African Journal of Urology

CASE REPORTS Open Access

Intracellular bacterial communities in patient with recurrent urinary tract infection caused by *Staphylococcus* spp and *Streptococcus agalactiae*: a case report and literature review

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Abstract

Background: Urinary tract infections (UTI) are among the most frequent pathologies worldwide. Uropathogenic *Escherichia coli* (UPEC) is the leading etiological agent; however, depending on the patient's characteristics, the etiology may include some atypical pathogens. Some pathogenic bacteria can internalize in the urothelial and phagocytic cells complicating treatment and timely diagnosis.

Case presentation: We present a clinical case of a married female patient with urological alteration, constant catheterization, and urethral dilation with recurrent UTI for ten years, with five episodes per year and reports of negative urine culture. The microscopic analysis revealed intracellular bacterial communities (IBC) and pyocytes with active bacteria. A protocol was designed for the release of intracellular bacteria in urine samples; without the proposed treatment, the urine culture was negative. However, upon releasing the internalized bacteria, we obtained a polymicrobial urine culture. We isolated and identified *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus simulans*, and *Streptococcus agalactiae*. All microorganisms were sensitive to nitrofurans and sulfas. The patient is under treatment with nitrofurantoin and continuous follow-up by our workgroup.

Conclusions: It is essential to look for IBC and pyocytes with active bacteria in patients with recurrent UTIs to avoid false-negative urine culture results and provide timely treatment. Polymicrobial culture must be considered depending on the patient and clinical history.

Keywords: Intracellular bacterial communities, Recurrent UTI, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Staphylococcus* spp

1 Background

Urinary tract infections (UTI) are among the most common infectious diseases worldwide [1], being uropathogenic *Escherichia coli* (UPEC) the leading etiological agent. However, the etiology can differ depending on the type of patients and their clinical background. Other less frequent uropathogens are *Klebsiella pneumoniae*, *Streptococcus agalactiae*, *Proteus mirabilis*, *Salmonella* spp, *Staphylococcus* spp, *Enterococcus* spp, and *Candida* spp [2]. These microorganisms possess virulence and antimicrobial resistance characteristics that allow them to adapt to the urinary tract environment and successfully carry out their pathogenesis mechanism. In this sense, it is reported that some urinary pathogens such as UPEC, *K. pneumoniae*, *Staphylococcus* spp, *S. agalactiae*, and *Enterococcus faecalis* can internalize into the bladder

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epithelium, forming biofilm-like bacterial consortia called intracellular bacterial communities (IBC) [3–7].

IBCs are formed due to the interaction of the pathogen with receptors present on the cell surface leading to a rearrangement of the actin cytoskeleton, allowing the internalization of the pathogen in an endocytic vacuole from which they are subsequently released to gain access to the cell cytoplasm, where they replicate [8, 9]. IBCs are important because they have been associated with immune evasion, antimicrobial resistance, persistence in the urinary tract, and recurrence of UTIs [3, 9, 10]. In addition, IBCs have also been associated with false-negative urine culture results, which considerably complicates diagnosis and timely treatment [11].

Despite its importance, there are few reports of IBC in the urinary sediment of patients with urinary tract infections. Polymicrobial cultures are generally considered contamination. However, there is evidence detailing the importance of polymicrobial infections, the interaction of these mixed bacterial populations, and their impact on infection development and persistence [12].

2 Case presentation

A 40-year-old married female patient was diagnosed with urethral stenosis after a bladder catheterization performed ten years ago. Bilateral renal and bladder ultrasound with convex transducer showed bilateral non-obstructive renal lithiasis, a full bladder with a volume of 297 mL, slightly thickened wall of 6 mm post-void, without mural lesions, homogeneous and anechoic content, with ureteral jets with adequate strength and frequency. Post-micturition, the patient presented urinary retention of 44% (132 mL). The patient has been submitted monthly since 2016 for catheterization and urethral dilation procedure to treat the urethral stenosis. She reported frequent UTI symptoms (mainly dysuria and low volume micturition) since the first catheterization, with more than five acute episodes per year and poor response to antimicrobial treatment. The patient also referred to present previously negative urine culture results, despite the presence of leukocytes and bacteriuria. She also described having been under repeated antibiotic treatment with levofloxacin and cotrimoxazole with a transient improvement but frequent relapses.

Since we have previously observed false negative urine cultures due to the presence of IBC or biofilms [11], we suspected that the recurrence of UTI episodes in the patient is due to these so-called bacterial morphotypes.

3 Urinalysis

After aseptic directions, a urine sample was collected and sent to the Emerging Diseases Laboratory of the University of Sonora. The urine sample was examined using an URISPIN-U120 (Spinreact, Girona, Spain) with URIN-10 (Spinreact, Girona, Spain) dipsticks. For the detection of bacterial morphotypes, 10 mL of urine was centrifuged for 10 min at 400 × g, and the obtained urine sediment was examined microscopically using Sternheimer-Malbin stain [13]. Adherence and IBC were considered positives if bacteria attached to epithelial cells and bacteria inside of endosomes in epithelial cells, respectively, were observed [11].

In the chemical analysis (dipstick), negative results were observed for nitrites but positive for leukocyte esterase, proteins, and ketones. In the urinary sediment, we observed scarce planktonic bacteria, urothelial cells with the presence of intracellular bacterial communities (Fig. 1, Additional file: 1) and coccoid bacteria adhered to the cell surface, scarce renal tubular cells, and moderate pyocytes (10–15 /high power field) with active bacteria (Additional file: 2).

4 Urine sample processing for intracellular bacterial release

Since IBCs have been associated with false negatives in urine culture, we released the internalized bacterial cells by centrifuging 10 mL of urine at 400 × g for 10 min to obtain the sediment. Once the sediment was obtained, it was resuspended in a mixture of Luria–Bertani broth (LB), Triton 100x, and sterile distilled water (4.5 mL:0.5 mL:5 mL) or a mix of LB and sterile distilled water (5 mL:5 mL).

The suspension was shaken thoroughly for one minute and incubated for 1 h at 37 °C and then plated on MacConkey agar (for Gram-negatives bacteria), mannitol-salt agar (for *Staphylococcus* spp), blood agar (for phenotypic detection of hemolysins), and Mueller–Hinton agar (for CFU/mL count). All cultures were incubated for 24 h at 37 °C. The experiment was performed three times under sterile conditions, and the untreated urine sample was used as a control and plated on the same culture media.

5 Microbiological analysis

No bacterial growth was observed in the plates inoculated with the untreated urine sample and controls; however, bacterial growth was observed on the samples treated with the different mixtures (with and without triton 100x). The CFU/mL count in the treated samples was greater than 100,000 CFU/mL. Interestingly, no bacterial growth was observed on the MacConkey agar plate, but growth was observed on the Mueller–Hinton agar, mannitol-salt agar (polymicrobial: yellow and pink colonies), and blood agar (polymicrobial: colonies greater than 5 mm with a β-hemolytic halo and non-hemolytic colonies) plates.

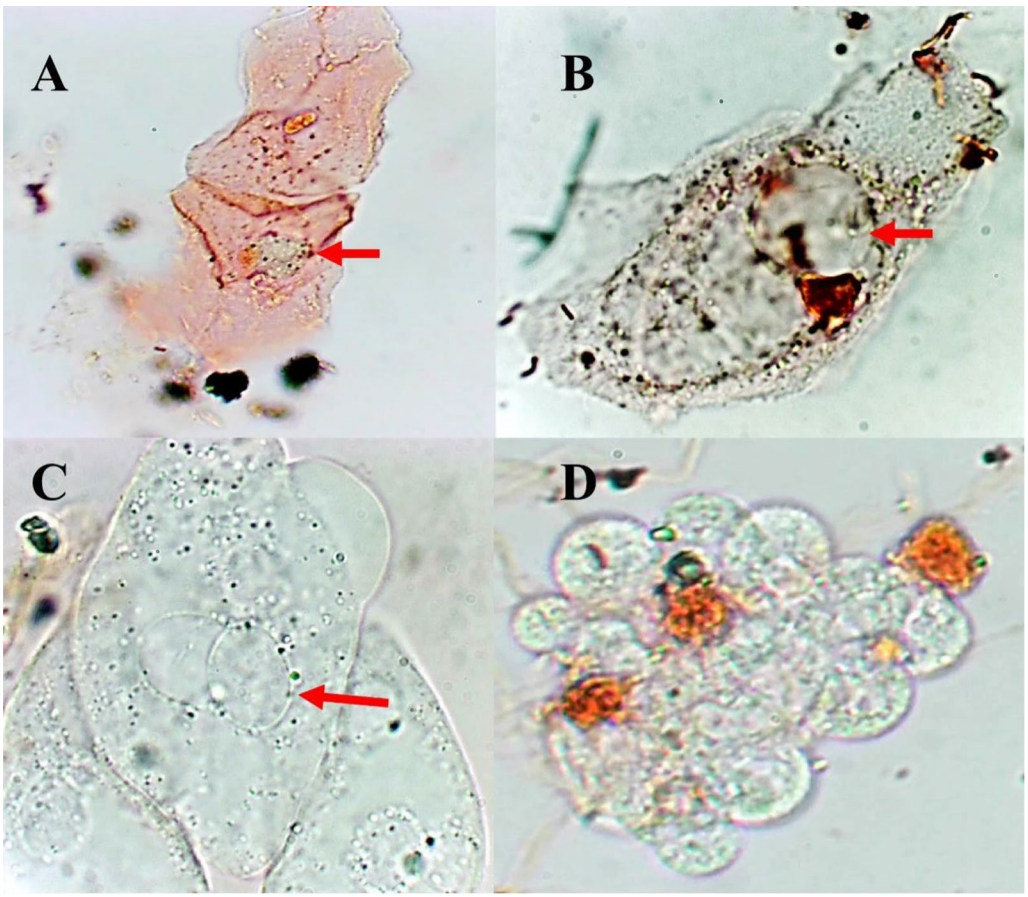

Fig. 1 Light field microscopy of patient's urinary sediment. Exfoliated cells were stain with Sternheimer-Malbin dye. **A**–**C** Bladder epithelial cells with superficial bacterial adherence and bacteria inside endosomes (red arrow). **D** Pyocytes with active bacteria

Gram staining was performed on the colonies obtained in the urine cultures. Gram-positives cocci grouped in clusters were observed in yellow or pink colonies from mannitol-salt agar and in non-hemolytic colonies from blood agar, while Gram-positive cocci in sets of 3–5 bacteria grouped in chains were observed in hemolytic colonies from blood agar. Given the colonial morphology in the culture media and the bacterial cell morphology in the Gram stain, we suspected that the isolated microorganisms were *S. aureus* (yellow colonies on mannitol-salt agar), *Staphylococcus* spp (pink colonies on blood and mannitol-salt agar), and *Streptococcus* spp (hemolytic colonies on blood agar).

Since these are polymicrobial cultures and considering their importance [12, 14], two mannitol-fermenting colonies (yellow colonies) and two non-fermenting colonies (pink colonies) were taken from mannitol-salt agar. Similarly, two hemolytic and two non-hemolytic colonies were taken from blood agar. The eight selected colonies were seeded on individual plates of mannitol-salt agar or blood agar (according to their origin) and incubated for 24 h at 37 °C; its purity was confirmed based on the colonial morphology and they were cryopreserved for later use. For the identification of the isolated microorganisms, catalase, coagulase, and oxidase tests were used [15]. All the isolated bacteria were negative for oxidase, the suspected *S. aureus,* and *Staphylococcus* spp, but not the *Streptococcus* spp were positive for catalase, and those that showed typical *S. aureus* morphology on mannitol-salt agar were positives for coagulase. Due to the colonial morphology on blood agar, specific antisera (Slidex Strepto A & Slidex Strepto B, Biomerieux, Spain) were used to differentiate *Streptococcus pyogenes* (Lancefield Group A) from *Streptococcus agalactiae* (Lancefield Group B) [16]; the agglutination test indicated that the β-hemolytic microorganism obtained was *Streptococcus agalactiae*.

The obtained clinical isolates and their antibiotic resistance profiles were again identified using the MicroScan AutoScan 4 automated equipment (Siemens Health care Diagnostics Ltd. Mexico) with specific panels for Gram-positive microorganisms and following the

manufacturer's directions. The identification of *S. agalactiae* (the two selected hemolytic colonies) and *S. aureus* (the two selected mannitol-fermenting colonies) was confirmed. In addition, the non-mannitol-fermenting and non-hemolytic colonies were identified as *Staphylococcus epidermidis* (two isolates) and *Staphylococcus simulans* (two isolates), respectively. At this stage of the study, we have identified *S. aureus*, *S. epidermidis*, S. *simulans*, and *S. agalactiae* as the etiologic agents of UTI in the patient.

Regarding antibiotic resistance, the identified isolates were multidrug resistant (MDR); all *Staphylococcus* spp (including one isolate of *S. aureus*) isolates showed resistance to amoxicillin/clavulanic acid, ampicillin/sulbactam, ciprofloxacin, ampicillin, and penicillin. Resistance to ceftriaxone, levofloxacin, and moxifloxacin was observed in all isolates, except in one isolate of *S. aureus*, while gentamicin resistance was found only in one isolate of *S. epidermidis* (Table 1). Synercid resistance was observed in *S. aureus* (SA2), which was also resistant to oxacillin and clindamycin (surrogate antibiotic for methicillin) [17], *S. agalactiae* (SGB) isolates, were resistant mainly to ciprofloxacin, clindamycin, erythromycin, and levofloxacin. On the other hand, all pathogens were sensitive to cotrimoxazole, nitrofurantoin, rifampicin, tetracycline, daptomycin, and vancomycin. Additionally, we determined methicillin resistance on the identified *S. aureus* strains by Kirby–Bauer disk diffusion test, according to CLSI guidelines [17], and was observed that *S. aureus* strain 2 (SA2) was resistant, but *S. aureus* strain 1 (SA1) was susceptible. The patient is currently under treatment with nitrofurantoin and in a periodic inspection by the Emerging Diseases Laboratory of the University of Sonora. Monthly, the patient provides a urine sample to the laboratory, and a urinalysis and urine cultures are performed.

6 Discussion

Urinary tract infections are mainly caused by UPEC; however, atypical pathogens have been reported mainly in patients with anatomical, functional, hormonal, or immunological compromises [18, 19]. Polymicrobial urine cultures are usually considered as contamination due to the process of urine specimen collection and are often discarded. However, they have now gained relevance due to probable polymicrobial interactions and their effect on the development and persistence of UTIs, as well as, on antimicrobial resistance associated with

Table 1 Antibiotic resistance results of isolated pathogens

Antibiotic	SA 1	SA 2	SE 1	SE 2	SS 1	SS 2	SGB 1	SGB 2
AMC	S	R	R	R	R	R	S	S
AMS	S	R	R	R	R	R	S	S
AMP	R	R	R	R	R	R	S	S
CRO	S	R	R	R	R	R	S	S
CIP	R	R	R	R	R	R	R	R
CLI	R	R	R	R	R	R	R	R
CX	S	R	ND	ND	ND	ND	ND	ND
DAP	S	S	S	S	S	S	S	S
ERY	R	R	R	R	R	R	R	R
GM	S	S	S	R	S	S	S	R
LEV	R	S	R	R	R	R	R	R
LNZ	S	S	S	S	S	S	S	S
MOX	R	S	R	R	R	R	R	S
NIT	S	S	S	S	S	S	S	S
OXA	R	R	ND	ND	ND	ND	ND	ND
PEN	R	R	R	R	R	R	S	S
RIF	S	S	S	S	S	S	S	S
SYN	S	R	S	S	S	S	S	S
TET	S	S	S	S	S	S	S	S
SXT	S	S	S	S	S	S	S	S
VAN	S	S	S	S	S	S	S	S

AMC Amoxicillin/Clavulanic acid, *AMS* Ampicillin/Sulbactam, *AMP* Ampicillin, *CRO* Ceftriaxone, *CIP* Ciprofloxacin, *CLI* Clindamycin, *DAP* Daptomycin, *ERY* Erythromycin, *GM* Gentamicin, *LEV* Levofloxacin, *LNZ* Linezolid, *MOX* Moxifloxacin, *NIT* Nitrofurantoin, *OXA* Oxacillin, *PEN* Penicillin, *RIF* Rifampicin, *SYN* Synercid, *TET* Tetracycline, *SXT* Cotrimoxazole, *VAN* Vancomycin, *SA Staphylococcus aureus*, *SE Staphylococcus epidermidis*, *SS Staphylococcus simulans*, *SGB Streptococcus agalactiae*, *R* Resistant, *S* Sensible, *ND* Not determined

mixed bacterial biofilms and modulation of the host immune response [12, 20–22].

We present a clinical case of polymicrobial UTI caused by *S. aureus*, *S. epidermidis*, *S. simulans*, and *S. agalactiae* in a patient with urological alteration and urinary retention. The patient had recurrent urinary tract infections over the last ten years with more than five episodes per year. In previous urine cultures, she reported negative results (< 100,000 CFU/mL) despite the presence of bacteria and leukocytes in the urinary sediment. She also said having been previously under constant antibiotic treatment with levofloxacin and cotrimoxazole with no improvement in her symptoms. In the analysis of the urinary sediment, the presence of pyocytes with active bacteria and intracellular bacterial communities in the bladder urothelium was observed. This finding could explain the constant reports of negative urine culture, since it has been reported that IBCs, besides being associated with immune evasion, antimicrobial resistance and persistence in the urinary tract, they are also associated with false negatives in urine culture [3, 11, 23]. Considering the above and to avoid a reduced CFU/mL count in the urine culture, we released the internalized bacteria using a mixture of Triton 100X, sterile distilled water, and mechanical agitation. We observed that treated samples presented CFU/mL counts indicative of UTI, while untreated samples showed no microbial growth. These results are similar to those previously reported by our workgroup in cases of recurrent UTIs caused by IBC-forming UPEC [11]. Interestingly, in this case, no Gram-negative bacteria were observed, we obtained polymicrobial cultures, and the identified microorganisms were *S. aureus*, *S. epidermidis*, *S. simulans*, and *S. agalactiae*. *S. aureus* is an etiologic agent of UTI; however, *S. epidermidis*, *S. simulans*, and *S. agalactiae* are less frequent, and some (*S. epidermidis*) are commonly considered contaminants related to the sample collection process [24]. However, there are reports of immune-compromised patients or with comorbidities with infectious processes caused by these microorganisms, which are mainly multidrug resistant. Table 2 shows some reports of infectious processes caused by *S. epidermidis*, *S. simulans*, and *S. agalactiae* and their resistance profiles.

Clinical isolates reported by other authors present resistance profiles similar to the isolates reported in this study, with high resistance mainly to β-lactam antibiotics (only in *Staphylococcus* spp. isolates), fluoroquinolones, clindamycin, and erythromycin. One of the *S. aureus* isolates (SA2) was resistant to cefoxitin, an antibiotic implemented for the detection of methicillin-resistant *S. aureus* (MRSA) [17, 34]. In this regard, MRSA is characterized by multidrug resistance and represents a major problem, mainly in healthcare-associated infections; in addition, clinical isolates of *S. aureus*, including MRSA isolated from our patient, not only presented resistance to clindamycin (surrogated antibiotic to methicillin) and oxacillin, but also one of them (SA2) was resistant to synercid, which is a mixture of streptogramins A and B (quinupristin and dalfopristin) with synergistic activity, that is used in cases of multidrug-resistant Gram-positive infectious processes. Resistance to these drugs involves the presence of *mecA*, *mecB,* or *mecC* genes (methicillin resistance) [35, 36] and methylations in the 23S rRNA subunit or the presence of genes, such as *vgbA* or *vgB* coding for a lactonase (streptogramin B), efflux pumps and acetylases (streptogramin A) [37–39], so it would be interesting to search for these genetic elements in the obtained clinical isolates.

Polymicrobial cultures are commonly considered as contamination; however, recently, in Japan, it was

Table 2 Reports of infectious processes caused by *S. epidermidis*, *S. simulans*, and *Streptococcus agalactiae*

Pathogen	Risk factor	Infection	Antibiotic Resistance	MDR	Refs.
SE	Blood malignancies, myelomas, diabetes, and hospitalized patients	Sepsis, ventriculitis, prosthetic-join infection	CIP, SYN, GEN	Yes	[25]
SE	Hospitalized patients	RTI's, UTI's, WI's	PEN, TET, ERY, SXT, CEF	Yes	[26]
SE	VUR	UTI	CRO	NR	[27]
SS	Geriatric patient, ureteral and renal stones, animal exposure	UTI	Fluoroquinolones	NR	[28]
SS	Geriatric patient	Pleural empyema	AMS, CEF, CIP, OXA, TET	Yes	[29]
SS	Elderly, animal exposure	Abscess, osteomyelitis foot	AMP, CIP, CLI, OXA, PEN, CRO	Yes	[30]
SGB	Diabetes	UTI's	TET, CIP	No	[31]
SGB	Pregnant women, HIV, diabetes	UTI's, none	CRO, ERY, CIP, CLI, TET	Yes	[32]
SGB	None and catheterized patients	UTI's	Macrolides, LVX, TET	Yes	[33]

SE Staphylococcus epidermidis, *SS Staphylococcus simulans*, *SGB Streptococcus* group B, *RTI's* Respiratory tract infection, *UTI's* Urinary tract infections, *WI's* Wound infection, *VUR* Vesicoureteral reflux, *MDR* Multidrug resistance, *CIP* Ciprofloxacin, *SYN* Synercid, *GM* Gentamicin, PEN Penicillin, *TET* Tetracycline, *ERY* Erythromycin, *SXT* Cotrimoxazole, *CEF* Cefazolin, *CRO* Ceftriaxone, *AMS* Ampicillin/Sulbactam, *OXA* Oxacillin, *CLI* Clindamycin, *LVX* Levofloxacin

observed that in patients with polymicrobial UTI, there is an increased risk of recurrence of infection after antimicrobial treatment, and it is proposed that it should be confirmed that the patient does not present risk factors associated with complicated UTI [22]. This is coincident with our report, since the patient presents urological alteration, urinary retention, and constant therapeutic failure, which allows us to classify her infectious process as a complicated UTI. In addition, the patient is submitted monthly to urethral catheterization and dilation processes as a treatment for urethral stenosis. In this sense, another study conducted in France reported a higher prevalence of polymicrobial urine cultures in catheterized patients compared to patients without a urinary catheter [40]. It is also mentioned that the use of antibiotics in patients with polymicrobial UTI represents a risk, given their ineffectiveness and the possibility of the emergence of antibiotic resistance due to selective pressure, which favors multidrug resistance.

A probable explanation for the observed etiology is that during manipulation of the patient's urogenital tract for treatment of urethral stenosis, these microorganisms gained access to the bladder and caused disease since, except for *S. aureus*, these bacteria are commonly found in the intestinal or genitourinary microbiota; however, it has been reported that they can form intracellular bacterial communities, and clinical cases of UTI caused by these infrequent uropathogens have been documented in patients with urological disorders and in pregnant women [5–7, 28, 41]. Therefore, they are not ruled out as the possible etiological agents of recurrent UTI.

In the case of *S. aureus*, its origin could be the bladder catheterization process that caused the stenosis in the patient, since one of the most frequent routes of entry of this pathogen to the urinary tract is through the use of prolonged catheterization. Interestingly, in addition to the IBC, pyocytes with active bacteria (10–15 cells per field) were observed. It is known that *S. aureus*, through the expression of fibronectin-binding adhesins (FnBPA or FnBPB), can internalize in epithelial cells and in non-professional phagocytic cells [42–44]. In addition, it is known that it can survive inside phagocytic cells and lyse them by the production of toxins such as leukocidin AB [6, 45]. This could explain the high number of endocytic vacuoles with bacteria in the bladder urothelium, as well as the high number of pyocytes present in the urinary sediment, along with the positive leukocyte esterase result in the urinalysis report.

Similarly, the negative urine culture results could be due to the presence of pyocytes with active bacteria and IBC. Therefore, it is important to consider modifying the urine culture protocol in urine samples with similar characteristics to avoid positive urine culture bias and facilitate timely treatment of UTIs. Interestingly, all the isolated microorganisms were resistant to quinolones (levofloxacin and ciprofloxacin); however, they were sensitive to the cotrimoxazole under which the patient was being treated; the persistence of these pathogens in the urinary tract despite being sensitive to cotrimoxazole could be due to its ability to internalize in urothelial and phagocytic cells which could provide protection against the antibacterial agent. To our knowledge, this is the first report of IBC in patients with recurrent polymicrobial and complicated UTIs in Mexico.

7 Conclusions

It is necessary to modify the established protocols for urinalysis and urine culture and include the search for intracellular bacteria, either in urothelial cells or the presence of pyocytes with active bacteria in the urinary sediment of patients with recurrent UTIs. Likewise, if no improvement is observed after treatment, constant follow-up of these patients is recommended, and a periodic search for IBC or endocytic vacuoles that could explain the therapeutic failure should be performed. Additionally, due to reports of unusual pathogens causing UTIs, it is important to not discard atypical microorganisms in UTIs and take them into consideration depending on the characteristics of the patients.

Abbreviations

UPEC: Uropathogenic Escherichia coli; IBC: Intracellular bacterial communities; CFU/mL: Colony forming units per milliliter; UTI: Urinary tract infection; LB: Luria–Bertani Broth.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12301-022-00314-6.

Additional file 1. Evidence of intracellular bacterial communities in the patient's urinary sediment. Presence of endocytic vacuole with motile bacteria.

Additional file 2. Presence of pyocytes with active bacteria in the urinary sediment of the patient with UTI.

Acknowledgements

The authors are pleased to acknowledge the Departamento de ciencias Químico-Biológicas y Agropecuarias, and División de Ciencias e Ingenierías from Universidad de Sonora.

Author contributions

BVE was involved in conceptualization, methodology, formal analysis, investigation, and writing—original draft; VD helped in writing—review and editing and resources; MPP contributed to writing—review and editing and resources; CL was involved in writing—review and editing and resources; BMMG helped in writing—review and editing, resources, project administration, and supervision. All authors have read and approved the final manuscript.

Funding
This research did not receive a grant from any funding agency in the public, commercial, or not-for-profit sectors.

Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
Patient was informed about the protocol to be followed during the urine sample analysis. Her participation was requested. The patient's personal data are kept under anonymity. In addition, the patient is under periodic examination by the workgroup.

Consent for publication
Written informed consent was obtained from the patient for publication of this case report and accompanying images.

Competing interests
The authors report no conflicts of interest.

Received: 26 May 2022 Accepted: 30 August 2022
Published online: 22 September 2022

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