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Evidence of bacterial imprints in different types of non-struvite kidney stones

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Abstract

Background Recent studies of renal lithiasis identified bacterial imprints in apatite phosphate stones and mixed calcium oxalate/apatite phosphate stones, neither of which contained struvite.

Methods This cross-sectional observational study examined 903 stones that were collected from 844 patients during the course of 1 year. All stones were initially examined by stereoscopic microscopy. Stone fragments were then examined by scanning electron microscopy + microanalysis by X-ray dispersive energy and by Fourier-transform infrared spectroscopy. When bacterial imprints were detected, biochemical and bacteriological analysis of the patient's urine was performed.

Results We found 8 renal stones that had bacterial imprints but no struvite. All 8 stones contained hydroxyapatite, and the imprints were located in this region. Five stones contained hydroxyapatite as the major component, two stones were mixed hydroxyapatite/calcium oxalate dihydrate stones, one was a papillary calcium oxalate monohydrate stone in which bacterial imprints were located at Randall's plaque and the other was a cavity calcium oxalate monohydrate stone that contained hydroxyapatite in the central core with bacterial imprints.

Conclusion We identified bacterial imprints in different types of renal stones that lacked struvite, including papillary stones, and these imprints were always present in a hydroxyapatite matrix. Notably, a urinary pH above 6.0 favors the formation of apatite phosphates and the growth of bacteria. Our findings point to the importance of controlling urinary pH to prevent bacteria-mediated calculogenic processes.

Clinical trial number Not applicable.

Keywords Renal calculi, Urine, Urinary tract infection, Non-urease producing bacteria, Bacterial imprints

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Introduction

Infection stones of the urinary system account for 5 to 15% of all urinary stones, and most of them consist of struvite, also known as magnesium ammonium phosphate or triple phosphate [1]. More precisely, with elevated urine pH due to infection of the urinary tract, the patient becomes prone to form magnesium ammonium phosphate hexahydrate (MAP or struvite), carbonated calcium phosphate apatite (carbonated apatite or CA), amorphous carbonated calcium phosphate (ACCP) or whitlockite (Wk) stones [2–3]. Although the prevalence of struvite stones (which are always attributable to urinary tract infections) is lower in industrialized countries [4], these stones are becoming more common in these regions, possibly because of increases in resistance to antibiotics. The formation of a struvite stone begins when bacterial urease catalyzes the hydrolysis of urea into ammonia and CO_2 [5]; ammonia is then ionized into the ammonium ion, which significantly alkalizes the urine, leading to the transformation of CO_2 into carbonate. These conditions lead to a high supersaturation of struvite and calcium phosphocarbonate (apatite), causing them to crystallize into stones [1]. Stones will grow at different rates, some slow and some fast, depending on the type of stone and urinary conditions, which may change over time (they may be different at the time of stone formation than at the time of stone removal). Recently, it was found that bacteria also play a role in the specific morphogenesis of struvite by control of nucleation and shape of the growing crystal [6].

A study showed that stones consisting of calcium phosphocarbonate but without struvite crystals had clear evidence of bacterial imprints [7]. Because phosphate stones without struvite can have a high carbonation of apatite and bacterial imprints [7], this suggests that microorganisms with weak ureolytic activity may contribute to the formation of these stones. Furthermore, other studies found that bacterial activity was associated with stones that lacked carbonated apatite, such as those mainly composed of calcium oxalate [8] or a mixture of oxalate and calcium phosphate [9].

In this paper we present a study of bacterial imprints in different types of stones that contained carbonated apatite but lacked struvite.

Patients and methods

This cross-sectional observational study examined 903 renal stones from 844 patients who had renal lithiasis and were recruited over a period of 1 year. This study was carried out in the Urology Service of the Son Espases University Hospital in collaboration with the Laboratory of Renal Lithiasis Research and Biobank of Renal Calculi (BICUIB) of the University of the Balearic Islands, and was approved by the Research Committee of Son Espases University Hospital (protocol code: CI-885/24).

The 903 renal stones were examined following the protocol established by the Laboratory of Renal Lithiasis Research [8]. In general, the examination began by observing the calculus using stereoscopic microscopy (MOTIC SMZ-161, MoticEurope, Barcelona, Spain) to identify areas that required subsequent analysis. Fragments were then selected for further study by scanning electron microscopy (SEM; TM4000 Plus II, Hitachi, Tokyo, Japan) with microanalysis by X-ray dispersive energy (RX, Quantax 75 EDS microanalyzer, Bruker, Berlin, Germany), and by Fourier-transform infrared spectroscopy (FTIR, Bruker Hyperion IR spectrometer, Bruker, Berlin, Germany). If an entire calculus was available, it was divided in half, and then into as many fragments necessary for evaluation by SEM + RX and FTIR. The methodology currently used for SEM consists of placing the stone or its fragments on a sample holder and fixation with adhesive conductive tape, and there is no need for gold sputter coating.

Biochemical and bacteriological analysis of urine samples was performed following the standard protocol used for stone-forming patients in our institution. Thus, 2-h fasting urine was collected first in the morning, and the pH, creatinine, and Ca were then determined. A 24-h urine sample was also collected for evaluation of diuresis and measurements of Ca, Mg, urate, citrate phosphate, creatinine, and oxalate. At least 30 mL of midstream urine specimen was cultured in CPSE bioMérieux media (bioMérieux, Lyon, France) from each patient before surgical stone removal. Using the semiquantitative method, 10^3 colony-forming units (CFUs) per mL of urine was considered as significant bacteriuria. The bacterial pathogens were identified by standard microbiological techniques, such as colony morphology, Gram staining, and several biochemical tests.

Results

The 844 included patients generated 903 kidney stones that we classified into 10 different groups, as previously described [10] (Table 1). A total of 3.9% of the stones consisted of hydroxyapatite, 11.6% were mixed hydroxyapatite/calcium oxalate dihydrate, and 2.4% were struvite. The most common type of stone was calcium oxalate dihydrate with or without hydroxyapatite (34.4%). Previous studies demonstrated that a stone with a high hydroxyapatite content but without struvite can sometimes have bacterial imprints, thus demonstrating bacterial activity by non-ureolytic bacteria [8, 11].

Five of the 844 patients generated 8 renal stones that lacked struvite but had bacterial imprints in the hydroxyapatite region (Table 2). Thus, non-struvite stones with

Table 1 Types of renal calculi generated by the 844 patients

Type	Number	Percentage
Papillary calcium oxalate monohydrate	140	15.5%
Non-papillary calcium oxalate monohydrate	211	23.4%
Calcium oxalate dihydrate	206	22.8%
Apatite	35	3.9%
Brushite	20	2.2%
Magnesium ammonium phosphate	22	2.4%
Calcium oxalate dihydrate/apatite (mixed)	105	11.6%
Uric acid	117	12.9%
Uric acid/calcium oxalate monohydrate (mixed)	45	5.0%
Cystine	2	0.2%
TOTAL	**903**	

Table 2 General characteristics of patients who generated non-struvite calculi with bacterial imprints and the corresponding renal calculi

	Patient 1 (2 stones)	Patient 2 (5 stones)	Patient 3 (2 stones)	Patient 4 (2 stones)	Patient 5 (1 stone)
Age, years	45	71	60	47	75
Sex	Female	Female	Female	Female	Female
Medical history	Not relevant	Dyslipidemia, hyperuricemia	Hepatitis B	Brain tumor	Not relevant
Pharmacological treatment	none	Alopurinol	Levocetirizine Paracetamol Ibuprofen	Escitalopram Lorazepam	none
Previous infection	*E. coli* (20 months before)	*K. pneumonia* (2.6 months before)	None	None	*K. pneumonia* (3 months before)
Stones without bacterial imprints [a]		- MAP + HAP - HAP - MAP		- MAP	
Stones with bacterial imprints [b]	- **Non-papillary COM** - HAP + COM (minor)	- **Papillary COM** - HAP + COM (minor)	- HAP + COD (minor) - **Mixed HAP + COD**	**- HAP + COM (minor)**	**- HAP + COM (minor) + COD (minor)**
Surgery	Ureteroscopy	Ureteroscopy, ESWL, RIRS	Ureteroscopy	PCNL, ESWL	Ureteroscopy
Blood culture [c]	Negative	ND	Negative	ND	*K. pneumoniae*
Urine culture [c]	*E. coli*	*K. pneumoniae*	*E. coli*	*E. coli*	*K. pneumoniae*

[a] Stones without bacterial imprints collected during different dates

[b] Boldfaced stones are shown in Fig. 1

[c] From a sample obtained at the time an imprinted stone was collected

COM: calcium oxalate monohydrate, COD: calcium oxalate dihydrate, HAP: hydroxyapatite / carbonated apatite, MAP: magnesium ammonium phosphate, ND: not determined, ESWL: extracorporeal shock wave lithotripsy, RIRS: Retrograde intrarenal surgery, PCNL: percutaneous nephrolithotomy

bacterial imprints were present in 0.6% of all patients (5/844) and accounted for 0.9% (8/903) of all stones.

Patient 1 received a flexible ureteroscopy of the right ureter. The extracted stone consisted of non-papillary calcium oxalate monohydrate (COM), and the center consisted of carbonated apatite and had evidence of bacterial imprints (Fig. 1A and B). A new flexible ureteroscopy was performed for removal of a second calyceal stone. The extracted stone was a mix of carbonated apatite and COM in a minor amount, and as previously, there were bacterial imprints in the carbonated apatite matrix.

Patient 2, who had a history of multiple carbonated apatite kidney stones since 2006, generated a papillary COM stone. This stone had deposits of sodium urate at the junction with the papilla, close to Randall's plaque (calcium phosphocarbonate), and there were bacterial imprints in the carbonated apatite deposit (Fig. 1C, D and E). A second stone that was generated at a later time consisted of carbonated apatite (major component) and COM (minor component). The hydroxyapatite mass of this second stone also had bacterial imprints.

Patient 3 generated a carbonated apatite stone, with calcium oxalate dihydrate (COD) as a minor component. This stone had numerous bacterial imprints in the hydroxyapatite matrix (Fig. 1F). This patient also generated a second mixed COD/apatite phosphate stone that had bacterial imprints in the hydroxyapatite mass.

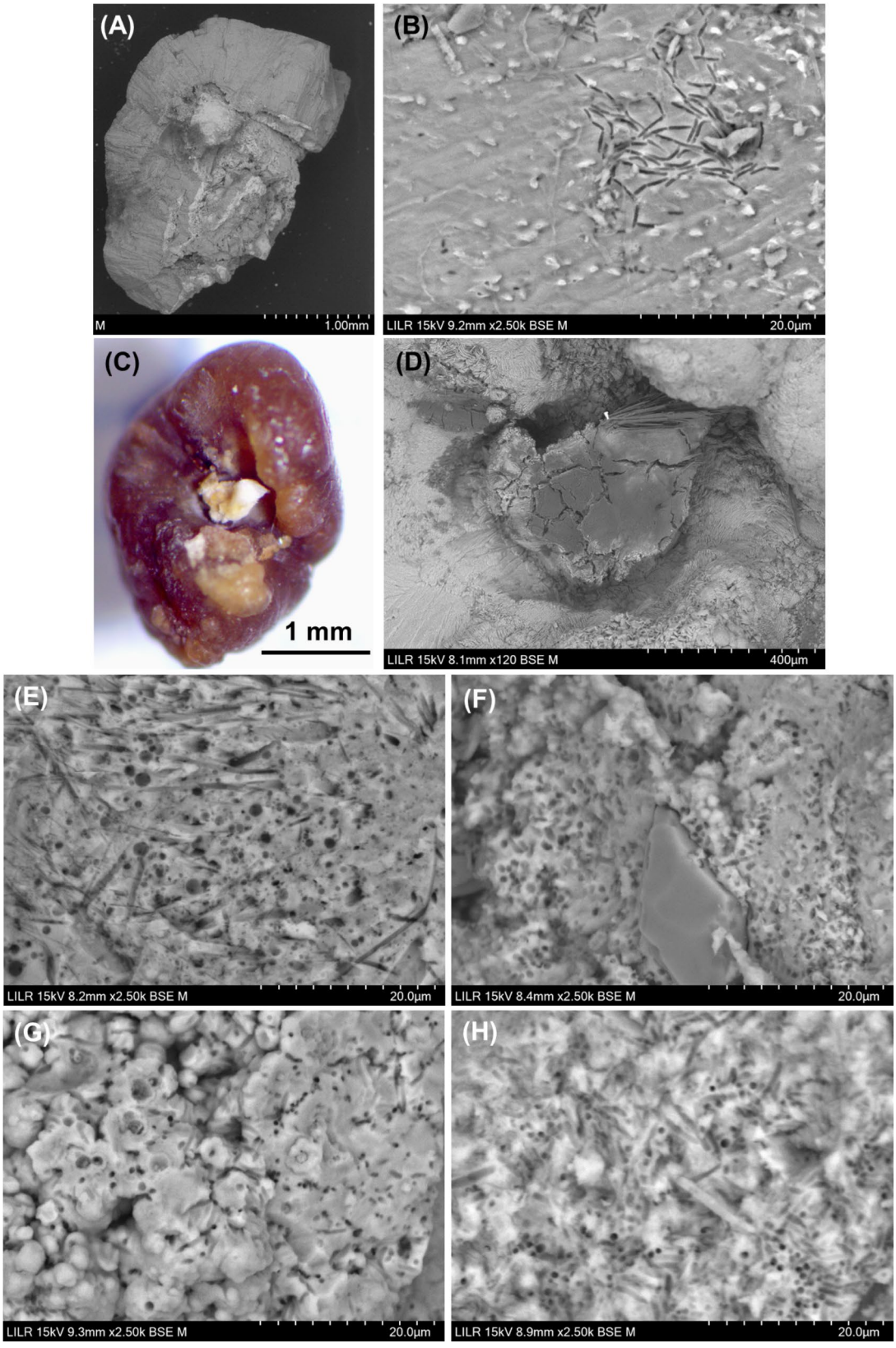

Fig. 1 (See legend on next page.)

(See figure on previous page.)

Fig. 1 Five of the eight non-struvite renal calculi that had bacterial imprints. **Patient 1**: (**A**) SEM section of a non-papillary COM calculus with a HAP central core. (**B**) Detail of the HAP core with bacterial imprints. **Patient 2**: (**C**) Stereoscopic microscopy of a papillary COM calculus. (**D**) SEM image of the calculus anchor point to renal papilla, with Randall's plaque and sodium urate crystals next to the plaque. (**E**) SEM of HAP from Randall's plaque with bacterial imprints. **Patient 3**: (**F**) Detail of the HAP matrix containing bacterial imprints with some COD crystals from the HAP + COD mixed calculus. **Patient 4**: (**G**) SEM of the HAP matrix with bacterial imprints in the HAP + COD mixed calculus. **Patient 5**: (**H**) SEM of the HAP matrix with bacterial imprints in the HAP calculus. COM: calcium oxalate monohydrate, COD: calcium oxalate dihydrate, HAP: Hydroxyapatite/ carbonated apatite, MAP: magnesium ammonium phosphate

Patient 4 had a previous struvite stone and also generated a carbonated apatite stone, with COM as a minor component, that had bacterial imprints. A second intervention using extracorporeal shock-wave lithotripsy led to expulsion of fragments of a mixed calculus of carbonated apatite and COM with bacterial imprints (Fig. 1G).

Patient 5 formed a stone that was removed by ureteroscopy. This stone consisted of phosphate apatite, with COM and COD as minor components, and had numerous bacterial imprints (Fig. 1H).

Discussion

The formation of renal stones consisting of pure struvite and or significant amounts of struvite is attributed to urinary infection by ureolytic bacteria [1]. In addition, stones with a small proportion of struvite may suggest an *E. coli* infection, because this species has weak urease activity [1]. However, there may be doubts about the infectious origin of stones when struvite is not present in a hydroxyapatite/calcium phosphocarbonate calculus. Recent studies showed that bacterial imprints were clearly present in some carbonated apatite stones, confirming that these stones developed in the presence of a bacterial infection [7], although not all apatite phosphate stones have an infectious origin. In fact, all of the 5 patients presented here who had stones with bacterial imprints had urinary infections by *E. coli* or *K. pneumoniae*. The reason for the absence of bacterial imprints in some struvite stones that are generated by ureolytic bacteria is uncertain, but may be attributed to the large size of struvite crystals [12]. In particular, bacteria can be trapped by a deposit of small apatite crystals or spherulites but may not be retained by large struvite crystals, making it difficult to identify imprints in struvite stones. Notably, the formation of carbonated apatite calculi requires a urine pH greater than 6, at which apatite phosphates are insoluble. It is also important to consider that urine always contains nitrate, and that ureolytic bacteria reduce nitrate to nitrite. In fact, an acidic urine is required for the conversion of nitrite to nitric oxide (NO) and other nitrogen compounds that are toxic to a variety of microorganisms. Renal bacteria generally require a urinary pH above 6 [11]. A urinary pH greater than 6 reduces the solubility of apatite phosphates [11] and favors bacterial development. For this reason, urinary stone formation in patients with distal renal tubular acidosis, a disorder that causes a persistent urinary pH higher than 6, is relatively common [1].

Although there is a clear relationship between struvite and apatite phosphate stones with renal infection, the relationship between calcium oxalate stones with renal infection is not so clear. Notably, our Patient 1 first presented with a mixed COM/ carbonated apatite stone with bacterial imprints, and then with a cavity COM stone whose apatite phosphate core had bacterial imprints (Fig. 1). Before the study period, our Patient 2 presented with two struvite stones, one of them with carbonated apatite, and a carbonated apatite stone, none of them with evidence of bacterial imprints. During the study period, she presented with a papillary COM stone that had deposits of sodium urate in the area of union with the papilla, and the hydroxyapatite deposit (corresponding to Randall's plaque) had bacterial imprints, and a carbonated apatite stone with bacterial imprints and a layer of COM on the surface. Our Patient 3 presented with a carbonated apatite stone that had bacterial imprints and a minor amount of COD, and later presented with a mixed stone of COD and carbonated apatite with bacterial imprints. Our Patient 4 presented with a stone of struvite, and then with a stone of carbonated apatite with bacterial imprints and a superficial layer of COM. Our Patient 5 had a phosphate apatite stone, with COM and COD as minor components, and phosphate apatite had bacterial imprints.

Our results demonstrated an association between urinary tract infection and the formation of stones containing COM or COD. However, all of the calcium oxalate calculi contained apatite phosphate, which probably functioned as a heterogeneous nucleant (inducer) of calcium oxalate crystallization. Therefore, a high urinary pH (pH > 6) was needed for the formation of these stones and this also favors colonization by bacteria. The organic matrix generated by these bacteria could also facilitate adhesion of the stone to the renal epithelium, thus favoring its retention [13]. Furthermore, *E. coli* infection can damage renal tubular epithelial cells [14], and this could increase the risk of papillary stone formation. There is in vitro evidence that *E. coli*, *K. pneumoniae*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* can significantly promote the growth and aggregation of calcium oxalate crystals [15, 16]. Bacterial membranes are known to be able to easily nucleate calcium phosphate crystals [17]. In fact, our Patient 2 generated a typical papillary

calculus, and our finding of bacterial imprints on Randall's plaque indicated the presence of bacteria where the stone was attached to the papilla. Furthermore, the presence of sodium urate crystals in this area indicated that the urinary pH was greater than 6.0 at the time of stone formation, compatible with the presence of bacteria. The presence of bacteria in this area of the epithelium could explain the tissue injury. In addition, because the contact of tissue with urine of a high pH can lead to the formation of hydroxyapatite deposits, this may induce the development of COM and formation of a typical papillary calculus. Obviously, this proposed mechanism for the development of papillary COM stones does not begin within the papillary tissue; instead, these stones seem to form as a consequence of an external alteration of the epithelium that covers the papilla. Further studies are needed to confirm this hypothesis.

Previous research reported that *E. coli* was the most common organism isolated from urine cultures and stone cultures of renal calculi consisting of mixed calcium oxalate/ carbonated apatite [9, 18]. It is important to consider that, although very few patients had non-struvite stones that contained bacterial imprints (0.6% of patients, 0.9% of stones), the percentage of struvite stones was 2.4%. It must be considered that the bacterial imprints in a non-struvite stone containing phosphate-apatite are usually not extensive, in that they are only on small areas of the stone and may go unnoticed. Thus, our identification of only 9 stones with bacterial imprints should be considered the minimum number, and is one of the main limitations of the present study.

Conclusion

Infection stones of the urinary system were traditionally considered to have struvite, a substrate generated by ureolytic bacteria. These bacteria generate significant amounts of ammonium and carbonate ions, which results in the supersaturation of struvite and may be accompanied by the generation of insoluble apatite phosphates, which is also greater at a high urinary pH. Moreover, previous in vitro studies demonstrated the ability of organic substances and secretory products generated by non-ureolytic bacteria to induce the development of crystals composed of calcium oxalate or calcium phosphates. It is therefore important to consider that a urinary pH above 6.0 favors the formation of apatite phosphates and can support the growth of bacteria.

Author contributions

F.G. and A.C.-B. conceived and supervised the study; J.G., JL.B., V.T. and E.P. collected the samples and the clinical histories of patients; F.G., A.C.-B., F.J. and B.I. processed the samples; F.G., A.C.-B. and E.P. analyzed the results and wrote the manuscript; F.G. and A.C.-B. acquired resources and funding. All authors reviewed the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

The study was approved by the Research Committee of Son Espases University Hospital (protocol code CI-885/24). Informed consent to participate was obtained from each participant in the study. The study was carried out in accordance with guidelines and regulations stated by Declaration of Helsinki.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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