รายงานวิจัยฉบับสมบูรณ์

โครงการ

ความชุกของเชื้อแบคทีเรียก่อโรคในระบบทางเดินปัสสาวะ รูปแบบความไวของยาปฏิชีวนะและการสร้างไบโอฟิล์มของแบคทีเรียที่แยกได้จากปัสสาวะและก้อนนิ่วของผู้ป่วยโรคนิ่วไตที่มีการติดเชื้อในระบบทางเดินปัสสาวะ

Prevalence of uropathogenic bacteria, antibiotic susceptibility patterns and bacterial biofilm formation in urine and stone matrix of kidney stone patients with urinary tract infection

โดย

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สัญญาเลขที่ **MRG5380061**

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โครงการ
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Prevalence of uropathogenic bacteria, antibiotic susceptibility patterns and bacterial biofilm formation in urine and stone matrix of kidney stone patients with urinary tract infection

ผู้วิจัย
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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

โครงการวิจัยเรื่อง “ความชุกของเชื้อแบคทีเรียก่อโรคในระบบทางเดินปัสสาวะ รูปแบบความไวของยาปฏิชีวนะและการสร้างไบโอฟิล์มของแบคทีเรียที่แยกได้จากปัสสาวะและก้อนนิ่วของผู้ป่วยโรคนิ่วไตที่มีการติดเชื้อในระบบทางเดินปัสสาวะ” ได้ดำเนินการวิจัยจนสำเร็จลุล่วงไปด้วยดี

ผู้วิจัยใคร่ขอขอบพระคุณ ศาสตราจารย์นายแพทย์ วิศิษฏ์ ทองบุญเกิด นักวิจัยที่ปรึกษาที่กรุณาให้คำปรึกษา ข้อเสนอแนะ และความช่วยเหลือในด้านต่างๆ ตลอดการทำวิจัยครั้งนี้ ตลอดจนสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) สำนักงานคณะกรรมการการอุดมศึกษา (สกอ.) และมหาวิทยาลัยขอนแก่น ที่ให้ทุนสนับสนุนโครงการวิจัยนี้ และขอขอบพระคุณคณะเทคนิคการแพทย์ มหาวิทยาลัยขอนแก่น ที่ให้ความอนุเคราะห์สถานที่ เครื่องมือและอุปกรณ์ต่างๆ ในการทำวิจัย และขอขอบพระคุณอาสาสมัครทุกท่านที่กรุณาให้ความอนุเคราะห์ตัวอย่างปัสสาวะและก้อนนิ่ว ตลอดจนบุคลากรของหน่วยศัลยกรรมโรคนิ่วไต โรงพยาบาลขอนแก่น จังหวัดขอนแก่น ที่ให้ความอนุเคราะห์เก็บตัวอย่างที่ใช้ในการศึกษาครั้งนี้

ราตรี ทวิชากรตระกูล

มิถุนายน 2555

สารบัญ

บทคัดย่อ

รหัสโครงการ : MRG5380061

ชื่อโครงการ : ความชุกของเชื้อแบคทีเรียก่อโรคในระบบทางเดินปัสสาวะ รูปแบบความไวของยาปฏิชีวนะและการสร้างไบโอฟิล์มของแบคทีเรียที่แยกได้จากปัสสาวะและก้อนนิ่วของผู้ป่วยโรคนิ่วไตที่มีการติดเชื้อในระบบทางเดินปัสสาวะ

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การติดเชื้อในทางเดินปัสสาวะมักจะพบร่วมกับโรคนิ่วไต โดยเฉพาะอย่างยิ่งโรคนิ่วชนิดสตรูไวท์ ซึ่งมักจะมีสาเหตุมาจากการติดเชื้อแบคทีเรียที่สามารถย่อยสลายยูเรียได้ เช่น เชื้อ *Proteus mirabilis* อย่างไรก็ตามจากการสังเกตของคณะผู้วิจัย พบว่าการติดเชื้อแบคทีเรียที่สามารถย่อยสลายยูเรียได้ อาจไม่ใช่สาเหตุของการนิ่วในประเทศไทย ดังนั้นคณะผู้วิจัยจึงได้มีความสนใจในการจำแนกชนิดของจุลชีพในก้อนนิ่วทุกชนิด โดยทำการศึกษาเชื้อแบคทีเรียที่แยกได้จากปัสสาวะและก้อนนิ่วของผู้ป่วยโรคนิ่วไตจำนวน 100 ราย เป็นเพศชาย 59 ราย และหญิง 41 ราย ที่เข้ารับการรักษาโรคนิ่วไตด้วยการผ่าตัดเอาก้อนนิ่วออก จากผลการศึกษาพบว่าสามารถแยกเชื้อแบคทีเรียได้จากปัสสาวะและ/หรือก้อนนิ่ว จำนวน 45 isolates จากผู้ป่วย จำนวน 36 ราย ซึ่งองค์ประกอบทางเคมีของผู้ป่วยเหล่านี้ แบ่งเป็น infection induced stone จำนวน 8 ราย และ metabolic stone จำนวน 28 ราย ส่วนการวิเคราะห์องค์ประกอบทางเคมีพบก้อนนิ่วชนิดแคลเซียมออกซาเลตมากที่สุดทั้งในผู้ป่วยที่สามารถและไม่สามารถแยกเชื้อแบคทีเรียได้ คิดเป็น 64 และ 75% ตามลำดับ และเชื้อแบคทีเรียที่พบได้บ่อยที่สุดทั้งในปัสสาวะและก้อนนิ่ว (ทั้งในใจกลาง และรอบนอกของก้อนนิ่ว) ได้แก่ *Escherichia coli* (คิดเป็น 1/3 ของเชื้อแบคทีเรียที่แยกได้ทั้งหมด) ยิ่งไปกว่านั้นยังพบความสัมพันธ์ไปในทิศทางเดียวกันระหว่างชนิดของเชื้อแบคทีเรียที่แยกได้จากปัสสาวะและก้อนนิ่วอย่างมีนัยสำคัญทางสถิติ ($r=0.860$, $P<0.001$) ซึ่งเชื้อแบคทีเรียเหล่านี้ส่วนใหญ่ดื้อต่อสารต้านจุลชีพหลายชนิดร่วมกัน นอกจากนี้ยังพบเชื้อแบคทีเรียที่สามารถย่อยสลายยูเรียได้และไม่ได้ คิดเป็น 31 และ 69% ตามลำดับ ยิ่งไปกว่าการศึกษาการสร้างไบโอฟิล์มของเชื้อแบคทีเรียที่พบได้บ่อย 3 อันดับต้นๆ ในปัสสาวะและก้อนนิ่ว ได้แก่ *Escherichia coli*, *Enterococcus faecalis* และ *Klebsiella* pneumoniae พบว่า 44.44 และ 42.88% ของเชื้อ *Escherichia coli* สามารถสร้างไบโอฟิล์มได้จากเชื้อที่แยกได้จากปัสสาวะและก้อนนิ่วตามลำดับ ส่วน *Enterococcus faecalis* พบว่า 60 และ 50% สามารถสร้างไบโอฟิล์มได้จากเชื้อที่แยกได้จากปัสสาวะและก้อนนิ่วตามลำดับ ในขณะที่ *Klebsiella pneumoniae* พบการสร้างไบโอฟิล์มเฉพาะในก้อน คิดเป็น 66.67% จากข้อมูลเหล่านี้แสดงให้เห็นว่า เชื้อแบคทีเรียที่สามารถย่อยสลายยูเรียได้ไม่ได้เป็นเชื้อสาเหตุหลักที่แยกได้จากปัสสาวะและก้อนนิ่วของผู้ป่วยโรคนิ่วไต ในขณะที่ความสามารถในการสร้างไบโอฟิล์มได้อาจเป็นปัจจัยสำคัญอย่างหนึ่งของการเกิดโรคนิ่วไตที่เกี่ยวข้องกับการติดเชื้อในทางเดินปัสสาวะทางภาคตะวันออกเฉียงเหนือของประเทศไทย ซึ่งจำเป็นต้องศึกษาวิจัยต่อไปในอนาคต

คำหลัก : kidney stone; antibiotic susceptibility; prevalence; uropathogenic bacteria; biofilm formation

Abstract

Project Code : MRG5380061

Project Title : Prevalence of uropathogenic bacteria, antibiotic susceptibility patterns and bacterial biofilm formation in urine and stone matrix of kidney stone patients with urinary tract infection

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Project Period : 2010-2012 (2 years)

Urinary tract infections (UTIs) are generally known to be associated with nephrolithiasis, particularly struvite stone, in which the most common microbe found is urea-splitting bacterium, i.e. *Proteus mirabilis*. However, our observation indicated that it might not be the case of stone formers in Thailand. We therefore extensively characterized microorganisms associated with all types of kidney stones. A total of 100 kidney stone formers (59 males and 41 females) admitted for elective percutaneous nephrolithotomy were recruited and microorganisms isolated from catheterized urine and cortex and nidus of their stones were analyzed. From 100 stone formers recruited, 36 cases had a total of 45 bacterial isolates cultivated from their catheterized urine and/or stone matrices. Among these 36 cases, chemical analysis by Fourier-transformed infrared spectroscopy revealed that 8 had the previously classified "infection-induced stones", whereas the other 28 cases had the previously classified "metabolic stones". Calcium oxalate (in either pure or mixed form) was the most common and found in 64 and 75% of the stone formers with and without bacterial isolates, respectively. *Escherichia coli* was the most common bacterium (approximately 1/3 of all bacterial isolates) found in urine and stone matrices (both nidus and periphery). Linear regression analysis showed significant correlation (r=0.860; p<0.001) between bacterial types in urine and stone matrices. Multidrug resistance was frequently found in these isolated bacteria. Moreover, urea test revealed that only 31% were urea-splitting bacteria, whereas the majority (69%) had negative urea test. Moreover, the biofilm-producing bateria in the three most common bacteria found in both urine and stone matrices samples were *Escherichia coli*, *Enterococcus faecalis* and *Klebsiella* pneumoniae. Among these, 44.44 and 42.88% of the *Escherichia coli* strains were positive for biofilm production in urine and stone matrices, respectively. In addition, 60 and 50% of the *Enterococcus faecalis* strains were also positive for biofilm production in both samples. While 66.67% of the *Klebsiella pneumoniae* strains was found only in stone matrices. Our data indicate that microorganisms are associated with almost all chemical types of kidney stones and urea-splitting bacteria are not the major causative microorganisms found in urine and stone matrices of the stone formers in Thailand. The ability of biofilm production may be the one of important factors in stone formation with urinary tract infection. These data may lead to rethinking and a new roadmap for future research regarding the role of microorganisms in kidney stone formation.

Keywords : kidney stone; antibiotic susceptibility; prevalence; uropathogenic bacteria; biofilm formation

Executive Summary

Background: Nephrolithiasis is highly prevalent in the northeastern region of Thailand. Urinary tract infections (UTIs) are generally known to be associated with nephrolithiasis. The association between nephrolithiasis and UTIs can be either (i) kidney stone formation developed following UTIs (the so-called "infection-induced stones") or (ii) nephrolithiasis with subsequent UTIs as its complications (the so-called "stones with subsequent infections"). However, our observation indicated that it might not be the case of stone formers in Thailand. We therefore extensively characterized microorganisms associated with all types of kidney stones.

Methods: A total of 100 kidney stone formers (59 males and 41 females) admitted for elective percutaneous nephrolithotomy were recruited and microorganisms isolated from catheterized urine and cortex and nidus of their stones were analyzed. Identification of bacterial isolates was done by standard biochemical tests and PCR method. The antimicrobial susceptibility test was done by disc diffusion method. The chemical composition of stone was analyzed by Fourier-transformed infrared spectroscopy. The biofilm test was performed in the three most common bacteria found in both urine and stone matrices samples (*Escherichia coli*, *Enterococcus faecalis*, and *Klebsiella pneumoniae*) by light microscopy and confirmed by scanning electron microscopy.

Results: From 100 stone formers recruited, 36 cases had a total of 45 bacterial isolates cultivated from their catheterized urine and/or stone matrices. Among these 36 cases, chemical analysis revealed that 8 had the previously classified "infection-induced stones", whereas the other 28 cases had the previously classified "metabolic stones". Calcium oxalate (in either pure or mixed form) was the most common and found in 64 and 75% of the stone formers with and without bacterial isolates, respectively. *Escherichia coli* was the most common bacterium (approximately 1/3 of all bacterial isolates) found in urine and stone matrices (both nidus and periphery). Linear regression analysis showed significant correlation (r=0.860; p<0.001) between bacterial types in urine and stone matrices. Multidrug resistance was frequently found in these isolated bacteria. Moreover, urea test revealed that only 31% were urea-splitting bacteria, whereas the majority (69%) had negative urea test. Moreover, the biofilm-producing bateria in the three most common bacteria found in both urine and stone matrices samples were *Escherichia coli*, *Enterococcus faecalis* (*All Enterococcus* spp.strains were identified as *Enterococcus faecalis*), and *Klebsiella* pneumoniae, respectively (Table 8). Among these, 44.44 (4/9) and 42.88% (6/14) of the *Escherichia coli* strains were positive for biofilm production in urine and stone matrices, respectively. In addition, 60 (3/5) and 50% (1/2) of the *Enterococcus faecalis* strains were positive for biofilm production in urine and stone matrices, respectively. Whereas, 66.67% (2/3) of *Klebsiella pneumoniae* was found only in stone matrices.

Conclusions: Our data indicate that the prevalence of UTIs associated with nephrolithiasis is still high (36%) in the northeastern region of Thailand. In addition, UTIs are frequently associated with almost all chemical types of kidney stones, not only struvite, and *E. coli*, not urea-splitting bacteria, is the most common causative microorganism found in urine and stone matrices of the stone formers in Thailand. Based on their locales in the stone nidus, we hypothesized that the microorganisms found in both urine and stone matrices were not the entrapped bacteria from secondary UTIs, but were indeed the causative bacteria involved in the

stone formation and pathogenesis. These bacteria remained in the urine most likely due to multidrug resistance; thus, hardly to be eradicated and easily to be further entrapped in the stone periphery. The ability of biofilm production may be the one of important factors in stone formation with urinary tract infection. However, further extensive investigations of smaller stones and elucidations of the effects of microbes on stone formation and growth are crucial. These data may lead to rethinking and a new roadmap for future research regarding the causative role of microorganisms in kidney stone formation.

เนื้อหางานวิจัย

บทนำ

Kidney stone disease (nephrolithiasis) remains a common public health problem worldwide and is highly prevalent in the northeastern region of Thailand [1-3]. Urinary tract infections (UTIs) are well known to be associated with kidney stone formation[4]. Moreover, antimicrobial resistance has been frequently observed in bacteria isolated from stone formers with UTIs [5]. Although the association between nephrolithiasis and UTIs is generally known and frequently detected, its prevalence, causative microorganisms, and their antimicrobial susceptibility patterns remain under-investigated. Previous studies have reported that the most common type of kidney stones caused by UTIs is struvite stone containing magnesium ammonium phosphate and the most common microbe found in struvite stone is urea-splitting bacterium, i.e. *Proteus mirabilis* [6-8]. Urease and biofilm producing bacteria are also involved in a wide variety of microbial infections in the body, including catheter- and stone-associated urinary tract infections[9, 10]. However, our observation indicated that it might not be the case of stone formers in Thailand. We therefore re-evaluated and extensively characterized microorganisms isolated from urine and stone matrices of patients with all types of kidney stones chemically analyzed by Fourier-transformed infrared (FTIR) spectroscopy, and also examined antimicrobial susceptibility patterns of the isolated bacteria. Moreover, urea, citrate and biofilm tests were performed to classify these bacteria into urea-splitting, non-urea-splitting, citrate-utilizing non-citrate-utilizing, biofilm-producing, or non-biofilm-producing group.

วิธีการทดลอง

Ethics statement

All the experiments involved human subjects and clinical samples were conducted according to the principles expressed in the Declaration of Helsinki, and written informed consent was provided by the study participants. This study was reviewed and approved by the Institutional Ethical Committee at Khon Kaen University.

Subjects and sample collection

A total of 100 kidney stone formers (59 males and 41 females) with idiopathic etiology admitted to Khon Kaen Hospital for elective kidney stone removal by percutaneous nephrolithotomy because of large stone and/or obstruction were consecutively recruited during 2009-2010. All these subjects had no symptoms and signs of UTIs. The exclusion criteria included: (i) Underlying systemic diseases and secondary causes of nephrolithiasis (e.g. primary hyperparathyroidism, vitamin D excess, hyperthyroidism, renal tubular acidosis, etc.); (ii) Active UTIs or other infections within one year prior to admission; (iii) History of antibiotic treatment of UTIs within one year prior to admission; and (iv) No permission from the subjects. In addition, 30 healthy individuals who had no stones (15 males and 15 females) served as the negative controls for urine culture.

Sample preparation, isolation and identification of bacteria

Catheterized urine samples and stones were collected from all subjects. For the catheterized urine, the samples were collected before prophylactic antibiotic treatment routinely used for surgical stone removal. Stone samples were washed several times with deionized water and each stone was then divided into two parts, as symmetrical as possible, by horizontal or sagittal plane. For the first part, stone matrices were taken from “periphery” (cortex) and “nidus” (nucleus) portions by scraping. For the second part, the stone was crushed into powder by sterilized mortar and pestle, and was then used for bacterial culture and chemical analysis of the “whole stone”. The catheterized urine samples and all samples derived from three locales of individual stones (including periphery, nidus and whole stone) were then cultivated in blood and MacConkey agar (Oxoid; Hampshire, UK) at 37°C for 24 and 48 h, respectively. Positive bacterial cultures were considered when there were >1 x 10^3 colony forming units (CFU) per ml urine. Thereafter, all bacterial isolates in urine and stone matrices were identified by standard biochemical tests[11]. Moreover, *Enterococcus* spp. was identified species by PCR method (modified from [12, 13]).

Antimicrobial susceptibility test

All bacterial isolates were tested for antimicrobial susceptibility by the disc diffusion assay on Mueller Hinton agar (Oxoid) and incubated at 37°C for 24 h. The antibiotics used in individual 6-mm discs (Oxoid) included: amikacin (AK, 30 µg); ampicillin (AMP, 10 µg); cephalothin (CF, 30 µg);

sulfamethoxazole/trimethoprim (SXT, 1.25/23.75 µg); gentamicin (GM, 10 µg); norfloxacin (NOR, 10 µg); ofloxacin(OFX, 5 µg); cefotaxime (CTX, 30 µg); ceftazidime (CAZ, 30 µg); netilmicin (NET, 30 µg), oxacillin (OX, 1 µg); cefoxitin (FOX, 30 µg); fosfomycin (FOS, 50 µg); fusidic acid (FA, 10 µg), vancomycin (VA, 30 µg); penicillin (P, 10 U); tetracycline (TE, 30 µg); ciprofloxacin (CIP, 5 µg); and tazocin (TZP, 110 µg). Extended-spectrum β-lactamase (ESBL) producing bacteria (indicating resistance to third-generation cephalosporins) were also determined by double-disc diffusion test, following the standards of Clinical and Laboratory Standards Institute.

Analysis of chemical compositions of stones

The analysis of chemical compositions of each stone was done using stone powder derived from the second part of stone sections (as aforementioned) that was left after bacterial culture. Chemical analysis was performed using Fourier-transformed infrared (FTIR) spectroscopy as described previously [14].

Biofilm test

The biofilm test was performed in the three most common bacteria found in both urine and stone matrices samples; *Escherichia coli*, *Enterococcus faecalis*, and *Klebsiella pneumoniae*, respectively. *Escherichia coli* ATCC 25922 [15, 16], *Enterococcus faecalis* ATCC 29212 [17] and *Klebsiella pneumoniae* ATCC 700603 [18] were used as positive control.

Microscopic analysis of biofilm production by light microscopy

Bacterial strains were cultured in Tryptic soy broth, and incubated at 37°C for 24 h without shaking. After that the cultures were sub-cultured and adjusted to 0.5 McFarland standards in Tryptic soy broth. The biofilm production was performed by using the polystyrene 6-well culture dishes (Nunc®, Denmark) with glass coverslips. Five ml of Tryptic soy broth were added to each well simultaneously with 100 µl of the bacterial overnight culture. The culture dishes were incubated overnight at 37°C without shaking. After 24 h the wells were washed 3 times with phosphate buffer saline. Biofilm production was visualized by first fixing the bacteria with 10% (v/v) formalin for 10 min and then staining with 300 µl of 1% Crystal violet for 5 min, washing with phosphate buffer saline and air-drying. The cover slides were removed from the wells and observed by a light microscopy using a 40X objective lens of a Nikon ECLIPSE 80i microscope(modified from [19]).

Verification of biofilm production by scanning electron microscopy

Bacterial strains were cultured in Tryptic soy broth for 24 h at 37°C without shaking. After that the cultures were sub-cultured and adjusted to 0.5 McFarland standards in Tryptic soy broth. Biofilm production was assessed by sub-culturing 20 µl of an overnight Tryptic soy broth in 800 µl of Tryptic soy broth in each well of 6-well flat-bottom polystyrene microtitre plates (Nunc®, Denmark) containing glass coverslips. They were incubated for 48 h at 37°C. Then the samples were rinsed once with sterile water and fixed in 4%

formaldehyde overnight. The fixed samples were dehydrated with 25%, 50%, 75% and 96% ethanol for 20 minutes at room temperature and finally air-dried. The coverslips were removed from the wells and observed by Scanning electron microscopy using 1000X and 7000X objective lens of LEO SEM 1450VP microscope (modified from [20]).

Statistical analysis

All the quantitative data are reported as mean ± SEM, unless stated otherwise. Comparisons between the two groups of samples were performed by unpaired *t*-test, whereas correlation between two variables was determined by linear regression analysis using SPSS software (version 11.0) (SPSS Corporation; Chicago, IL). *P* values less than 0.05 were considered statistically significant.

ผลการทดลองและบทวิจารณ์

From a total of 100 stone formers, 36 (19 males and 17 females) had bacteria isolated from catheterized urine samples and/or stone matrices, whereas other 64 subjects (40 males and 24 females) had no microorganisms detected. Their demographic and clinical chemistry data are shown in Table 1. Statistical analysis showed no significant differences between stone formers with and without bacterial isolates in their gender, age, urine pH, WBC count, blood urea nitrogen, serum creatinine, K^+, Na^+, Cl^-, and HCO_3^- levels. Their stone sizes were also comparable (1.15 ± 0.07 vs. 1.21± 0.06 in width and 3.56 ± 0.29 vs. 3.12 ± 0.20 in length) (Table 1). Urine culture was also performed in 30 healthy normal individuals who had no stones (15 males and 15 females). The results showed negative bacterial culture in the urine of this negative control group.

From 36 stone formers, a total of 45 bacterial isolates were found. Among these, 6 isolates were detected only in urine samples (of 4 stone formers), 18 were found only in stone matrices (of 12 stone formers) and 21 were observed in both urine and stone matrices (of 20 stone formers) (Table 2). The top-three most common bacteria found in urine samples were *Escherichia coli*, *Enterococcus* spp., and *Klebsiella/Enterobacter* spp., respectively, and those found in stone matrices were *E. coli*, *P. mirabilis*, and *Klebsiella* spp., respectively (Table 3). Overall, *E. coli* was the most common bacterium found in both urine and stone matrices (approximately 1/3 of all bacterial isolates). Other bacteria found included *K. pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus* coagulase negative, *Citrobacter freundii*, *Acinetobacter baumannii*, *A. lwoffii*, *Citrobacter diversus*, *Salmonella* spp., and *Staphylococcus* coagulase positive (Table 3).

The association between nephrolithiasis and UTIs can be either (i) kidney stone formation developed following UTIs (the so-called “infection-induced stones”) or (ii) nephrolithiasis with subsequent UTIs as its complications (the so-called “stones with subsequent infections”) [6, 8]. Whether they are infection-induced stones or stones with subsequent infections, the stones themselves are the important source of secondary infection [4]. Therefore, complete eradication of the associated UTIs is possible after the stones have been removed [8, 21]. It has been thought that types of bacteria in the stone nidus implicate the causative microorganisms involved in stone formation and the pathogenesis of “infection-induced stones”, whereas those found in the stone periphery implicate microorganisms entrapped into the stones during secondary UTIs (“stones with subsequent infections”). The latter should have microorganisms found only in the periphery of stone matrices, not in the stone nidus. Moreover, types of bacteria found in the stone nidus of infection-induced stones should be different from those entrapped recently into the periphery of stones with subsequent infections. In our present study, it is very interesting that types of bacteria found in the stone nidus were almost identical to those found in the stone periphery, as well as the whole stone (Table 3). Moreover, linear regression analysis showed significant correlation (r = 0.860; p < 0.001) between types of bacteria found in catheterized urine samples and those of stone matrices (Figure 1). Among 20 stone formers who had bacterial isolates in both urine and stone matrices, 19 had exactly the same organisms found in both samples, whereas only one had different strains of *E. coli* found in urine and stone matrices. These data led to a hypothesis that the microorganisms found in both urine and stone matrices were not the entrapped bacteria from secondary UTIs, but were indeed the causative bacteria involved in the stone formation and pathogenesis. The data also implicate that they remained in these samples most likely due to resistance to antibiotics; thus, hardly to be eradicated and easily to be further entrapped in the stone periphery.

To address this, antimicrobial susceptibility assay was performed. The data revealed that the bacteria isolated from urine and stone matrices had multidrug resistance, and their antimicrobial resistance patterns are shown in Tables 4 and 5, respectively. From a total of 27 bacterial isolates detected in catheterized urine samples, 19 (approximately 70%) had antimicrobial resistance (mostly to multiple drugs) (Table 4). Similarly, from 39 bacterial isolates detected in stone matrices, 24 (approximately 62%) had antimicrobial resistance (mostly to multiple drugs) (Table 5). This is the first dataset demonstrating the high proportion and patterns of antimicrobial resistance in bacteria isolated from urine and stone matrices of the stone formers.

FTIR spectroscopic analysis revealed that of all 100 stone formers, 15 showed chemical compositions fitted into the previously classified or so-called “infection-induced stones”, whereas the majority (85 cases) had chemical compositions fitted into those previously classified as “metabolic stones” (Table 6). This proportion remained when compared these two stone groups among the 36 stone formers with positive bacterial isolates. Only 8 had chemical compositions fitted into the so-called “infection-induced stones” and only 5 were struvite (magnesium ammonium phosphate). Among all chemical compositions, calcium oxalate (in either pure or mixed form) was the most common and found in 64 and 75% of the stone formers with and without bacterial isolates, respectively. Comparing stone formers with versus without bacterial isolates, the three most common chemical compositions found in those with bacterial isolates included calcium oxalate mixed with phosphate, magnesium ammonium phosphate (struvite) and uric acid, whereas the three most commons for those without bacterial isolates were calcium oxalate mixed with phosphate, pure calcium oxalate and uric acid (Table 6).

In addition, urea test on the bacteria isolated from 36 stone formers revealed that the majority (25 of 36; approximately 69%) of microorganisms were non-urea-splitting bacteria, whereas only 31% (11 of 36) were urea-splitting (urease-producing) bacteria (Table 7). For citrate test, the majority (22 of 36; approximately 61%) of microorganisms were non-citrate-utilizing bacteria, whereas only 39% (14 of 36) were citrate-utilizing bacteria (Table 7).

Our present study indicates that the prevalence of UTIs associated with nephrolithiasis is still high (36%) in the northeastern region of Thailand (Table 1). This prevalence is even higher than that of a recent study done in Baghdad demonstrating that 24.4% of cases had bacterial isolates [5]. There were some contradictory results comparing our present study to the previous Baghdad study[5]. We found that the majority of stone formers who had positive bacterial isolates were males (Table 1), whereas they reported that females had a higher chance to have positive bacterial isolates[5]. Moreover, we also found that urea-splitting bacteria were not the major microorganisms found in our case series (Table 7), whereas they reported 74% of the isolated microorganisms were urea-splitting bacteria[5]. These contradictory results might be due to geographical differences and, more importantly, different pathogenic mechanisms of nephrolithiasis and its association with UTIs.

In the present study, among the 45 bacterial isolates found in 36 stone formers, the most common was *Escherichia coli* found in approximately 1/3 of all bacterial isolates. *P. mirabilis* was found only in 7.41 and 12.82% of bacteria isolated from urine samples and stone matrices, respectively (Table 3). This data was contradictory to that of the case series in Baghdad[5]. However, the data in our present study was consistent to that previously reported on the case series in southern part of Thailand, in which the two most common agents found in staghorn calculi were *Corynebacterium* spp. and *Escherichia coli*, whereas urease-producing bacteria were found only in 25%[22]. Interestingly, among urease-producing bacteria found in the latter study, only *Klebsiella* spp. and *Pseudomonas* spp. were isolated, whereas *P. mirabilis* was not detected[22].

In addition, hypocitraturia is a major metabolic abnormality found in stone formers in the northeastern part of Thailand[23, 24]. The information on citrate-utilizing bacteria in urine and stone matrices may be another important factor for kidney stone formation in this area. However, the majority of bacterial isolates found in our present study were non-citrate-utilizing bacteria (Table 7). These conflicting results on the citrate assay might implicate that hypocitraturia found in this population is likely due to metabolic causes, not due to citrate-utilizing bacteria.

Moreover, the biofilm-producing bateria in the three most common bacteria found in both urine and stone matrices samples were *Escherichia coli*, *Enterococcus faecalis* (*All Enterococcus* spp.strains were identified as *Enterococcus faecalis*), and *Klebsiella* pneumoniae (Table 8). Among these, 44.44 (4/9) and 42.88% (6/14) of the *Escherichia coli* strains were positive for biofilm production in urine and stone matrices, respectively. In addition, 60 (3/5) and 50% (1/2) of the *Enterococcus faecalis* strains were positive for biofilm production in urine and stone matrices, respectively. While 66.67% (2/3) of *Klebsiella pneumoniae* was found only in stone matrices. Some representative figures of both biofilm-producing bacteria and non biofilm-producing bacteria groups are shown in Figure 2-7. Moreover, we also found that biofilm-producing bacteria were the major microorganisms found in **metabolic stones** (Table 9), especially, in the *mix component stone* group. The difference of biofilm production may associate with the different stone types and their role in persistence of infection, multidrug resistance and survival in stones [20, 25].

Nevertheless, limitations of our present study should be noted. First, sizes of all stones obtained in our present study were relatively large (Table 1) (as the patients were recruited from those who were admitted to the hospital for elective kidney stone removal by percutaneous nephrolithotomy). Bacteria found in urine and stone matrices might promote formation and growth of these relatively large stones. On the other hand, these large stones are more susceptible for bacterial colonization. Therefore, extensive investigations of smaller stones are required to address this concern. Second, although all the subjects recruited into this study had no active UTIs or antibiotic treatment of UTIs within 12 months prior to the admission, their precise information and details of infections (particularly UTIs) and antibiotic treatments in the past (>12 months before the admission) are not available. Thus, it is inconclusive in our present study that when and how antibiotic resistance occurred.

Table 1: Demographic and clinical chemistry data of all stone formers with and without bacterial isolates.

Parameters	**Stone formers (total n=100)**		***P value***
	With bacterial isolates (n=36)	**Without bacterial isolates (n=64)**	
Gender (Male:Female)	19:17	40:24	NS
Age (years)	50.69 ± 2.20	54.14 ± 1.61	NS
Urine pH	6.22 ± 0.12	6.01 ± 0.10	NS
Blood tests			
WBC ($10^3/mm^3$)	7.96 ± 0.50	7.63 ± 0.31	NS
BUN (mg/dl)	16.56 ± 1.92	15.96 ± 2.10	NS
Creatinine (mg/dl)	1.38 ± 0.12	1.20 ± 0.07	NS
K (mEq/l)	3.98 ± 0.07	3.94 ± 0.06	NS
Na (mEq/l)	138.85 ± 0.93	139.98 ± 0.43	NS
Cl^- (mEq/l)	106.34 ± 1.33	106.81 ± 1.23	NS
HCO^{3-} (mEq/l)	27.10 ± 0.61	26.20 ± 0.37	NS
Stone size			
Width	1.15 ± 0.07	1.21 ± 0.06	NS
Length	3.56 ± 0.29	3.12 ± 0.20	NS

NS = Not significant

Table 2: Source and number of bacterial isolates detected in 36 stone formers.

Source / **Number**	**Detected only in urine**	**Detected only in stone matrix**	**Detected in both urine and stone matrix**	**Total number**
Number of cases with bacterial isolates	4	12	20	36
Number of bacterial isolates	6	18	21	45

Table 3: Types of bacteria isolated from urine and stone matrices.

Bacteria	**Number of bacterial isolates in urine (%)**	**Number of bacterial isolates in stone matrices**		
		Periphery	**Nidus**	**Whole stone (%)**
Escherichia coli	**9 (33.33)**	13	13	**14 (35.90)**
Enterococcus spp.	5 (18.52)	2	2	2 (5.13)
Klebsiella spp.	3 (11.11)	2	4	4 (10.26)
Enterobacter spp.	3 (11.11)	1	2	2 (5.13)
Proteus mirabilis	2 (7.41)	5	5	5 (12.82)
Klebsiella pneumoniae	1 (3.70)	2	1	3 (7.69)
Pseudomonas aeruginosa	1 (3.70)	2	2	2 (5.13)
Staphylococcus coagulase negative	1 (3.70)	1	1	1 (2.56)
Citrobacter freundii	1 (3.70)	1	1	1 (2.56)
Acinetobacter baumannii	1 (3.70)	1	1	1 (2.56)
Acinetobacter lwoffii	0 (0.00)	1	1	1 (2.56)
Citrobacter diversus	0 (0.00)	1	1	1 (2.56)
Salmonella spp.	0 (0.00)	1	1	1 (2.56)
Staphylococcus coagulase positive	0 (0.00)	0	1	1 (2.56)
Total	**27 (100)**	**33**	**36**	**39 (100)**

Table 4: Antimicrobial resistance of 27 bacterial isolates obtained from urine.

Bacteria	**Antimicrobial resistance**										
Enterobacteriaceae	**Number**	**AK (%)**	**AMP (%)**	**CF (%)**	**SXT (%)**	**GM (%)**	**NOR (%)**	**OFX (%)**	**CTX (%)**	**CAZ (%)**	**ESBL producer (%)**
Escherichia coli	9	0	67	44	56	22	33	44	22	22	22
Klebsiella spp.	3	33	100	100	67	100	100	100	67	67	0
Enterobacter spp.	3	67	100	100	67	67	67	67	67	67	0
Proteus mirabilis	2	0	0	0	0	0	0	0	0	0	0
Klebsiella pneumoniae	1	0	100	100	0	0	100	100	100	100	100
Citrobacter freundii	1	0	100	100	0	0	0	0	0	0	0
Non fermentative Gram-Negative Bacilli	**Number**	**AK (%)**	**SXT (%)**	**GM (%)**	**OFX (%)**	**CTX (%)**	**CAZ (%)**	**NET (%)**	**TZP (%)**		
Pseudomonas aeruginosa	1	100	0	100	100	100	0	100	100		
Acinetobacter baumannii	1	ND	ND	ND	ND	ND	ND	ND	ND		
Enterococcus	**Number**	**AMP (%)**	**GM (%)**	**VA (%)**	**P (%)**	**TE (%)**	**CIP (%)**				
Enterococcus spp.	5	0	20	0	0	80	40				
Staphylococcus	**Number**	**CF (%)**	**SXT (%)**	**GM (%)**	**OFX (%)**	**OX (%)**	**FOX (%)**	**FOS (%)**	**FA (%)**	**VA (%)**	
Staphylococcus coagulase negative	1	0	0	0	0	0	0	0	0	0	
Total bacterial isolates in urine	**27**										

ND = Not determined and Number = Number of bacterial isolates

Table 5: Antimicrobial resistance of 39 bacterial isolates obtained from stone matrices.

Bacteria	**Antimicrobial resistance**										
Enterobacteriaceae	**Number**	**AK (%)**	**AMP (%)**	**CF (%)**	**SXT (%)**	**GM (%)**	**NOR (%)**	**OFX (%)**	**CTX (%)**	**CAZ (%)**	**ESBL producer (%)**
Escherichia coli	14	0	57	36	57	14	21	29	14	14	14
Proteus mirabilis	5	0	20	0	20	0	0	0	0	0	0
Klebsiella spp.	4	0	100	75	50	75	75	75	50	50	0
Klebsiella pneumoniae	3	0	100	67	0	0	33	33	33	33	33
Enterobacter spp.	2	0	100	100	0	0	0	0	50	50	0
Citrobacter freundii	1	0	100	100	0	0	0	0	0	0	0
Citrobacter diversus	1	0	0	100	0	0	0	0	0	0	0
Salmonella spp.	1	0	0	0	0	0	0	0	0	0	0
Non fermentative Gram-Negative Bacilli	**Number**	**AK (%)**	**SXT (%)**	**GM (%)**	**OFX (%)**	**CTX (%)**	**CAZ (%)**	**NET (%)**	**TZP (%)**		
Pseudomonas aeruginosa	2	50	50	50	100	50	0	50	50		
Acinetobacter baumannii	1	ND	ND	ND	ND	ND	ND	ND	ND		
Acinetobacter lwoffii	1	0	0	100	100	100	0	100	0		
Enterococcus	**Number**	**AMP (%)**	**GM (%)**	**VA (%)**	**P (%)**	**TE (%)**	**CIP (%)**				
Enterococcus spp.	2	0	0	0	0	50	0				
Staphylococcus	**Number**	**CF (%)**	**SXT (%)**	**GM (%)**	**OFX (%)**	**OX (%)**	**FOX (%)**	**FOS (%)**	**FA (%)**	**VA (%)**	
Staphylococcus coagulase negative	1	0	0	0	0	0	0	0	0	0	
Staphylococcus coagulase positive	1	0	0	0	0	0	0	0	0	0	
Total bacterial isolates in stone	**39**										

ND = Not determined and Number = Number of bacterial isolates

Table 6: Chemical compositions of all kidney stones (n=100).

Chemical composition of stone	**Stone formers with bacterial isolates (from a total of 36)**		**Stone formers without bacterial isolates (from a total of 64)**		**Total number (from a total of 100)**		**Stone size (width x length) (cm)**
	N	**%**	**N**	**%**	**N**	**%**	
Previously classified as "infection-induced stones"	***8***	***22.22***	***7***	***10.94***	***15***	***15***	***1.55±0.18 x 4.17±0.39***
Magnesium ammonium phosphate (Struvite)	5	*13.89*	4	*6.25*	9	*9*	1.24±0.13 x 4.51±0.48
Calcium phosphate/carbapatite	3	*8.33*	2	*3.13*	5	*5*	1.90±0.40 x 3.80±0.80
Calcium phosphate/oxalate/carbapatite	0	*0*	1	*1.56*	1	*1*	2.50 x 3.00
Previously classified as "metabolic stones"	***28***	***77.78***	***57***	***89.06***	***85***	***85***	***1.12±0.04 x 3.12±0.17***
Calcium oxalate/phosphate	18	*50.00*	33	*51.56*	51	*51*	1.10±0.06 x 2.88±0.23
Calcium oxalate	2	*5.56*	13	*20.32*	15	*15*	1.12±0.09 x 2.93±0.43
Uric acid	5	*13.89*	9	*14.06*	14	*14*	1.27±0.14 x 4.00±0.40
Calcium oxalate/uric acid	3	*8.33*	1	*1.56*	4	*4*	1.05±0.05 x 3.90±0.49
Calcium phosphate	0	*0*	1	*1.56*	1	*1*	0.70 x 2.80

Table 7: Urea and citrate tests in 36 stone formers with bacterial isolates.

Chemical composition of stone	Stone formers with bacterial isolates (total n=36)			
	Urea test		Citrate test	
	Number of urea-splitting bacteria (n=11)	Number of non-urea-splitting bacteria (n=25)	Number of citrate-utilizing bacteria (n=14)	Number of non-citrate-utilizing bacteria (n=22)
Previously classified as "infection-induced stones" (n=8)	***5***	***3***	***3***	***5***
Magnesium ammonium phosphate (Struvite) (n=5)	4	1	1	4
Calcium phosphate/carbapatite (n=3)	1	2	2	1
Calcium phosphate/oxalate/carbapatite (n=0)	0	0	0	0
Previously classified as "metabolic stones" (n=28)	***6***	***22***	***11***	***17***
Calcium oxalate/phosphate (n=18)	4	14	7	11
Calcium oxalate (n=2)	0	2	0	2
Uric acid (n=5)	1	4	3	2
Calcium oxalate/uric acid (n=3)	1	2	1	2
Calcium phosphate (n=0)	0	0	0	0

Table 8: Biofilm test in the top-three most common bacteria found in both urine and stone matrices samples.

Bacteria	**Number of bacterial isolates in urine (%)**	**Number of bacterial isolates in stone matrices (%)**
Escherichia coli	4/9 (44.44)	6/14 (42.86)
Enterococcus faecalis	3/5 (60)	1/2 (50)
Klebsiella pneumoniae	0/1 (0)	2/3 (66.67)

Table 9: Biofilm test in stone matrices with *Escherichia coli*, *Enterococcus faecalis* and *Klebsiella* pneumoniae isolates.

Biochemical composition of stone	**Bacterial strains**					
	Escherichia coli **(n= 14)**		***Enterococcus faecalis*** **(n=2)**		***Klebsiella*** **pneumoniae (n=3)**	
	Number of BPB (n=6)	**Number of non-BPB (n=8)**	**Number of BPB (n=1)**	**Number of non-BPB (n=1)**	**Number of BPB (n=2)**	**Number of non-BPB (n=1)**
Infection-induced stones						
Magnesium ammonium phosphate (Struvite)		1				
Calcium phosphate/carbapatite	1					
Metabolic stones						
Calcium oxalate/phosphate	4	2	1	1	1	1
Calcium oxalate		2				
Uric acid		3				
Calcium oxalate/uric acid	1				1	

BPB = Biofilm-producing bacteria and Number = Number of bacterial isolates

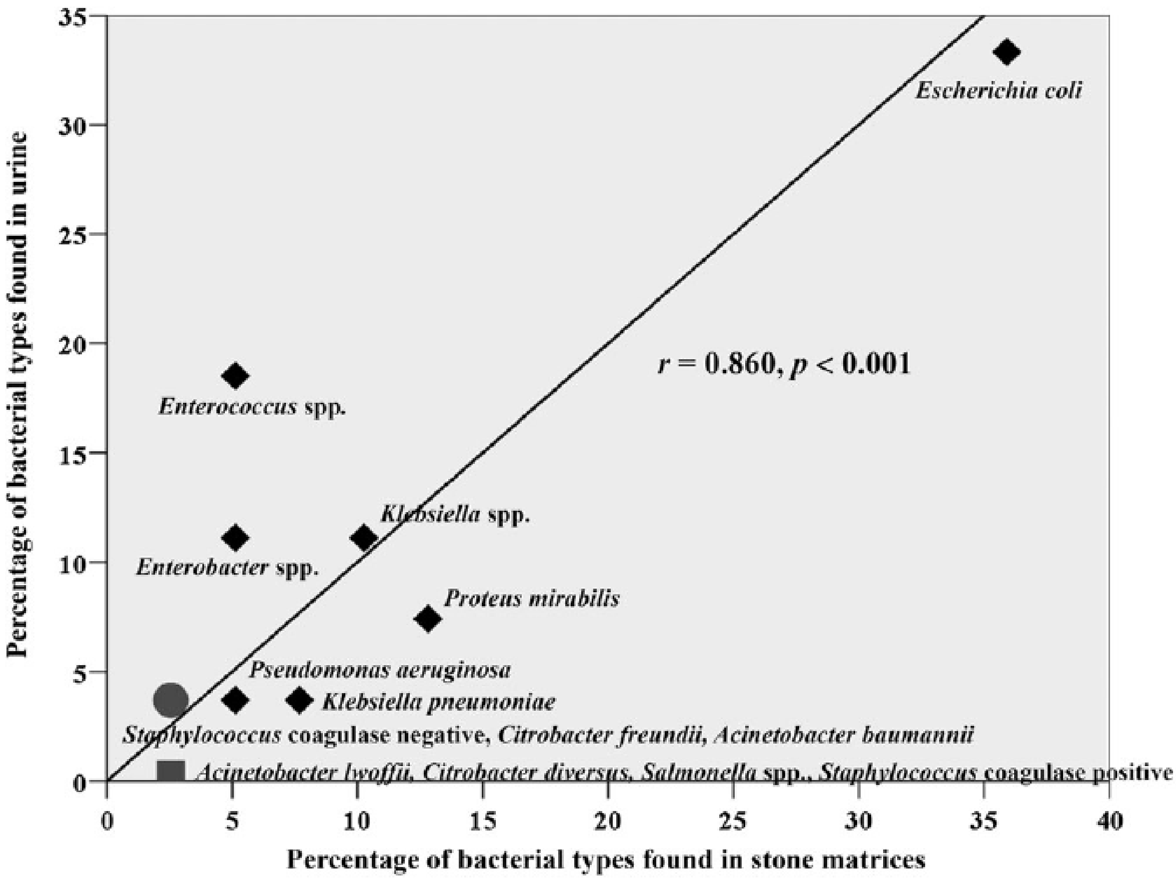

Figure 1. Linear regression analysis of types of bacteria found in urine and stone matrices.

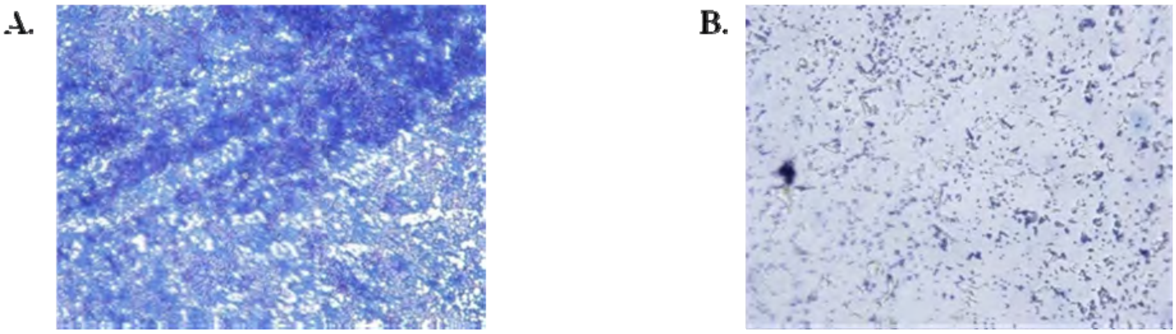

Figure 2. Two representative figures showing differentially expressed biofilm productions between biofilm-producing (A) and non-biofilm-producing *Escherichia coli* (B) by light microscopy (x40).

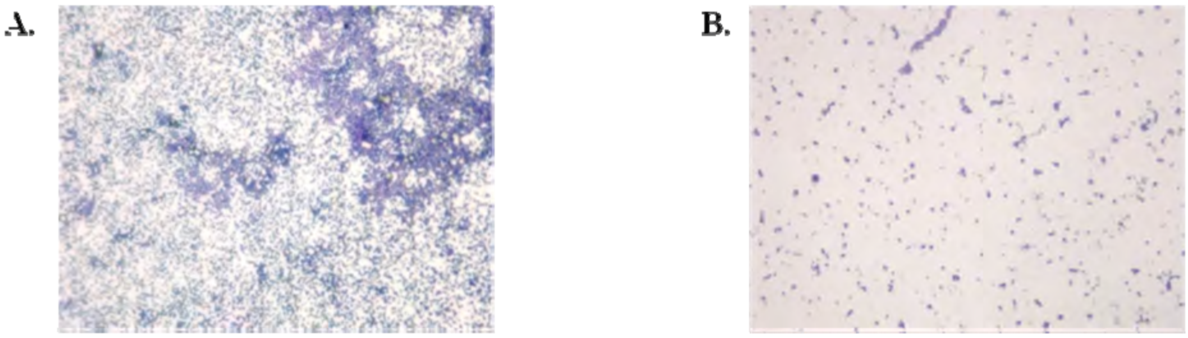

Figure 3. Two representative figures showing differentially expressed biofilm productions between biofilm-producing (A) and non-biofilm-producing *Enterococcus faecalis* (B) by light microscopy(x40).

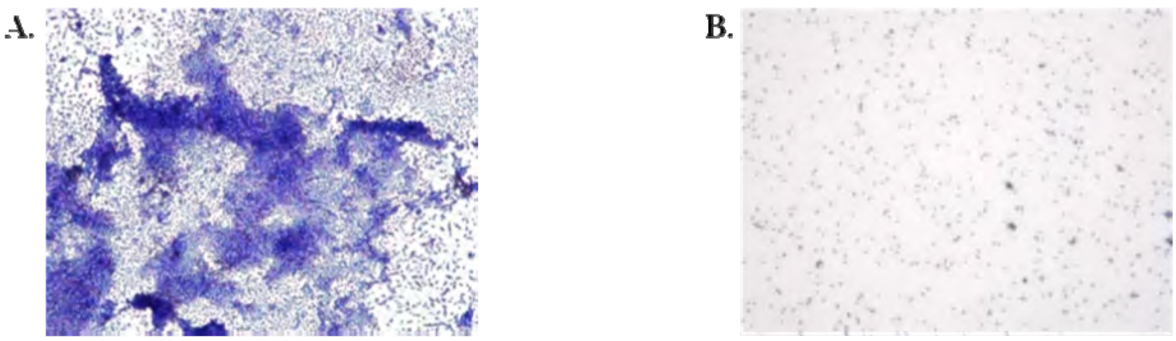

Figure 4. Two representative figures showing differentially expressed biofilm productions between biofilm-producing (A) and non-biofilm-producing *Klebsiella pneumoniae* (B) by light microscopy(x40).

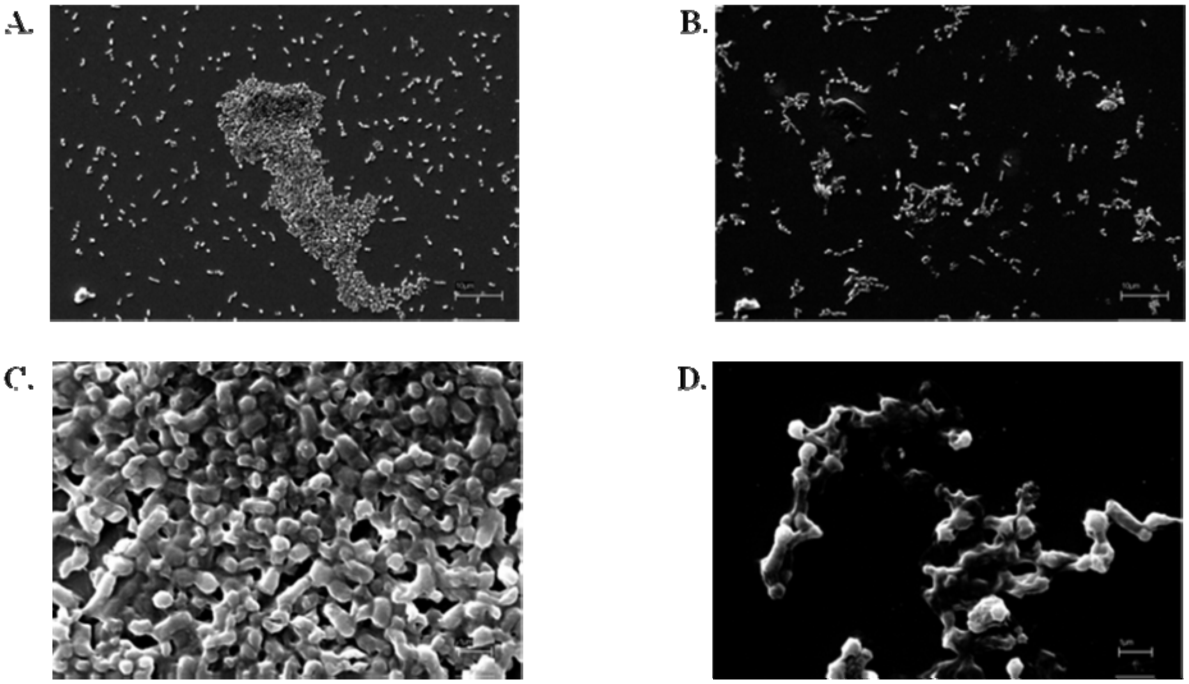

Figure 5. Four representative figures showing differentially expressed biofilm productions between biofilm-producing (A, C) and non-biofilm-producing *Escherichia coli* (B, D) a by scanning electron microscopy (A, B X1000, C, DX7000).

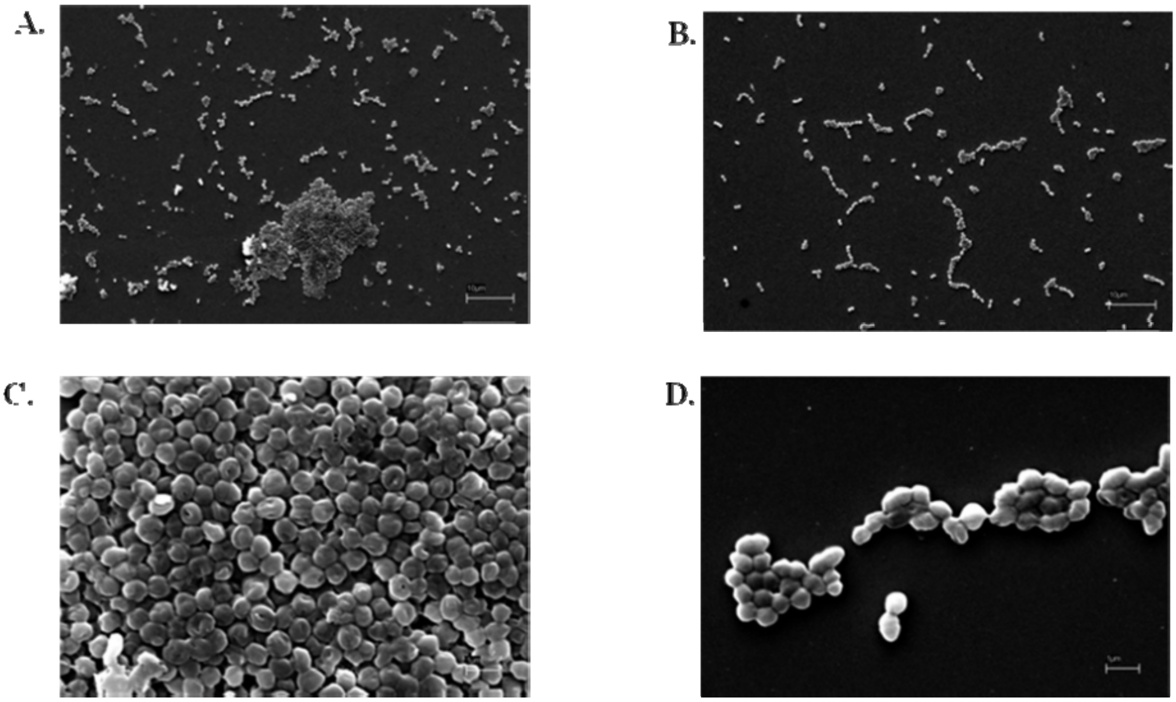

Figure 6. Four representative figures showing differentially expressed biofilm productions between biofilm-producing (A, C) and non-biofilm-producing *Enterococcus faecalis* (B, D) by scanning electron microscopy(A, B X1000, C, DX7000).

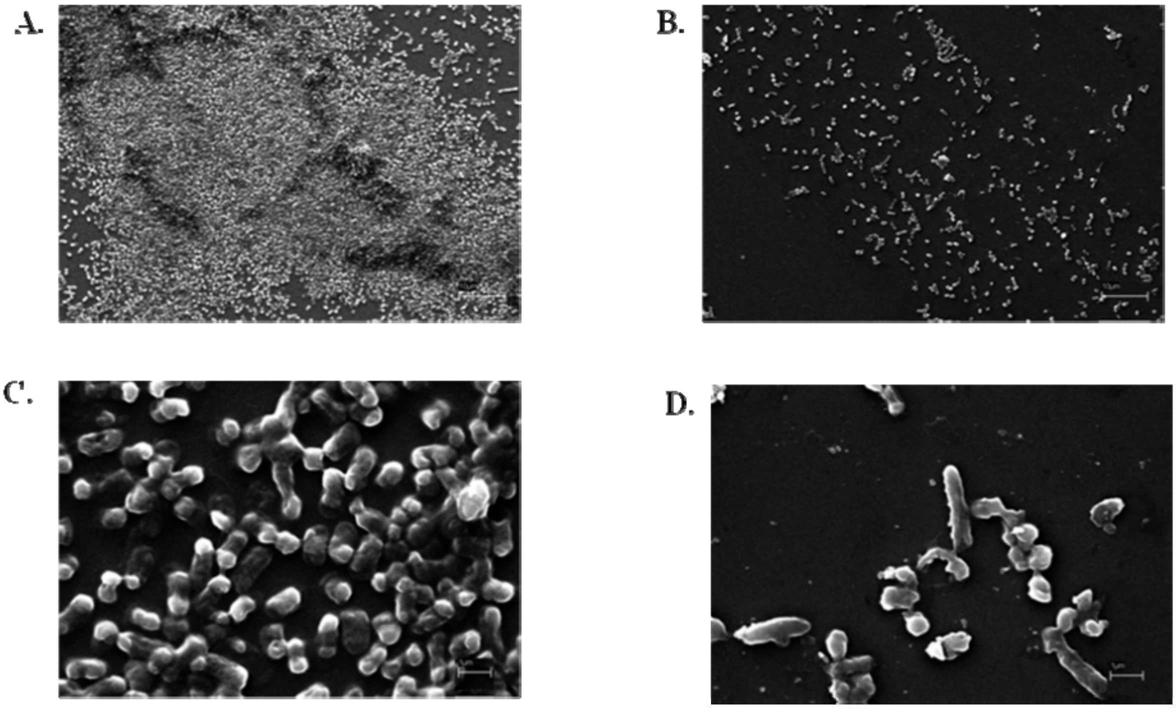

Figure 7. Four representative figures showing differentially expressed biofilm productions between biofilm-producing (A, C) and non-biofilm-producing *Klebsiella pneumoniae* (B, D) by scanning electron microscopy (A, B X1000, C, DX7000).

บทสรุป

Our data indicate that the prevalence of UTIs associated with nephrolithiasis is still high (36%) in the northeastern region of Thailand. In addition, UTIs are frequently associated with almost all chemical types of kidney stones, not only struvite, and *E. coli*, not urea-splitting bacteria, is the most common causative microorganism found in urine and stone matrices of the stone formers in Thailand. Based on their locales in the stone nidus, we hypothesized that the microorganisms found in both urine and stone matrices were not the entrapped bacteria from secondary UTIs, but were indeed the causative bacteria involved in the stone formation and pathogenesis. These bacteria remained in the urine most likely due to multidrug resistance; thus, hardly to be eradicated and easily to be further entrapped in the stone periphery. The ability of biofilm production may be the one of important factors in stone formation with urinary tract infection. However, further extensive investigations of smaller stones and elucidations of the effects of microbes on stone formation and growth are crucial. These data may lead to rethinking and a new roadmap for future research regarding the causative role of microorganisms in kidney stone formation.

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1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

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Original Article

Extensive characterizations of bacteria isolated from catheterized urine and stone matrices in patients with nephrolithiasis

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ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ สำหรับผลงานวิจัยปีที่ 2

อยู่ในขั้นตอนการเตรียมต้นฉบับเพื่อตีพิมพ์

2. การนำผลงานวิจัยไปใช้ประโยชน์

- เชิงวิชาการ (<u>มีการพัฒนาการเรียนการสอน</u>/สร้างนักวิจัยใหม่)

  ใช้ในการเรียนการสอนนักศึกระดับบัณฑิตศึกษา หัวข้อ ความสัมพันธ์ระหว่างโรคนิ่วไตกับการติดเชื้อในระบบทางเดินปัสสาวะ ในรายวิชา 463 794 ปัญหาพิเศษทางจุลชีววิทยาคลินิก หลักสูตรวิทยาศาสตรมหาบัณฑิต สาขาวิทยาศาสตร์การแพทย์ คณะเทคนิคการแพทย์ มหาวิทยาลัยขอนแก่น

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Original Article

Extensive characterizations of bacteria isolated from catheterized urine and stone matrices in patients with nephrolithiasis

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Abstract
Background. Urinary tract infections are generally known to be associated with nephrolithiasis, particularly struvite stone, in which the most common microbe found is urea-splitting bacterium, i.e. *Proteus mirabilis*. However, our observation indicated that it might not be the case of stone formers in Thailand. We therefore extensively characterized microorganisms associated with all types of kidney stones.
Methods. A total of 100 kidney stone formers (59 males and 41 females) admitted for elective percutaneous nephrolithotomy were recruited and microorganisms isolated from catheterized urine and cortex and nidus of their stones were analyzed.
Results. From 100 stone formers recruited, 36 cases had a total of 45 bacterial isolates cultivated from their catheterized urine and/or stone matrices. Among these 36 cases, chemical analysis by Fourier-transformed infrared spectroscopy revealed that 8 had the previously classified 'infection-induced stones', whereas the other 28 cases had the previously classified 'metabolic stones'. Calcium oxalate (in either pure or mixed form) was the most common and found in 64 and 75% of the stone formers with and without bacterial isolates, respectively. *Escherichia coli* was the most common bacterium (approximately one-third of all bacterial isolates) found in urine and stone matrices (both nidus and periphery). Linear regression analysis showed significant correlation ($r = 0.860$, $P < 0.001$) between bacterial types in urine and stone matrices. Multidrug resistance was frequently found in these isolated bacteria. Moreover, urea test revealed that only 31% were urea-splitting bacteria, whereas the majority (69%) had negative urea test.
Conclusions. Our data indicate that microorganisms are associated with almost all chemical types of kidney stones and urea-splitting bacteria are not the major causative microorganisms found in urine and stone matrices of the stone formers in Thailand. These data may lead to rethinking and a new roadmap for future research regarding the role of microorganisms in kidney stone formation.

Keywords: antibiotic susceptibility; prevalence; stone matrix; urine; uropathogenic bacteria

Introduction

Kidney stone disease (nephrolithiasis) remains a common public health problem worldwide and is highly prevalent in the northeastern region of Thailand [1–3]. Urinary tract infections (UTIs) are well known to be associated with kidney stone formation [4]. Moreover, antimicrobial resistance has been frequently observed in bacteria isolated from stone formers with UTIs [5]. Although the association between nephrolithiasis and UTIs is generally known and frequently detected, its prevalence, causative microorganisms and their antimicrobial susceptibility patterns remain under-investigated. Previous studies have reported that the most common type of kidney stones caused by UTIs is struvite stone containing magnesium ammonium phosphate and the most common microbe found in struvite stone is urea-splitting bacterium, i.e. *Proteus mirabilis* [6–8]. However, our observation indicated that it might not be the case of stone formers in Thailand. We therefore re-evaluated and extensively characterized microorganisms isolated from urine and stone matrices of patients with all types of kidney stones, chemically analyzed by Fourier-transformed infrared (FTIR) spectroscopy and also examined antimicrobial susceptibility patterns of the isolated bacteria. Moreover, urea and citrate tests were performed to classify these bacteria into urea-splitting, non-urea-splitting, citrate-utilizing or non-citrate-utilizing group.

Materials and methods

Ethics statement

All the experiments involved human subjects and clinical samples were conducted according to the principles expressed in the Declaration of Helsinki, and written informed consent was provided by the study participants. This study was reviewed and approved by the Institutional Ethical Committee at Khon Kaen University.

Subjects and sample collection

A total of 100 kidney stone formers (59 males and 41 females) with idiopathic etiology admitted to Khon Kaen Hospital for elective kidney stone removal by percutaneous nephrolithotomy because of large stone and/or obstruction were consecutively recruited during 2009–10. All these subjects had no symptoms and signs of UTIs. The exclusion criteria included (i) underlying systemic diseases and secondary causes of nephrolithiasis (e.g. primary hyperparathyroidism, vitamin D excess, hyperthyroidism, renal tubular acidosis, etc.), (ii) active UTIs or other infections within 1 year prior to admission, (iii) history of antibiotic treatment of UTIs within 1 year prior to admission and (iv) no permission from the subjects. In addition, 30 healthy individuals who had no stones (15 males and 15 females) served as the negative controls for urine culture.

Sample preparation, isolation and identification of bacteria

Catheterized urine samples and stones were collected from all subjects. For the catheterized urine, the samples were collected before prophylactic antibiotic treatment routinely used for surgical stone removal. Stone samples were washed several times with deionized water and each stone was then divided into two parts, as symmetrical as possible, by horizontal or sagittal plane. For the first part, stone matrices were taken from 'periphery' (cortex) and 'nidus' (nucleus) portions by scraping. For the second part, the stone was crushed into powder by sterilized mortar and pestle and was then used for bacterial culture and chemical analysis of the 'whole stone'. The catheterized urine samples and all samples derived from three locales of individual stones (including periphery, nidus and whole stone) were then cultivated in blood and MacConkey agar (Oxoid, Hampshire, UK) at 37°C for 24 and 48 h, respectively. Positive bacterial cultures were considered when there were $>1 \times 10^5$ colony forming units/mL urine. Thereafter, all bacterial isolates in urine and stone matrices were identified by standard biochemical tests [9].

Antimicrobial susceptibility test

All bacterial isolates were tested for antimicrobial susceptibility by the disc diffusion assay on Mueller-Hinton agar (Oxoid) and incubated at 37°C for 24 h. The antibiotics used in individual 6-mm discs (Oxoid) included: amikacin (AK, 30 μg); ampicillin (AMP, 10 μg); cephalothin (CF, 30 μg); sulfamethoxazole/trimethoprim (SXT, 1.25/23.75 μg); gentamicin (GM, 10 μg); norfloxacin (NOR, 10 μg); ofloxacin (OFX, 5 μg); cefotaxime (CTX, 30 μg); ceftazidime (CAZ, 30 μg); netilmicin (NET, 30 μg), oxacillin (OX, 1 μg); cefoxitin (FOX, 30 μg); fosfomycin (FOS, 50 μg); fusidic acid (FA, 10 μg), vancomycin (VA, 30 μg); penicillin (P, 10 U); tetracycline (TE, 30 μg); ciprofloxacin (CIP, 5 μg) and tazocin (TZP, 110 μg). Extended-spectrum β-lactamase-producing bacteria (indicating resistance to third-generation cephalosporins) were also determined by double-disc diffusion test, following the standards of Clinical and Laboratory Standards Institute.

Analysis of chemical compositions of stones

The analysis of chemical compositions of each stone was done using stone powder derived from the second part of stone sections (as aforementioned) that was left after bacterial culture. Chemical analysis was performed using FTIR spectroscopy as described previously [10].

Statistical analysis

All the quantitative data are reported as mean ± SEM, unless stated otherwise. Comparisons between the two groups of samples were performed by unpaired *t*-test, whereas correlation between two variables was determined by linear regression analysis using SPSS software (version 11.0; SPSS Corporation, Chicago, IL). P-values <0.05 were considered statistically significant.

Results and discussion

From a total of 100 stone formers, 36 (19 males and 17 females) had bacteria isolated from catheterized urine samples and/or stone matrices, whereas other 64 subjects (40 males and 24 females) had no microorganisms detected. Their demographic and clinical chemistry data are shown in Table 1. Statistical analysis showed no significant differences between stone formers with and without bacterial isolates in their gender, age, urine pH, white blood cell count, blood urea nitrogen, serum creatinine, K^+, Na^+, Cl^- and HCO_3^- levels. Their stone sizes were also comparable (1.15 ± 0.07 versus 1.21± 0.06 in width and 3.56 ± 0.29 versus 3.12 ± 0.20 in length) (Table 1). Urine culture was also performed in 30 healthy normal individuals who had no stones (15 males and 15 females). The results showed negative bacterial culture in the urine of this negative control group.

From 36 stone formers, a total of 45 bacterial isolates were found. Among these, 6 isolates were detected only in urine samples (of 4 stone formers), 18 were found only in stone matrices (of 12 stone formers) and 21 were observed in both urine and stone matrices (of 20 stone formers) (Table 2). The top three most common bacteria found in

Table 1. Demographic and clinical chemistry data of all stone formers with and without bacterial isolates[a]

	Stone formers (total n = 100)		
Parameters	With bacterial isolates (n = 36)	Without bacterial isolates (n = 64)	P-value
Gender (male:female)	19:17	40:24	NS
Age (years)	50.69 ± 2.20	54.14 ± 1.61	NS
Urine pH	6.22 ± 0.12	6.01 ± 0.10	NS
Blood tests			
WBC (10^3/mm^3)	7.96 ± 0.50	7.63 ± 0.31	NS
BUN (mg/dL)	16.56 ± 1.92	15.96 ± 2.10	NS
Creatinine (mg/dL)	1.38 ± 0.12	1.20 ± 0.07	NS
K (mEq/L)	3.98 ± 0.07	3.94 ± 0.06	NS
Na (mEq/L)	138.85 ± 0.93	139.98 ± 0.43	NS
Cl^- (mEq/L)	106.34 ± 1.33	106.81 ± 1.23	NS
HCO_3^- (mEq/L)	27.10 ± 0.61	26.20 ± 0.37	NS
Stone size			
Width	1.15 ± 0.07	1.21 ± 0.06	NS
Length	3.56 ± 0.29	3.12 ± 0.20	NS

[a]NS, not significant.

Table 2. Source and number of bacterial isolates detected in 36 stone formers

Source / Number	Detected only in urine	Detected only in stone matrix	Detected in both urine and stone matrix	Total number
Number of cases with bacterial isolates	4	12	20	36
Number of bacterial isolates	6	18	21	45

urine samples were *Escherichia coli*, *Enterococcus* spp. and *Klebsiella/Enterobacter* spp., respectively, and those found in stone matrices were *E. coli*, *P. mirabilis* and *Klebsiella* spp., respectively (Table 3). Overall, *E. coli* was the most common bacterium found in both urine and stone matrices (approximately one-third of all bacterial isolates). Other bacteria found included *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus* coagulase negative, *Citrobacter freundii*, *Acinetobacter baumannii*, *Acinetobacter lwoffii*, *Citrobacter diversus*, *Salmonella* spp. and *Staphylococcus* coagulase positive (Table 3).

Table 3. Types of bacteria isolated from urine and stone matrices

Bacteria	Number of bacterial isolates in urine (%)	Number of bacterial isolates in stone matrices		
		Periphery	Nidus	Whole stone (%)
E. coli	9 (33.33)	13	13	14 (35.90)
Enterococcus spp.	5 (18.52)	2	2	2 (5.13)
Klebsiella spp.	3 (11.11)	2	4	4 (10.26)
Enterobacter spp.	3 (11.11)	1	2	2 (5.13)
Proteus mirabilis	2 (7.41)	5	5	5 (12.82)
Klebsiella pneumoniae	1 (3.70)	2	1	3 (7.69)
Pseudomonas aeruginosa	1 (3.70)	2	2	2 (5.13)
Staphylococcus coagulase negative	1 (3.70)	1	1	1 (2.56)
Citrobacter freundii	1 (3.70)	1	1	1 (2.56)
Acinetobacter baumannii	1 (3.70)	1	1	1 (2.56)
Acinetobacter lwoffii	0 (0.00)	1	1	1 (2.56)
Citrobacter diversus	0 (0.00)	1	1	1 (2.56)
Salmonella spp.	0 (0.00)	1	1	1 (2.56)
Staphylococcus coagulase positive	0 (0.00)	0	1	1 (2.56)
Total	27 (100)	33	36	39 (100)

The association between nephrolithiasis and UTIs can be either (i) kidney stone formation developed following UTIs (the so-called 'infection-induced stones') or (ii) nephrolithiasis with subsequent UTIs as its complications (the so-called 'stones with subsequent infections') [6, 7]. Whether they are infection-induced stones or stones with subsequent infections, the stones themselves are the important source of secondary infection [4]. Therefore, complete eradication of the associated UTIs is possible after the stones have been removed [6, 11]. It has been thought that types of bacteria in the stone nidus implicate the causative microorganisms involved in stone formation and the pathogenesis of 'infection-induced stones', whereas those found in the stone periphery implicate microorganisms entrapped into the stones during secondary UTIs (stones with subsequent infections). The latter should have microorganisms found only in the periphery of stone matrices, not in the stone nidus. Moreover, types of bacteria found in the stone nidus of infection-induced stones should be different from those entrapped recently into the periphery of stones with subsequent infections. In our present study, it is very interesting that types of bacteria found in the stone nidus were almost identical to those found in the stone periphery, as well as the whole stone (Table 3). Moreover, linear regression analysis showed significant correlation ($r = 0.860$, $P < 0.001$) between types of bacteria found in catheterized urine samples and those of stone matrices (Figure 1). Among 20 stone formers who had bacterial isolates in both urine and stone matrices, 19 had exactly the same organisms found in both samples, whereas 1 had different strains of *E. coli* found in urine and stone matrices. These data led to a hypothesis that the microorganisms found in both urine and stone matrices were not the entrapped bacteria from secondary UTIs but were indeed the causative bacteria involved in the stone formation and pathogenesis. The data also implicate that

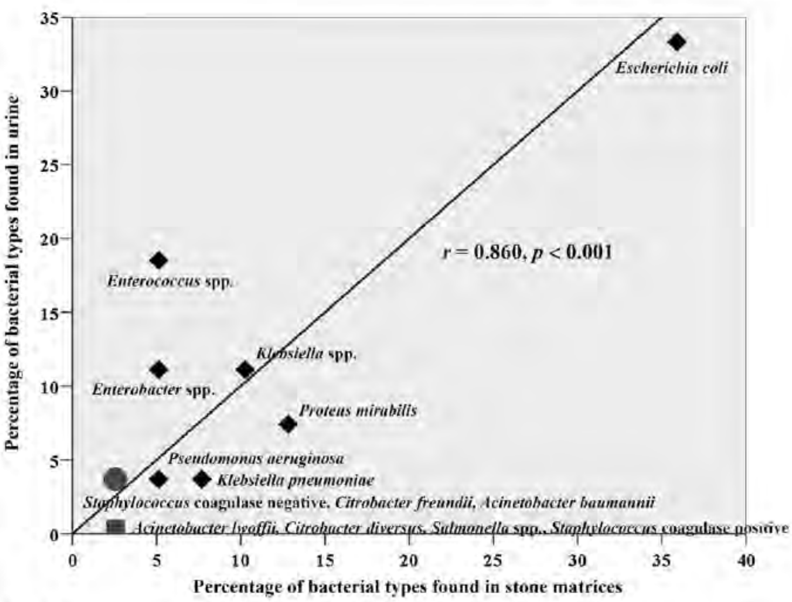

Fig. 1. Linear regression analysis of types of bacteria found in urine and stone matrices.

they remained in these samples most likely due to resistance to antibiotics, thus hardly to be eradicated and easily to be further entrapped in the stone periphery.

To address this, antimicrobial susceptibility assay was performed. The data revealed that the bacteria isolated from urine and stone matrices had multidrug resistance, and their antimicrobial resistance patterns are shown in Tables 4 and 5, respectively. From a total of 27 bacterial isolates detected in catheterized urine samples, 19 (~70%) had antimicrobial resistance (mostly to multiple drugs) (Table 4). Similarly, from 39 bacterial isolates detected in stone matrices, 24 (~62%) had antimicrobial resistance (mostly to multiple drugs) (Table 5). This is the first dataset demonstrating the high proportion and patterns of antimicrobial resistance in bacteria isolated from urine and stone matrices of the stone formers.

FTIR spectroscopic analysis revealed that of all 100 stone formers, 15 showed chemical compositions fitted into

Table 4. Antimicrobial resistance of 27 bacterial isolates obtained from urine[a]

Bacteria	Antimicrobial resistance										
Enterobacteriaceae	Number	AK (%)	AMP (%)	CF (%)	SXT (%)	GM (%)	NOR (%)	OFX (%)	CTX (%)	CAZ (%)	ESBL producer (%)
E. coli	9	0	67	44	56	22	33	44	22	22	22
Klebsiella spp.	3	33	100	100	67	100	100	100	67	67	0
Enterobacter spp.	3	67	100	100	67	67	67	67	67	67	0
Proteus mirabilis	2	0	0	0	0	0	0	0	0	0	0
Klebsiella pneumoniae	1	0	100	100	0	0	100	100	100	100	100
Citrobacter freundii	1	0	100	100	0	0	0	0	0	0	0
Non-fermentative Gram-negative bacilli	Number	AK (%)	SXT (%)	GM (%)	OFX (%)	CTX (%)	CAZ (%)	NET (%)	TZP (%)		
Pseudomonas aeruginosa	1	100	0	100	100	100	0	100	100		
Acinetobacter baumannii	1	ND	ND	ND	ND	ND	ND	ND	ND		
Enterococcus	Number	AMP (%)	GM (%)	VA (%)	P (%)	TE (%)	CIP (%)				
Enterococcus spp.	5	0	20	0	0	80	40				
Staphylococcus	Number	CF (%)	SXT (%)	GM (%)	OFX (%)	OX (%)	FOX (%)	FOS (%)	FA (%)	VA (%)	
Staphylococcus coagulase negative	1	0	0	0	0	0	0	0	0	0	
Total bacterial isolates in urine	27										

[a]ND, not determined; number, number of bacterial isolates.

Table 5. Antimicrobial resistance of 39 bacterial isolates obtained from stone matrices[a]

Bacteria	Antimicrobial resistance										
Enterobacteriaceae	Number	AK (%)	AMP (%)	CF (%)	SXT (%)	GM (%)	NOR (%)	OFX (%)	CTX (%)	CAZ (%)	ESBL producer (%)
E. coli	14	0	57	36	57	14	21	29	14	14	14
Proteus mirabilis	5	0	20	0	20	0	0	0	0	0	0
Klebsiella spp.	4	0	100	75	50	75	75	75	50	50	0
Klebsiella pneumoniae	3	0	100	67	0	0	33	33	33	33	33
Enterobacter spp.	2	0	100	100	0	0	0	0	50	50	0
Citrobacter freundii	1	0	100	100	0	0	0	0	0	0	0
Citrobacter diversus	1	0	0	100	0	0	0	0	0	0	0
Salmonella spp.	1	0	0	0	0	0	0	0	0	0	0
Non-fermentative Gram-negative bacilli	Number	AK (%)	SXT (%)	GM (%)	OFX (%)	CTX (%)	CAZ (%)	NET (%)	TZP (%)		
Pseudomonas aeruginosa	2	50	50	50	100	50	0	50	50		
Acinetobacter baumannii	1	ND	ND	ND	ND	ND	ND	ND	ND		
Acinetobacter lwoffii	1	0	0	100	100	100	0	100	0		
Enterococcus	Number	AMP (%)	GM (%)	VA (%)	P (%)	TE (%)	CIP (%)				
Enterococcus spp.	2	0	0	0	0	50	0				
Staphylococcus	Number	CF (%)	SXT (%)	GM (%)	OFX (%)	OX (%)	FOX (%)	FOS (%)	FA (%)	VA (%)	
Staphylococcus coagulase negative	1	0	0	0	0	0	0	0	0	0	
Staphylococcus coagulase positive	1	0	0	0	0	0	0	0	0	0	
Total bacterial isolates in stone	39										

[a]ND, not determined; number, number of bacterial isolates.

the previously classified or so-called 'infection-induced stones', whereas the majority (85 cases) had chemical compositions fitted into those previously classified as 'metabolic stones' (Table 6). This proportion remained when compared these two stone groups among the 36 stone formers with positive bacterial isolates. Only eight had chemical compositions fitted into the so-called 'infection-induced stones' and only five were struvite (magnesium ammonium phosphate). Among all chemical compositions, calcium oxalate (in either pure or mixed form) was the most common and found in 64 and 75% of the stone formers with and without bacterial isolates, respectively. Comparing stone formers with versus without bacterial isolates, the three most common chemical compositions found in those with bacterial isolates included calcium oxalate mixed with phosphate, magnesium ammonium phosphate (struvite) and uric acid, whereas the three most common chemical compositions for those without bacterial isolates were calcium oxalate mixed with phosphate, pure calcium oxalate and uric acid (Table 6).

In addition, urea test on the bacteria isolated from 36 stone formers revealed that the majority (25 of 36, ~69%) of microorganisms were non-urea-splitting bacteria, whereas only 31% (11 of 36) were urea-splitting (urease-producing) bacteria (Table 7). For citrate test, the majority (22 of 36, ~61%) of microorganisms were non-citrate-utilizing bacteria, whereas only 39% (14 of 36) were citrate-utilizing bacteria (Table 7).

Our present study indicates that the prevalence of UTIs associated with nephrolithiasis is still high (36%) in the northeastern region of Thailand (Table 1). This prevalence is even higher than that of a recent study done in Baghdad demonstrating that 24.4% of cases had bacterial isolates [5]. There were some contradictory results comparing our present study to the previous Baghdad study [5]. We found that the majority of stone formers who had positive bacterial isolates were males (Table 1), whereas they reported that females had a higher chance to have positive bacterial isolates [5]. Moreover, we also found that urea-splitting bacteria were not the major microorganisms found in our case series (Table 7), whereas they reported 74% of the isolated microorganisms were urea-splitting bacteria [5]. These contradictory results might be due to geographical differences and, more importantly, different pathogenic mechanisms of nephrolithiasis and its association with UTIs.

Table 6. Chemical compositions of all kidney stones ($n = 100$)

Chemical composition of stone	Stone formers with bacterial isolates (from a total of 36)		Stone formers without bacterial isolates (from a total of 64)		Total number (from a total of 100)		Stone size (width × length) (cm)
	N	%	*N*	%	*N*	%	
Previously classified as 'infection-induced stones'	8	22.22	7	10.94	15	15	1.55 ± 0.18 × 4.17 ± 0.39
Magnesium ammonium phosphate (Struvite)	5	13.89	4	6.25	9	9	1.24 ± 0.13 × 4.51 ± 0.48
Calcium phosphate/carbapatite	3	8.33	2	3.13	5	5	1.90 ± 0.40 × 3.80 ± 0.80
Calcium phosphate/oxalate/carbapatite	0	0	1	1.56	1	1	2.50 × 3.00
Previously classified as 'metabolic stones'	28	77.78	57	89.06	85	85	1.12 ± 0.04 × 3.12 ± 0.17
Calcium oxalate/phosphate	18	50.00	33	51.56	51	51	1.10 ± 0.06 × 2.88 ± 0.23
Calcium oxalate	2	5.56	13	20.32	15	15	1.12 ± 0.09 × 2.93 ± 0.43
Uric acid	5	13.89	9	14.06	14	14	1.27 ± 0.14 × 4.00 ± 0.40
Calcium oxalate/uric acid	3	8.33	1	1.56	4	4	1.05 ± 0.05 × 3.90 ± 0.49
Calcium phosphate	0	0	1	1.56	1	1	0.70 × 2.80

Table 7. Urea and citrate tests in 36 stone formers with bacterial isolates

Chemical composition of stone	Stone formers with bacterial isolates (total $n = 36$)			
	Urea test		Citrate test	
	Number of urea-splitting bacteria ($n = 11$)	Number of non-urea-splitting bacteria ($n = 25$)	Number of citrate-utilizing bacteria ($n = 14$)	Number of non-citrate-utilizing bacteria ($n = 22$)
Previously classified as 'infection-induced stones' ($n = 8$)	5	3	3	5
Magnesium ammonium phosphate (Struvite) ($n = 5$)	4	1	1	4
Calcium phosphate/carbapatite ($n = 3$)	1	2	2	1
Calcium phosphate/oxalate/carbapatite ($n = 0$)	0	0	0	0
Previously classified as 'metabolic stones' ($n = 28$)	6	22	11	17
Calcium oxalate/phosphate ($n = 18$)	4	14	7	11
Calcium oxalate ($n = 2$)	0	2	0	2
Uric acid ($n = 5$)	1	4	3	2
Calcium oxalate/uric acid ($n = 3$)	1	2	1	2
Calcium phosphate ($n = 0$)	0	0	0	0

In the present study, among the 45 bacterial isolates found in 36 stone formers, the most common was *E. coli* found in approximately one-third of all bacterial isolates. *P. mirabilis* was found only in 7.41 and 12.82% of bacteria isolated from urine samples and stone matrices, respectively (Table 3). This data were contradictory to that of the case series in Baghdad [5]. However, the data in our present study were consistent to that previously reported on the case series in southern part of Thailand, in which the two most common agents found in staghorn calculi were *Corynebacterium* spp. and *E. coli*, whereas urease-producing bacteria were found only in 25% [12]. Interestingly, among urease-producing bacteria found in the latter study, only *Klebsiella* spp. and *Pseudomonas* spp. were isolated, whereas *P. mirabilis* was not detected [12].

In addition, hypocitraturia is a major metabolic abnormality found in stone formers in the northeastern part of Thailand [13, 14]. The information on citrate-utilizing bacteria in urine and stone matrices may be another important factor for kidney stone formation in this area. However, the majority of bacterial isolates found in our present study were non-citrate-utilizing bacteria (Table 7). These conflicting results on the citrate assay might implicate that hypocitraturia found in this population is likely due to metabolic causes, not due to citrate-utilizing bacteria.

Nevertheless, limitations of our present study should be noted. First, sizes of all stones obtained in our present study were relatively large (Table 1) (as the patients were recruited from those who were admitted to the hospital for elective kidney stone removal by percutaneous nephrolithotomy). Bacteria found in urine and stone matrices might promote formation and growth of these relatively large stones. On the other hand, these large stones are more susceptible for bacterial colonization. Therefore, extensive investigations of smaller stones are required to address this concern. Second, although all the subjects recruited into this study had no active UTIs or antibiotic treatment of UTIs within 12 months prior to the admission, their precise information and details of infections (particularly UTIs) and antibiotic treatments in the past (>12 months before the admission) are not available. Thus, it is inconclusive in our present study that when and how antibiotic resistance occurred.

In summary, our data indicate that the prevalence of UTIs associated with nephrolithiasis is still high (36%) in the northeastern region of Thailand. In addition, UTIs are frequently associated with almost all chemical types of kidney stones, not only struvite, and *E. coli*, not urea-splitting bacteria, is the most common causative microorganism found in urine and stone matrices of the stone formers in Thailand. Based on their locales in the stone nidus, we hypothesized that the microorganisms found in both urine and stone matrices were not the entrapped bacteria from secondary UTIs but were indeed the causative bacteria involved in the stone formation and pathogenesis. These bacteria remained in the urine most likely due to multidrug resistance, thus hardly to be eradicated and easily to be further entrapped in the stone periphery. However, further extensive investigations of smaller stones and elucidations of the effects of microbes on stone formation and growth are crucial. These data may lead to rethinking and a new roadmap for future research regarding the causative role of microorganisms in kidney stone formation.

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Original Article

Extensive characterizations of bacteria isolated from catheterized urine and stone matrices in patients with nephrolithiasis

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Abstract

Background. Urinary tract infections are generally known to be associated with nephrolithiasis, particularly struvite stone, in which the most common microbe found is urea-splitting bacterium, i.e. *Proteus mirabilis*. However, our observation indicated that it might not be the case of stone formers in Thailand. We therefore extensively characterized microorganisms associated with all types of kidney stones.

Methods. A total of 100 kidney stone formers (59 males and 41 females) admitted for elective percutaneous nephrolithotomy were recruited and microorganisms isolated from catheterized urine and cortex and nidus of their stones were analyzed.

Results. From 100 stone formers recruited, 36 cases had a total of 45 bacterial isolates cultivated from their catheterized urine and/or stone matrices. Among these 36 cases, chemical analysis by Fourier-transformed infrared spectroscopy revealed that 8 had the previously classified 'infection-induced stones', whereas the other 28 cases had the previously classified 'metabolic stones'. Calcium oxalate (in either pure or mixed form) was the most common and found in 64 and 75% of the stone formers with and without bacterial isolates, respectively. *Escherichia coli* was the most common bacterium (approximately one-third of all bacterial isolates) found in urine and stone matrices (both nidus and periphery). Linear regression analysis showed significant correlation ($r = 0.860$, $P < 0.001$) between bacterial types in urine and stone matrices. Multidrug resistance was frequently found in these isolated bacteria. Moreover, urea test revealed that only 31% were urea-splitting bacteria, whereas the majority (69%) had negative urea test.

Conclusions. Our data indicate that microorganisms are associated with almost all chemical types of kidney stones and urea-splitting bacteria are not the major causative microorganisms found in urine and stone matrices of the stone formers in Thailand. These data may lead to rethinking and a new roadmap for future research regarding the role of microorganisms in kidney stone formation.

Keywords: antibiotic susceptibility; prevalence; stone matrix; urine; uropathogenic bacteria

Introduction

Kidney stone disease (nephrolithiasis) remains a common public health problem worldwide and is highly prevalent in the northeastern region of Thailand [1–3]. Urinary tract infections (UTIs) are well known to be associated with kidney stone formation [4]. Moreover, antimicrobial resistance has been frequently observed in bacteria isolated from stone formers with UTIs [5]. Although the association between nephrolithiasis and UTIs is generally known and frequently detected, its prevalence, causative microorganisms and their antimicrobial susceptibility patterns remain under-investigated. Previous studies have reported that the most common type of kidney stones caused by UTIs is struvite stone containing magnesium ammonium phosphate and the most common microbe found in struvite stone is urea-splitting bacterium, i.e. *Proteus mirabilis* [6–8]. However, our observation indicated that it might not be the case of stone formers in Thailand. We therefore re-evaluated and extensively characterized microorganisms isolated from urine and stone matrices of patients with all types of kidney stones, chemically analyzed by Fourier-transformed infrared (FTIR) spectroscopy and also examined antimicrobial susceptibility patterns of the isolated bacteria. Moreover, urea and citrate tests were performed to classify these bacteria into urea-splitting, non-urea-splitting, citrate-utilizing or non-citrate-utilizing group.

Materials and methods

Ethics statement

All the experiments involved human subjects and clinical samples were conducted according to the principles expressed in the Declaration of Helsinki, and written informed consent was provided by the study participants. This study was reviewed and approved by the Institutional Ethical Committee at Khon Kaen University.

Subjects and sample collection

A total of 100 kidney stone formers (59 males and 41 females) with idiopathic etiology admitted to Khon Kaen Hospital for elective kidney stone removal by percutaneous nephrolithotomy because of large stone and/or obstruction were consecutively recruited during 2009–10. All these subjects had no symptoms and signs of UTIs. The exclusion criteria included (i) underlying systemic diseases and secondary causes of nephrolithiasis (e.g. primary hyperparathyroidism, vitamin D excess, hyperthyroidism, renal tubular acidosis, etc.), (ii) active UTIs or other infections within 1 year prior to admission, (iii) history of antibiotic treatment of UTIs within 1 year prior to admission and (iv) no permission from the subjects. In addition, 30 healthy individuals who had no stones (15 males and 15 females) served as the negative controls for urine culture.

Sample preparation, isolation and identification of bacteria

Catheterized urine samples and stones were collected from all subjects. For the catheterized urine, the samples were collected before prophylactic antibiotic treatment routinely used for surgical stone removal. Stone samples were washed several times with deionized water and each stone was then divided into two parts, as symmetrical as possible, by horizontal or sagittal plane. For the first part, stone matrices were taken from 'periphery' (cortex) and 'nidus' (nucleus) portions by scraping. For the second part, the stone was crushed into powder by sterilized mortar and pestle and was then used for bacterial culture and chemical analysis of the 'whole stone'. The catheterized urine samples and all samples derived from three locales of individual stones (including periphery, nidus and whole stone) were then cultivated in blood and MacConkey agar (Oxoid, Hampshire, UK) at 37°C for 24 and 48 h, respectively. Positive bacterial cultures were considered when there were $>1 \times 10^3$ colony forming units/mL urine. Thereafter, all bacterial isolates in urine and stone matrices were identified by standard biochemical tests [9].

Antimicrobial susceptibility test

All bacterial isolates were tested for antimicrobial susceptibility by the disc diffusion assay on Mueller-Hinton agar (Oxoid) and incubated at 37°C for 24 h. The antibiotics used in individual 6-mm discs (Oxoid) included: amikacin (AK, 30 μg); ampicillin (AMP, 10 μg); cephalothin (CF, 30 μg); sulfamethoxazole/trimethoprim (SXT, 1.25/23.75 μg); gentamicin (GM, 10 μg); norfloxacin (NOR, 10 μg); ofloxacin (OFX, 5 μg); cefotaxime (CTX, 30 μg); ceftazidime (CAZ, 30 μg); netilmicin (NET, 30 μg), oxacillin (OX, 1 μg); cefoxitin (FOX, 30 μg); fosfomycin (FOS, 50 μg); fusidic acid (FA, 10 μg), vancomycin (VA, 30 μg); penicillin (P, 10 U); tetracycline (TE, 30 μg); ciprofloxacin (CIP, 5 μg) and tazocin (TZP, 110 μg). Extended-spectrum β-lactamase-producing bacteria (indicating resistance to third-generation cephalosporins) were also determined by double-disc diffusion test, following the standards of Clinical and Laboratory Standards Institute.

Analysis of chemical compositions of stones

The analysis of chemical compositions of each stone was done using stone powder derived from the second part of stone sections (as aforementioned) that was left after bacterial culture. Chemical analysis was performed using FTIR spectroscopy as described previously [10].

Statistical analysis

All the quantitative data are reported as mean ± SEM, unless stated otherwise. Comparisons between the two groups of samples were performed by unpaired *t*-test, whereas correlation between two variables was determined by linear regression analysis using SPSS software (version 11.0; SPSS Corporation, Chicago, IL). P-values <0.05 were considered statistically significant.

Results and discussion

From a total of 100 stone formers, 36 (19 males and 17 females) had bacteria isolated from catheterized urine samples and/or stone matrices, whereas other 64 subjects (40 males and 24 females) had no microorganisms detected. Their demographic and clinical chemistry data are shown in Table 1. Statistical analysis showed no significant differences between stone formers with and without bacterial isolates in their gender, age, urine pH, white blood cell count, blood urea nitrogen, serum creatinine, K^+, Na^+, Cl^- and HCO_3^- levels. Their stone sizes were also comparable (1.15 ± 0.07 versus 1.21± 0.06 in width and 3.56 ± 0.29 versus 3.12 ± 0.20 in length) (Table 1). Urine culture was also performed in 30 healthy normal individuals who had no stones (15 males and 15 females). The results showed negative bacterial culture in the urine of this negative control group.

From 36 stone formers, a total of 45 bacterial isolates were found. Among these, 6 isolates were detected only in urine samples (of 4 stone formers), 18 were found only in stone matrices (of 12 stone formers) and 21 were observed in both urine and stone matrices (of 20 stone formers) (Table 2). The top three most common bacteria found in

Table 1. Demographic and clinical chemistry data of all stone formers with and without bacterial isolates[a]

Parameters	Stone formers (total $n = 100$) With bacterial isolates ($n = 36$)	Stone formers (total $n = 100$) Without bacterial isolates ($n = 64$)	P-value
Gender (male:female)	19:17	40:24	NS
Age (years)	50.69 ± 2.20	54.14 ± 1.61	NS
Urine pH	6.22 ± 0.12	6.01 ± 0.10	NS
Blood tests			
WBC (10^3/mm^3)	7.96 ± 0.50	7.63 ± 0.31	NS
BUN (mg/dL)	16.56 ± 1.92	15.96 ± 2.10	NS
Creatinine (mg/dL)	1.38 ± 0.12	1.20 ± 0.07	NS
K (mEq/L)	3.98 ± 0.07	3.94 ± 0.06	NS
Na (mEq/L)	138.85 ± 0.93	139.98 ± 0.43	NS
Cl^- (mEq/L)	106.34 ± 1.33	106.81 ± 1.23	NS
HCO_3^- (mEq/L)	27.10 ± 0.61	26.20 ± 0.37	NS
Stone size			
Width	1.15 ± 0.07	1.21 ± 0.06	NS
Length	3.56 ± 0.29	3.12 ± 0.20	NS

[a]NS, not significant.

Table 2. Source and number of bacterial isolates detected in 36 stone formers

Number \ Source	Detected only in urine	Detected only in stone matrix	Detected in both urine and stone matrix	Total number
Number of cases with bacterial isolates	4	12	20	36
Number of bacterial isolates	6	18	21	45

urine samples were *Escherichia coli*, *Enterococcus* spp. and *Klebsiella/Enterobacter* spp., respectively, and those found in stone matrices were *E. coli*, *P. mirabilis* and *Klebsiella* spp., respectively (Table 3). Overall, *E. coli* was the most common bacterium found in both urine and stone matrices (approximately one-third of all bacterial isolates). Other bacteria found included *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus* coagulase negative, *Citrobacter freundii*, *Acinetobacter baumannii*, *Acinetobacter lwoffii*, *Citrobacter diversus*, *Salmonella* spp. and *Staphylococcus* coagulase positive (Table 3).

Table 3. Types of bacteria isolated from urine and stone matrices

Bacteria	Number of bacterial isolates in urine (%)	Number of bacterial isolates in stone matrices		
		Periphery	Nidus	Whole stone (%)
E. coli	9 (33.33)	13	13	14 (35.90)
Enterococcus spp.	5 (18.52)	2	2	2 (5.13)
Klebsiella spp.	3 (11.11)	2	4	4 (10.26)
Enterobacter spp.	3 (11.11)	1	2	2 (5.13)
Proteus mirabilis	2 (7.41)	5	5	5 (12.82)
Klebsiella pneumoniae	1 (3.70)	2	1	3 (7.69)
Pseudomonas aeruginosa	1 (3.70)	2	2	2 (5.13)
Staphylococcus coagulase negative	1 (3.70)	1	1	1 (2.56)
Citrobacter freundii	1 (3.70)	1	1	1 (2.56)
Acinetobacter baumannii	1 (3.70)	1	1	1 (2.56)
Acinetobacter lwoffii	0 (0.00)	1	1	1 (2.56)
Citrobacter diversus	0 (0.00)	1	1	1 (2.56)
Salmonella spp.	0 (0.00)	1	1	1 (2.56)
Staphylococcus coagulase positive	0 (0.00)	0	1	1 (2.56)
Total	27 (100)	33	36	39 (100)

The association between nephrolithiasis and UTIs can be either (i) kidney stone formation developed following UTIs (the so-called 'infection-induced stones') or (ii) nephrolithiasis with subsequent UTIs as its complications (the so-called 'stones with subsequent infections') [6, 7]. Whether they are infection-induced stones or stones with subsequent infections, the stones themselves are the important source of secondary infection [4]. Therefore, complete eradication of the associated UTIs is possible after the stones have been removed [6, 11]. It has been thought that types of bacteria in the stone nidus implicate the causative microorganisms involved in stone formation and the pathogenesis of 'infection-induced stones', whereas those found in the stone periphery implicate microorganisms entrapped into the stones during secondary UTIs (stones with subsequent infections). The latter should have microorganisms found only in the periphery of stone matrices, not in the stone nidus. Moreover, types of bacteria found in the stone nidus of infection-induced stones should be different from those entrapped recently into the periphery of stones with subsequent infections. In our present study, it is very interesting that types of bacteria found in the stone nidus were almost identical to those found in the stone periphery, as well as the whole stone (Table 3). Moreover, linear regression analysis showed significant correlation ($r = 0.860$, $P < 0.001$) between types of bacteria found in catheterized urine samples and those of stone matrices (Figure 1). Among 20 stone formers who had bacterial isolates in both urine and stone matrices, 19 had exactly the same organisms found in both samples, whereas 1 had different strains of *E. coli* found in urine and stone matrices. These data led to a hypothesis that the microorganisms found in both urine and stone matrices were not the entrapped bacteria from secondary UTIs but were indeed the causative bacteria involved in the stone formation and pathogenesis. The data also implicate that

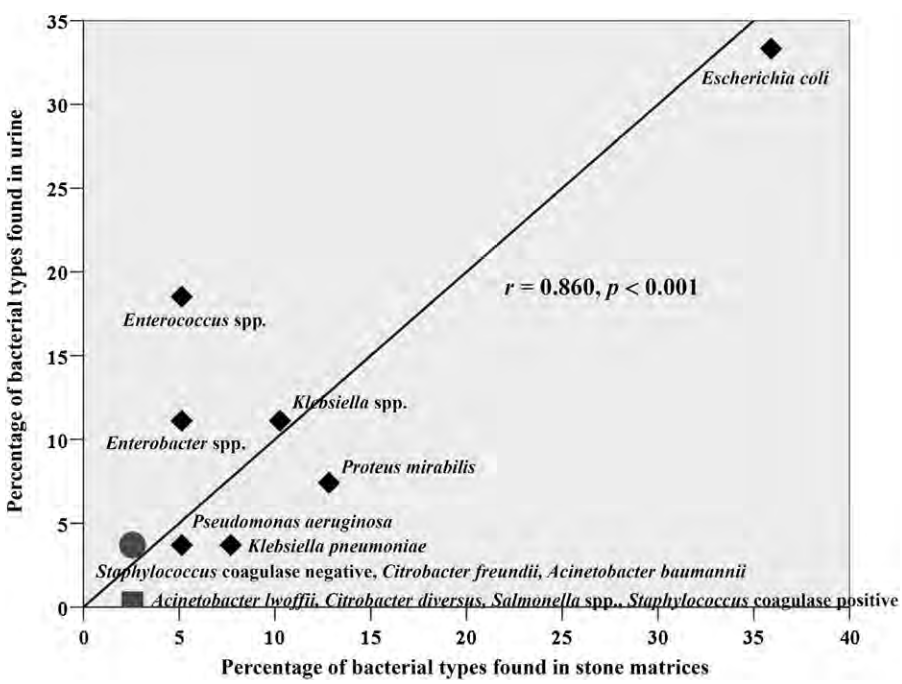

Fig. 1. Linear regression analysis of types of bacteria found in urine and stone matrices.

they remained in these samples most likely due to resistance to antibiotics, thus hardly to be eradicated and easily to be further entrapped in the stone periphery.

To address this, antimicrobial susceptibility assay was performed. The data revealed that the bacteria isolated from urine and stone matrices had multidrug resistance, and their antimicrobial resistance patterns are shown in Tables 4 and 5, respectively. From a total of 27 bacterial isolates detected in catheterized urine samples, 19 (~70%) had antimicrobial resistance (mostly to multiple drugs) (Table 4). Similarly, from 39 bacterial isolates detected in stone matrices, 24 (~62%) had antimicrobial resistance (mostly to multiple drugs) (Table 5). This is the first dataset demonstrating the high proportion and patterns of antimicrobial resistance in bacteria isolated from urine and stone matrices of the stone formers.

FTIR spectroscopic analysis revealed that of all 100 stone formers, 15 showed chemical compositions fitted into

Table 4. Antimicrobial resistance of 27 bacterial isolates obtained from urine[a]

Bacteria	Antimicrobial resistance										
Enterobacteriaceae	Number	AK (%)	AMP (%)	CF (%)	SXT (%)	GM (%)	NOR (%)	OFX (%)	CTX (%)	CAZ (%)	ESBL producer (%)
E. coli	9	0	67	44	56	22	33	44	22	22	22
Klebsiella spp.	3	33	100	100	67	100	100	100	67	67	0
Enterobacter spp.	3	67	100	100	67	67	67	67	67	67	0
Proteus mirabilis	2	0	0	0	0	0	0	0	0	0	0
Klebsiella pneumoniae	1	0	100	100	0	0	100	100	100	100	100
Citrobacter freundii	1	0	100	100	0	0	0	0	0	0	0
Non-fermentative Gram-negative bacilli	Number	AK (%)	SXT (%)	GM (%)	OFX (%)	CTX (%)	CAZ (%)	NET (%)	TZP (%)		
Pseudomonas aeruginosa	1	100	0	100	100	100	0	100	100		
Acinetobacter baumannii	1	ND	ND	ND	ND	ND	ND	ND	ND		
Enterococcus	Number	AMP (%)	GM (%)	VA (%)	P (%)	TE (%)	CIP (%)				
Enterococcus spp.	5	0	20	0	0	80	40				
Staphylococcus	Number	CF (%)	SXT (%)	GM (%)	OFX (%)	OX (%)	FOX (%)	FOS (%)	FA (%)	VA (%)	
Staphylococcus coagulase negative	1	0	0	0	0	0	0	0	0	0	
Total bacterial isolates in urine	27										

[a]ND, not determined; number, number of bacterial isolates.

Table 5. Antimicrobial resistance of 39 bacterial isolates obtained from stone matrices[a]

Bacteria	Antimicrobial resistance										
Enterobacteriaceae	Number	AK (%)	AMP (%)	CF (%)	SXT (%)	GM (%)	NOR (%)	OFX (%)	CTX (%)	CAZ (%)	ESBL producer (%)
E. coli	14	0	57	36	57	14	21	29	14	14	14
Proteus mirabilis	5	0	20	0	20	0	0	0	0	0	0
Klebsiella spp.	4	0	100	75	50	75	75	75	50	50	0
Klebsiella pneumoniae	3	0	100	67	0	0	33	33	33	33	33
Enterobacter spp.	2	0	100	100	0	0	0	0	50	50	0
Citrobacter freundii	1	0	100	100	0	0	0	0	0	0	0
Citrobacter diversus	1	0	0	100	0	0	0	0	0	0	0
Salmonella spp.	1	0	0	0	0	0	0	0	0	0	0
Non-fermentative Gram-negative bacilli	Number	AK (%)	SXT (%)	GM (%)	OFX (%)	CTX (%)	CAZ (%)	NET (%)	TZP (%)		
Pseudomonas aeruginosa	2	50	50	50	100	50	0	50	50		
Acinetobacter baumannii	1	ND	ND	ND	ND	ND	ND	ND	ND		
Acinetobacter lwoffii	1	0	0	100	100	100	0	100	0		
Enterococcus	Number	AMP (%)	GM (%)	VA (%)	P (%)	TE (%)	CIP (%)				
Enterococcus spp.	2	0	0	0	0	50	0				
Staphylococcus	Number	CF (%)	SXT (%)	GM (%)	OFX (%)	OX (%)	FOX (%)	FOS (%)	FA (%)	VA (%)	
Staphylococcus coagulase negative	1	0	0	0	0	0	0	0	0	0	
Staphylococcus coagulase positive	1	0	0	0	0	0	0	0	0	0	
Total bacterial isolates in stone	39										

[a]ND, not determined; number, number of bacterial isolates.

the previously classified or so-called 'infection-induced stones', whereas the majority (85 cases) had chemical compositions fitted into those previously classified as 'metabolic stones' (Table 6). This proportion remained when compared these two stone groups among the 36 stone formers with positive bacterial isolates. Only eight had chemical compositions fitted into the so-called 'infection-induced stones' and only five were struvite (magnesium ammonium phosphate). Among all chemical compositions, calcium oxalate (in either pure or mixed form) was the most common and found in 64 and 75% of the stone formers with and without bacterial isolates, respectively. Comparing stone formers with versus without bacterial isolates, the three most common chemical compositions found in those with bacterial isolates included calcium oxalate mixed with phosphate, magnesium ammonium phosphate (struvite) and uric acid, whereas the three most common chemical compositions for those without bacterial isolates were calcium oxalate mixed with phosphate, pure calcium oxalate and uric acid (Table 6).

In addition, urea test on the bacteria isolated from 36 stone formers revealed that the majority (25 of 36, ~69%) of microorganisms were non-urea-splitting bacteria, whereas only 31% (11 of 36) were urea-splitting (urease-producing) bacteria (Table 7). For citrate test, the majority (22 of 36, ~61%) of microorganisms were non-citrate-utilizing bacteria, whereas only 39% (14 of 36) were citrate-utilizing bacteria (Table 7).

Our present study indicates that the prevalence of UTIs associated with nephrolithiasis is still high (36%) in the northeastern region of Thailand (Table 1). This prevalence is even higher than that of a recent study done in Baghdad demonstrating that 24.4% of cases had bacterial isolates [5]. There were some contradictory results comparing our present study to the previous Baghdad study [5]. We found that the majority of stone formers who had positive bacterial isolates were males (Table 1), whereas they reported that females had a higher chance to have positive bacterial isolates [5]. Moreover, we also found that urea-splitting bacteria were not the major microorganisms found in our case series (Table 7), whereas they reported 74% of the isolated microorganisms were urea-splitting bacteria [5]. These contradictory results might be due to geographical differences and, more importantly, different pathogenic mechanisms of nephrolithiasis and its association with UTIs.

Table 6. Chemical compositions of all kidney stones ($n = 100$)

Chemical composition of stone	Stone formers with bacterial isolates (from a total of 36)		Stone formers without bacterial isolates (from a total of 64)		Total number (from a total of 100)		Stone size (width × length) (cm)
	N	%	*N*	%	*N*	%	
Previously classified as 'infection-induced stones'	8	22.22	7	10.94	15	15	1.55 ± 0.18 × 4.17 ± 0.39
Magnesium ammonium phosphate (Struvite)	5	13.89	4	6.25	9	9	1.24 ± 0.13 × 4.51 ± 0.48
Calcium phosphate/carbapatite	3	8.33	2	3.13	5	5	1.90 ± 0.40 × 3.80 ± 0.80
Calcium phosphate/oxalate/carbapatite	0	0	1	1.56	1	1	2.50 × 3.00
Previously classified as 'metabolic stones'	28	77.78	57	89.06	85	85	1.12 ± 0.04 × 3.12 ± 0.17
Calcium oxalate/phosphate	18	50.00	33	51.56	51	51	1.10 ± 0.06 × 2.88 ± 0.23
Calcium oxalate	2	5.56	13	20.32	15	15	1.12 ± 0.09 × 2.93 ± 0.43
Uric acid	5	13.89	9	14.06	14	14	1.27 ± 0.14 × 4.00 ± 0.40
Calcium oxalate/uric acid	3	8.33	1	1.56	4	4	1.05 ± 0.05 × 3.90 ± 0.49
Calcium phosphate	0	0	1	1.56	1	1	0.70 × 2.80

Table 7. Urea and citrate tests in 36 stone formers with bacterial isolates

Chemical composition of stone	Stone formers with bacterial isolates (total $n = 36$)			
	Urea test		Citrate test	
	Number of urea-splitting bacteria ($n = 11$)	Number of non-urea-splitting bacteria ($n = 25$)	Number of citrate-utilizing bacteria ($n = 14$)	Number of non-citrate-utilizing bacteria ($n = 22$)
Previously classified as 'infection-induced stones' ($n = 8$)	5	3	3	5
Magnesium ammonium phosphate (Struvite) ($n = 5$)	4	1	1	4
Calcium phosphate/carbapatite ($n = 3$)	1	2	2	1
Calcium phosphate/oxalate/carbapatite ($n = 0$)	0	0	0	0
Previously classified as 'metabolic stones' ($n = 28$)	6	22	11	17
Calcium oxalate/phosphate ($n = 18$)	4	14	7	11
Calcium oxalate ($n = 2$)	0	2	0	2
Uric acid ($n = 5$)	1	4	3	2
Calcium oxalate/uric acid ($n = 3$)	1	2	1	2
Calcium phosphate ($n = 0$)	0	0	0	0

In the present study, among the 45 bacterial isolates found in 36 stone formers, the most common was *E. coli* found in approximately one-third of all bacterial isolates. *P. mirabilis* was found only in 7.41 and 12.82% of bacteria isolated from urine samples and stone matrices, respectively (Table 3). This data were contradictory to that of the case series in Baghdad [5]. However, the data in our present study were consistent to that previously reported on the case series in southern part of Thailand, in which the two most common agents found in staghorn calculi were *Corynebacterium* spp. and *E. coli*, whereas urease-producing bacteria were found only in 25% [12]. Interestingly, among urease-producing bacteria found in the latter study, only *Klebsiella* spp. and *Pseudomonas* spp. were isolated, whereas *P. mirabilis* was not detected [12].

In addition, hypocitraturia is a major metabolic abnormality found in stone formers in the northeastern part of Thailand [13, 14]. The information on citrate-utilizing bacteria in urine and stone matrices may be another important factor for kidney stone formation in this area. However, the majority of bacterial isolates found in our present study were non-citrate-utilizing bacteria (Table 7). These conflicting results on the citrate assay might implicate that hypocitraturia found in this population is likely due to metabolic causes, not due to citrate-utilizing bacteria.

Nevertheless, limitations of our present study should be noted. First, sizes of all stones obtained in our present study were relatively large (Table 1) (as the patients were recruited from those who were admitted to the hospital for elective kidney stone removal by percutaneous nephrolithotomy). Bacteria found in urine and stone matrices might promote formation and growth of these relatively large stones. On the other hand, these large stones are more susceptible for bacterial colonization. Therefore, extensive investigations of smaller stones are required to address this concern. Second, although all the subjects recruited into this study had no active UTIs or antibiotic treatment of UTIs within 12 months prior to the admission, their precise information and details of infections (particularly UTIs) and antibiotic treatments in the past (>12 months before the admission) are not available. Thus, it is inconclusive in our present study that when and how antibiotic resistance occurred.

In summary, our data indicate that the prevalence of UTIs associated with nephrolithiasis is still high (36%) in the northeastern region of Thailand. In addition, UTIs are frequently associated with almost all chemical types of kidney stones, not only struvite, and *E. coli*, not urea-splitting bacteria, is the most common causative microorganism found in urine and stone matrices of the stone formers in Thailand. Based on their locales in the stone nidus, we hypothesized that the microorganisms found in both urine and stone matrices were not the entrapped bacteria from secondary UTIs but were indeed the causative bacteria involved in the stone formation and pathogenesis. These bacteria remained in the urine most likely due to multidrug resistance, thus hardly to be eradicated and easily to be further entrapped in the stone periphery. However, further extensive investigations of smaller stones and elucidations of the effects of microbes on stone formation and growth are crucial. These data may lead to rethinking and a new roadmap for future research regarding the causative role of microorganisms in kidney stone formation.

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