibiotics within 3 months preceding the study. The baseline sample from this patient showed no ampicillin-resistant isolates. Eighteen patients were randomized to the 5-day regimen and 13 to the 10-day regimen. Adherence to the treatment was 100% in all 31 patients. Seventy-nine percent (23/29) were women and 21% (6/29) men. The median age was 38 years (IQR 25–45).

Ecological disturbances in the faecal microbiota

All samples yielded rich growth on the control blood agar plates. Duplicate cultures of the total set of selective agar plates of five series of patients showed high reproducibility. The remaining samples were not cultured in duplicate. No significant differences regarding the numbers of patients with detectable ampicillin resistance (9 versus 7, 14 versus 11, and 10 versus 8 species in sample 1, 2 and 3), or reduced susceptibility to third-generation cephalosporins (5 versus 5, 9 versus 6, and 10 versus 6 species in sample 1, 2 and 3) in the faecal microbiota were seen between the 5- and 10-day treatment groups. The following analyses were performed on available faecal samples from all included patients. There was a significant increase in the proportion of Enterobacterales resistant to ampicillin, from 16/31 (52%) to 25/29 (86%) ($P=0.007$), and in the proportion of Enterobacterales with decreased susceptibility to third-generation cephalosporins, from 10/31 (32%) to 15/29 (52%) ($P=0.034$), from baseline to directly after the last dose of penicillin V (sample 2). At follow-up the increase from baseline was no longer significant for any of these analyses. (Figures 1 and 2). There was new colonization with ampicillin-resistant Enterobacterales and non-Enterobacterales Gram-negative species after exposure to penicillin V. The number of unique species per sample within each patient significantly increased from baseline to directly after penicillin V treatment (samples 1 to 2; $P=0.003$). The increase remained significant at the follow-up (samples 1 to 3; $P=0.008$) (Figure 3). Thirteen of 29 patients had an increased growth of enterococci in sample 2, of whom 11 still had increased levels of enterococci in sample 3.

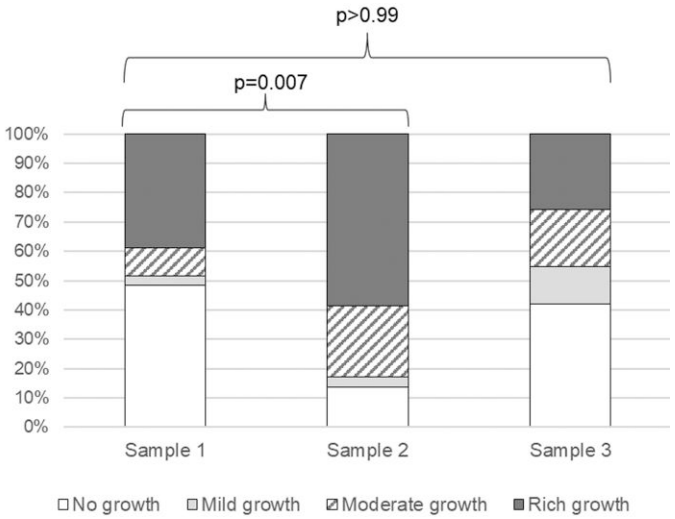

Figure 1. Proportion of ampicillin-resistant Enterobacterales in faecal samples from 29 patients treated with penicillin V. Results at baseline (sample 1), after the last dose of penicillin V (sample 2) and at follow-up 7–9 days after completed treatment (sample 3). The brackets show *P* values for dichotomized differences, whether colonized with target isolates or not, regardless of semiquantification (McNemar test).

In 3 patients the growth of enterococci was reduced in sample 2, whereas in 13 patients the levels of enterococci remained stable throughout the study period. No shifts among enterococcal species and no significant variations in the numbers of *Bacteroides* spp. were detected during the study period. Three patients were newly colonized with *C. albicans* in sample 2. One patient had moderate growth of toxin A- and B-positive *C. difficile*, ribotype 14 and sequence type 13, in sample 3.

Adverse events

No serious adverse events were reported during the study period for patients participating in the present sub-study. One patient had a *C. difficile* infection. This patient had 10 days of penicillin V treatment and suffered from diarrhoea and fatigue during the follow-up visit to the primary care centre. The patient recovered spontaneously within weeks and required no hospital care.

Discussion

Principal findings

The proportion of patients colonized with Enterobacterales resistant to ampicillin, and with Enterobacterales with reduced susceptibility to third-generation cephalosporins, increased significantly from baseline to after the last dose of penicillin V, but was not sustained. A significant increase in ampicillin-resistant Enterobacterales species and non-Enterobacterales Gram-negative species was observed after penicillin V exposure, which remained at follow-up. One patient had a *C. difficile* infection.

Strengths and limitations

The pragmatic design with real world patients seeking primary care, strengthens the external validity of the present study. This

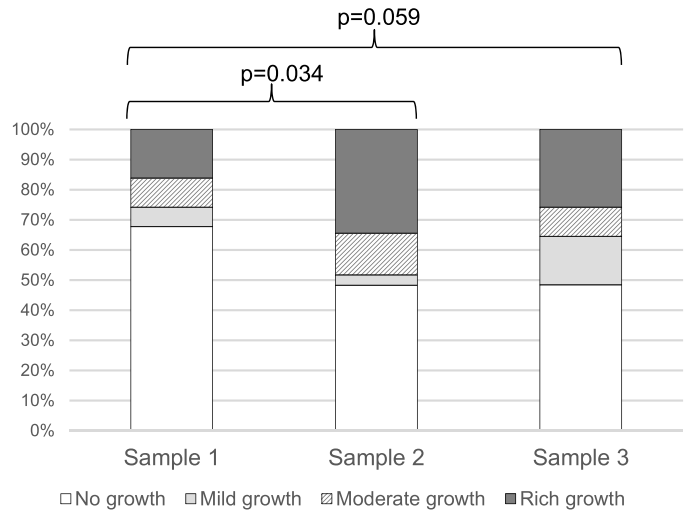

Figure 2. Proportion of Enterobacterales with decreased susceptibility to third-generation cephalosporins (isolated from CHROMagar C3GR medium) in faecal samples from 29 penicillin V-treated patients. Results at baseline (sample 1), after the last dose of penicillin V (sample 2) and at follow-up 7–9 days after completed treatment (sample 3). The brackets show *P* values for dichotomized differences, whether colonized with target isolates or not, regardless of semiquantification (McNemar test).

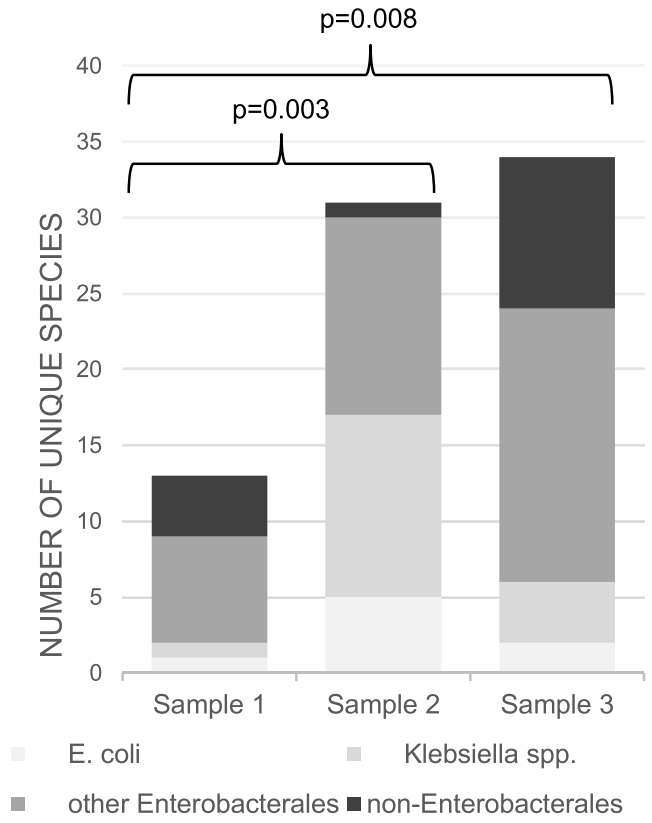

Figure 3. Distribution of faecal colonization with ampicillin-resistant *E. coli* and non-*E. coli* Gram-negative rods in penicillin V-treated patients. Species present both at baseline and in at least one of samples 2 and 3 within a single patient are excluded. The brackets show *P* values for differences in total number of ampicillin-resistant Enterobacterales and non-Enterobacterales species, unique within each patient at each sampling, between samples 1 and 2 (29 patients), and between samples 1 and 3 (31 patients), respectively (paired Wilcoxon signed rank test). 'Other Enterobacterales' include*: Citrobacter* spp., *Enterobacter* spp., *Hafnia* spp., *Raoultella* spp., *Pantoea* spp. and *Kluyvera* spp.; 'non-Enterobacterales' include: *Acinetobacter* spp., *Pseudomonas* spp., *Stenotrophomonas* spp. and *Aeromonas* spp.

study focused on clinically relevant parameters, such as emergence of resistance and colonization with *C. difficile*. The results are applicable in other healthcare settings where penicillin V is employed. One limitation was the low number of patients included in the present study. More patients and a longer follow-up period could have added more information about the duration and diversity of the alterations in the faecal microbiota. Despite this, the study achieved significant results. Another limitation was that the analyses of the microbiota were based on semiquantitative culture methods rather than quantitative methods. Although metagenomic analyses have several advantages, a strength of culture methods is the possibility to determine proportions of resistant strains within bacterial genera, families and orders.

Findings in relation to existing literature

To our knowledge, this is the first study of the effects of penicillin V treatment on the faecal microbiota specifically focusing on emergence of antimicrobial resistance. Two previous studies gave 800 mg penicillin V twice a day for 7 days, to 10 and 6 subjects, respectively.[13,14] In these studies, the faecal microbiota was relatively constant during the study. A third study giving 1 g penicillin V twice a day for 10 days to 10 subjects reported minor alterations in the aerobic faecal microbiota and an increase in *Clostridioides* species during penicillin V treatment.[15] In the present study, we found alterations such as increased growth of enterococci, and new colonization with non-*E. coli* Gram-negative species and with *C. albicans* directly after penicillin V exposure, all signs of ecological disturbances in the microbiota.[12] We also found one patient with a *C. difficile* infection, a condition that has not been previously reported during penicillin V treatment. A plausible explanation for the differences in results compared with earlier studies is the use of higher penicillin V daily dosages. The main study found that the 10-day group had a higher incidence and longer duration of diarrhoea, nausea and vaginal symptoms compared with the 5-day group.[20] Unfortunately, the number of patients in the present sub-study were too few to analyse potential differences in the ecological changes of the faecal microbiota between the treatment arms. It is difficult to compare the effects on faecal microbiota of penicillin V with the effects of other antibiotics commonly used for the treatment of pharyngotonsillitis. Studies on the effects of amoxicillin on faecal microbiota used different dosages of amoxicillin, and gave disparate results regarding changes in the faecal microbiota. The duration of the microbiota alterations has varied from 2 weeks to 6 months.[23]

In the present study, the emergence of species with reduced susceptibility to third-generation cephalosporins after penicillin

V treatment raises questions about what impact penicillin V might have on ESBL-producing species. Penicillin V's impact on the presence of ESBL in the faecal microbiota needs to be addressed in future studies. We also found new colonization of unusual potentially pathogenic Gram-negative species, not commonly detected in the intestinal microbiota and often considered potential pathogens. Their persistence in follow-up samples implies that even treatment with penicillin V can induce prolonged disturbances in the intestinal microbiota.

Implications

Penicillin V in currently recommended dosages had a marked ecological impact on the faecal microbiota in terms of a significant increase in Enterobacterales with resistance to ampicillin and a significantly reduced susceptibility to third-generation cephalosporins. The increased number of unusual potentially pathogenic Gram-negative rods remained during the follow-up period after exposure to penicillin V. These findings challenge the general perception of penicillin V as an ecologically safe agent. These results also indicate that penicillin V should be used with caution and prescribed only when the benefit to the patient clearly outweighs the potential risks of the treatment.

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Transparency declarations

None of the authors have any conflicts of interest to disclose. C.E., K.H., K.R., P.-D.S., P.E., M.T., G.S.S. and C.G.G. contributed to the study's concept. K.H., K.R., M.T. and P.-D.S. acted as regional investigators and contributed to the acquisition of data. Development of laboratory methodology was made by P.E. and C.E.. Statistical analysis and interpretation of data were performed by K.R. under supervision from C.E. and K.H. The original manuscript was drafted by K.R., P.E. and C.E. All authors were involved in revising the work critically and in the approval of the final manuscript.

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