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A new look at the etiology of interstitial cystitis/bladder pain syndrome: extraordinary cultivations

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Abstract

Purpose So far, studies have not clearly identified infectious agents as an etiological factor for interstitial cystitis (IC). Specific microbiological diagnosis for detecting the pathogen with higher sensitivity in IC may decrease the treatment costs and increase psychosocial health of the patients.

Methods A prospective clinical study was performed in 26 IC patients and 20 controls between April and September 2017. All participants were asked to give mid-stream urine sample for routine urine cultures. Followed by the negative results, symptomatic 26 patients were evaluated for L-form pathogen existence by extraordinary cultivation methods. Biopsy samples were taken from 19 patients with ulcerative lesions in the bladder while collecting sterile urine samples from all 26 patients. PG broth, 5% sheep blood agar, EMB, Sabouraud's dextrose, LEM, and GYPA were used. Followed by the 1st day inoculations, all inoculated PG broths were subcultured into the same solid media at the 2nd and 10th days in case of any growth after incubation of 24 h under 35–37 °C. The "O'Leary Sant Symptom and Problem Index" score forms were used to evaluate response to the appropriate treatment for those patients with documented pathogens.

Results Bacterial isolations were yielded from samples of 13 IC patients in PG broth. Eight (61.5%) *P. aeruginosa*, 2 (15.4%) *K. pneumoniae*, 2 (15.4%) *C. mucifaciens,* and 1 (7.7%) *E. faecalis* were isolated. Antibiotic susceptibility tests were performed. Somehow, the median symptom index and problem scores of those 13 IC patients were lower after the appropriate antibiotic treatment ($p < 0.05$).

Conclusions Extraordinary mediums with longer incubation periods may reveal a causative pathogen in the etiology of IC. Future culture techniques may have some value, because still some of IC/BPS patients are describing symptomatic relief by a group of antibiotics.

Keywords L-form · Interstitial cystitis · Bladder pain syndrome · Urine culture

Introduction

Interstitial cystitis/bladder pain syndrome (IC/BPS) is a clinically diagnosed problem with urethral/pelvic pain lasting more than 6 weeks. IC/BPS is mainly seen in females four-to-five times more than males. IC/BPS is usually presents with lower urinary tract symptoms (urgency, pollakiuria, nocturia, and dysuria) without a definitive positive urine culture [1]. The estimated prevalence of IC/BPS is 45/100.000 for women and 8/100.000 for men [2]. In USA, every year, 3.3 million women are admitted to a clinic with the diagnosis of IC/BPS [3]. IC/BPS is not only a clinical problem but also has a burden on social life. It was documented that 50% of patients with IC/BPS could not able to carry on day-time job and 70% of them had sleep arousal problems as well as dyspareunia [4].

In the etiology of IC/BPS, various theories were listed including glucosaminoglycan deficiency on the surface of bladder uroepithelium, increased uroepithelial permeability, increased mast cell activity and inflammatory response, autoimmunity, infections, diet, and environmental factors [1, 2]. Studies have not been able to demonstrate infectious agents as a definitive factor in the etiology of the disease [2].

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On the other hand, diagnostic workup includes urine cultures for aerobic bacteria, fungi, and tuberculosis to exclude any presence of infection. Sexually transmitted diseases (STD) such as *Chlamydia trachomatis* (CT), *Neisseria gonorrhoeae* (NG), *Mycoplasma genitalium* (MG), *Mycoplasma hominis* (MH), *Ureaplasma urealyticum* (UU), *Ureaplasma parvum* (UP), and *Trichomonas vaginalis* (TV) are also needed to be excluded for the diagnosis of IC/BPS.

To the best of our knowledge, studies have not focused on L-form bacteria in cases with IC/BPS. L-form bacteria are the cell wall-deficient state of bacteria and are not able to grow on routine culture medium [5]. In some cases, patients describe symptomatic relief after certain antibiotic treatments despite their negative urine cultures. We aimed to show the presence of L-form bacteria as well as maximize their culture in urine and bladder biopsy samples in patients with IC/BPS.

Materials and methods

Study participants

This prospective study was conducted on 51 participants admitted to Hacettepe University Department of Urology between April 2017 and September 2017. Informed consent was obtained from all participants before their enrollment into study. The study protocol was approved by the Hacettepe University Institutional Review Board (IRB) (GO 16/713-01).

The IC/BPS group was consisted of 26 patients (25 female and 1 male) which were diagnosed according to the American Urological Association (AUA) criteria [6], and the control group was consisted of 25 female participants. Participants in the control group were composed of healthy volunteers over the age of 18. The inclusion criteria for the control group were no known history of recurrent cystitis and no current urinary tract infection symptoms. The exclusion criteria were pregnancy, endometriosis, dysmenorrhea, history of pelvic radiation, urogynecologic malignancy, and history of urolithiasis.

For the evaluation of symptoms (nocturia, urgency, frequency, and pain), the "O'Leary Sant Symptom and Problem Index" score forms [7] were given to the IC/BPS group before the study. This form includes both problem and symptom index scores. Re-evaluation of scores was planned in cases of any defined pathogen and after its treatment.

Clinical specimens and microbiological methods

At the initial visit, mid-stream urine samples were collected from all participants and cultivated for facultative anaerobicic bacteria, fungi, and tuberculosis to rule out subjects with positive urine cultures. First-void urine samples were also collected for the evaluation of sexually transmitted disease (STD) pathogens via real-time polymerase chain reaction (Rt-PCR) (Allplex™ STI Essential Assay, Bio-Rad, USA).

Cystoscopy procedure

All participants in the IC/BPS group underwent cystoscopy procedure. At the beginning of cystoscopy, sterile urine sample was collected via 14F urethral catheter from each patient. Inoculation of 10 µl urine sample was immediately made into the liquid peptone–glucose (PG) medium at the operating room for investigation of pathogens and the rest of the urine sample was stored for Rt-PCR.

A flexible videocystoscopy (*Karl-Storz* 11272c1 model analog cystoscope) was performed in the IC/BPS group with the application of 5% lidocaine sterile gel lubricant under local anesthesia. An 80 mmHg pressure hydrodistension for 10 min was created in the IC/BPS group. When ulcerative lesions visualized, sterile punch biopsies were taken. Two tissue samples were taken from each patient. One sample was stored in a falcon tube with a 2 ml of sterile Tris EDTA buffer solution for Rt-PCR at − 20 °C to rule out any STD pathogen. The second sample was immediately inoculated into the liquid PG medium during the procedure.

During cystoscopy procedures, samples were also collected from each sterile instrument including cystoscope, forceps, and disinfectant solution to rule out any contamination.

All samples were immediately transferred to the microbiology laboratory under appropriate conditions to investigate the existence of any L-form pathogens.

Microbiological culture methods

All the urine and biopsy samples were inoculated for the screening of any L-form variant of *Escherichia coli*, *Klebsiella*, *Proteus*, *Enterococcus*, *Enterobacteria*, and *Staphylococcus* species in extraordinary culture mediums [7]. On the first day, all samples were inoculated into peptone–glucose (PG) (non-high osmotic medium) broth, 5% sheep blood agar, Eosin–Methylene Blue (EMB), Sabouraud's dextrose, L-form Egg Medium (LEM), and Glucose–Yeast–Peptone Agar (GYPA). All inoculated PG broths were subcultured into the same solid media on the 2nd and 10th days in case of any growth during the initial 24 h incubation. All media were checked for visible growth and in case of any growth Gram staining was performed. Pathogen species were identified with Matrix-Assisted Laser Desorption/Ionization–time of Flight Mass Spectrometry (MALDI-TOF MS) (Biomerieux Biosciences, France) only if quantitative documentation of ≥ 100.000 cfu/ml was made. All identified pathogens after

re-growth in PG medium have been filtered through a 0.2 µm pore-size filter and re-inoculated into the same solid media to identify any L-form variants.

All control samples which were collected from each sterile site and medical instrument were inoculated into Stuart's transport and PG media. They were all inoculated into 5% sheep blood, LEM, GYPA, EMB, and Sabouraud's dextrose agar as in the same protocol which we mentioned above.

Real-time polymerase chain reaction

Urine and biopsy samples collected during the cystoscopy procedure for Rt-PCR were stored at − 80 °C. NucliSENS easyMAG (Biomerieux Biosciences, France) automated nucleic acid extraction system was used for the isolation of nucleic acids. Rt-PCR was performed with Allplex TM STI Essential Assay CFX96TM Real-time PCR System (Bio-Rad, USA). For the internal control of each sample, 10 µl of ASTI IC was used. A total volume of 15 µl PCR master mix was prepared adding 5 µl 4X STI-EA MOM, 5 µl EM1 and 5 µl RNase-free water. 5 µl of each nucleic acid sample was added on 15 µl PCR master mix with a final volume of 20 µl.

Statistical analyses

All analyses were performed using SPSS software version 22.0 (Chicago, IL, USA) and GraphPad Prism 8.0 (GraphPad Software, California, USA). The Shapiro–Wilk test was used to assess the normality of the variables. Using the independent *t* samples test, we compared the numerical variables with normal distribution and the data presented as mean ± standard deviation. Wilcoxon signed-rank test was used to compare pre- and post-treatment "O'Leary Sant Symptom and Problem Index" score forms. A two-tailed *p* value of less than 0.05 was considered statistically significant.

Results

The demographic data of participants are summarized in Table 1. During the bacterial culture protocol, 13 cases among the IC/BPS group (13/26, 50%) showed positive bacterial isolations in PG broth. The positive culture results (urine and biopsy) of these patients are summarized in Table 2 and Fig. 1.

At first visit, four patients in the IC/BPS group had positive aerobicic urine cultures on 5% sheep blood agar and EMB agar; *E. coli* with 100.000 cfu/ml colony count grew in three of them and *K. pneumonia* with 70.000 cfu/ml in one patient (Table 1). Appropriate treatments were given according to the antimicrobial susceptibility testing. Though cell wall-deficient (L-form) variants cannot be eradicated with the use of cell wall acting antibiotics and further these antibiotics can force bacteria for alteration into the L-form morphology, we preferred oral fluoroquinolones for the treatment of these four patients [8]. Because of fluoroquinolone allergy, the patient with a positive *K. pneumoniae* urine culture was treated with oral cefuroxime. Control urine culture was found negative in all four patients at the end of appropriate antimicrobial treatment. The aforementioned four patients were included into study for further evaluation with cystoscopy and cultivation.

Table 1 The demographic data of the patients and participants

	IC/BPS group $n=26$	Control group $n=25$
Gender		
Female	25	25
Male	1	–
Age (years)	45.5 ± 10.5	36.55 ± 8.4
Duration of symptoms (months)	90.53 ± 50.4	–
Culture results of the first visit clean-catch urine samples (*n*)		
Negative	22	20
Escherichia coli	3	1
Klebsiella pneumoniae	1	–
Contaminated	–	4

IC interstitial cystitis, *BPS* bladder pain syndrome

Sterile punch biopsies were taken from the 19 patients; those ulcerative lesions were visualized during the cystoscopy procedure. Only the biopsy cultivation results of patient #1, #3, #10, and #12 were documented as *P. fluorescens*, *P. aeruginosa* and *E. faecalis*, *P. aeruginosa*, *K. pneumoniae*, respectively. As opposed to this, patient #7 showed a positive urine cultivation result for *P. aeruginosa*. Culture results of patient #3 showed co-existence of *E. faecalis* and *P. aeruginosa,* while patient #8 showed co-existences of *C. mucifaciens and P. aeruginosa.* Patient #3 also showed a positive result for *U. parvum* co-existence on Rt-PCR of the urine sample (Table 2). No biopsy sample was obtained from the patient #13 as cystoscopy was normal; however, *P. aeruginosa* was documented during on urine cultivations.

All identified isolates were re-inoculated into the PG liquid medium and these pathogens were filtered through 0.2 µm pore-size filter and subcultures with the PG liquid medium. No growth was observed in mediums after the filtering. The samples that were taken from the cystoscope, forceps, and disinfectant solution were also found culture negative.

In the control group, four of them showed contamination and one was diagnosed with asymptomatic bacteriuria which was documented for *E. coli* with 100.000 cfu/ml (Table 1). After the exclusion of these participants, same culture method used in the IC/BPS group was applied to

Table 2 The distribution of the positive culture (urine/biopsy) and Rt-PCR results of the IC/BPS group

Patient no.	First day culture results		Second day culture results		Tenth day culture results		Rt-PCR results	
	Urine	Biopsy	Urine	Biopsy	Urine	Biopsy	Urine	Biopsy
1	N	N	N	*P. fluorescens*	N	N	N	N
2	N	N	N	N	*C. mucifaciens*	*C. mucifaciens*	N	N
3	N	*P. aeruginosa*	N	*P. aeruginosa* *E. faecalis*	N	N	*U. parvum*	N
4	N	N	*P. aeruginosa*	*P. aeruginosa*	*P. aeruginosa*	*P. aeruginosa*	N	N
5	N	N	N	N	*P. aeruginosa*	*P. aeruginosa*	N	N
6	N	N	*P. aeruginosa*	*P. aeruginosa*	N	N	N	N
7	N	N	*P. aeruginosa*	N	N	N	N	N
8	*C. mucifaciens*	*C. mucifaciens*	N	*P. aeruginosa*	N	N	N	N
9	N	N	*K. pneumoniae*	*K. pneumoniae*	*K. pneumoniae*	N	N	N
10	N	N	N	*P. aeruginosa*	N	N	N	N
11	N	N	N	N	*P. aeruginosa*	*P. aeruginosa*	N	N
12	N	N	N	*K. pneumoniae*	N	*K. pneumoniae*	N	N
13	*P. aeruginosa*	–	*P. aeruginosa*	–	N	–	N	N

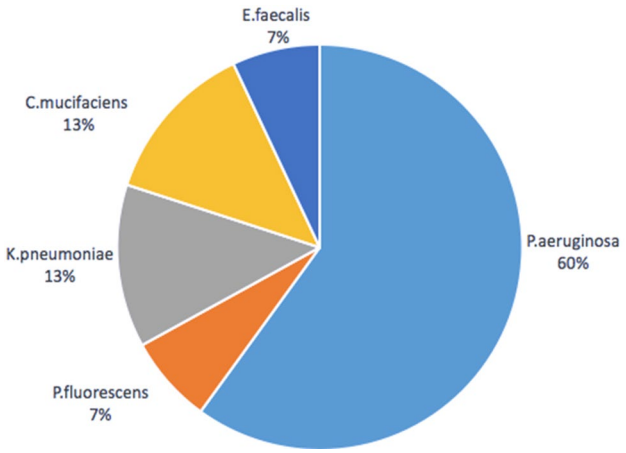

Fig. 1 The distribution of positive culture results (urine and biopsy) in the IC/BPS group ($n=13$)

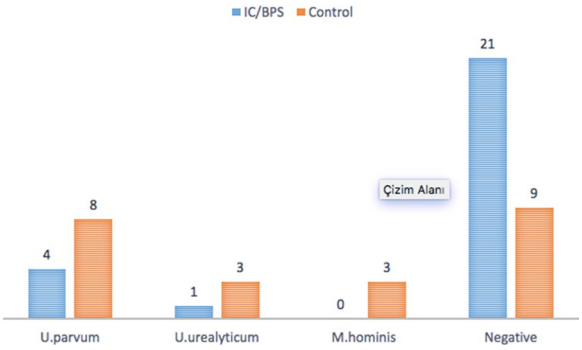

Fig. 2 Rt-PCR results of the study cohort (IC/BPS group $n=26$, control group $n=20$). Asterisk: one sample showed co-existence of *M. hominis* with *U. parvum*. Two other urine samples showed co-existences of *U. urealyticum* and *M. hominis*

the rest of the 20 control subjects. The urine cultures were negative in the control group.

The urine samples in 11 participants from the control and 5 patients from the IC/BPS group had positive real-time PCR results (Fig. 2). In the control group; one sample showed co-existence of *M. hominis* with *U. parvum.* Two other urine samples showed co-existences of *U. urealyticum* and *M. hominis* (Fig. 2). The distribution of *U. parvum* among the control ($n=8$) and IC/BPS ($n=4$) groups were not statistically significant ($p=0.09$). Among the IC/BPS group, none of the biopsy samples had a positive real-time PCR result.

In terms of symptom relief, patients with IC/BPS were requested to fill the "O'Leary Sant Symptom and Problem Index" (OPSI) form at their initial visit. The culture positive IC/BPS patients ($n=13$) were treated according to their antibiotic susceptibility testing. After completing antibiotic regimens, the same cohort was asked to refill the form. The median symptom index score dropped after the treatment (16.5 vs 12.5, $p=0.0039$) (Fig. 3). The median problem index score also dropped after the treatment (15 vs 11, $p=0.0039$) (Fig. 3).

Discussion

Our results showed that extension in the duration of incubation on 1st, 2nd, and 10th day re-inoculation into PG medium, which has a non-high osmotic property, may lead to identify the pathogen which is not identified in routine culture media. A routine urine culture method is

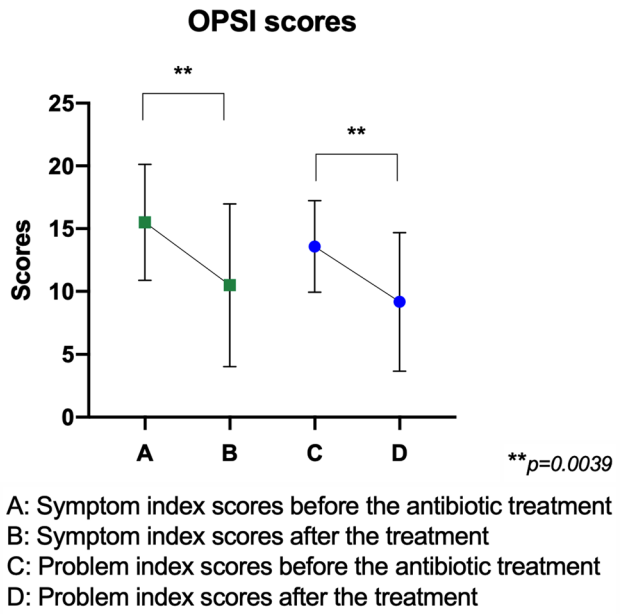

Fig. 3 OSPI scores of the IC/BPS group. A: Symptom index scores-before the antibiotic treatment. B: Symptom index scores after the-treatment. C: Problem index scores before the antibiotic treatment. D:Problem index scores after the treatment

processed under 35–37 °C for a 24 h period and cultures with < 10.000 cfu/ml growth reported as culture negative [9]. The etiology of IC/BPS is still uncertain and many studies could not identify specific microorganisms as a causative agent of the disease [2, 10]. So far, a few studies showed L-form variants in the etiology of chronic recurrent infections [11, 12]. In urinary tract infections, urine osmolarity of patients is generally expected to be higher than healthy people. Thus, we may catch an unexpected pathogen via manipulating the osmotic environment through different mediums. Furthermore, with antibiotics against cell wall synthesis, lytic enzymes and the addition of some amino acids, sucrose, or sodium chloride, an optimal osmotic equilibrium can be created for the isolation of L-form colonies. In this study, pathogen isolates were obtained using PG culture in patients with IC/BPS.

L-form *H. pylori* existence in cholecystectomy specimens was investigated by Wang et al. [11]. They inoculated the samples into a high osmotic LEM and non-high osmotic PG mediums [11]. The researchers further filtered the samples through 0.22 µm pore-size filters and re-inoculated into PG liquid medium where they showed stable form of *H. pylori* L-form variants [11]. Gutman et al. tried to isolate cell wall-deficient pathogens via filtering through 0.45 µm pore-size filters in patients with chronic urinary tract infection [12]. L-form variants of *E. coli*, *Klebsiella,* and *Enterococcus faecalis* were isolated in 11 out of 57 patients from the filtered urine samples [12]. In another study, 29% of the bladder biopsies in patients with IC/BPS were positive for Gram-negative bacterial 16S rRNA [13]. After filtering biopsy cultures through 0.22 µm pore size, they reported nucleic acid consisted filterable structures which was probably a cell wall-deficient state of a pathogen [13].

We cultivated our samples under a protocol to detect any existence of L-form state of commonly seen urinary tract pathogens. Zhang et al. also demonstrated existence of small self-replicating microbes as nanobacteria (NB) both in bladder biopsy and urine samples of IC/BPS patients [14]. With the use of special medium- and long-term cultivation methods, they showed NB in 11 bladder biopsy and 6 urine samples [14]. NB (+) patients received tetracycline treatment and filled the OPSI form before and after their treatments [14]. Similar to our study, symptom and problem index scores were decreased after completion of antibiotic regimens [14].

All cell wall-deficient morphologies such as L-form, biofilm form, small colony variant, and dormant cystic forms are not able to grow on a routine culture medium. *P. aeruginosa* and *K. pneumoniae* are some of the mostly seen pathogens in the urinary tract infections. Mulyukin et al. demonstrated some dormant cystic forms of persistent *P. aeruginosa* after the treatment of urinary tract infections with ciprofloxacin [15]. The persistent cells (dormant forms) can survive and even multiply under the antibiotic treatment. Furthermore, those dormant forms look like L-form variants in the culture medium. Mulyukin et al. reported that both ciprofloxacin sensitive and resistant dormant forms can survive as microcolonies leading to latent infections [15]. Bacterial density in the urine does not correlate with the number of bacterial cells in the bladder wall and significant numbers of bacteria can remain within the tissue [16]. In our IC/BPS group, *P. aeruginosa* was the most common identified pathogen followed by *K. pneumoniae* and *C. mucifaciens* (Fig. 1). We already know that *P. aeruginosa* is a common cause of nosocomial infections; therefore, we collected samples from cystoscope, forceps, and disinfectant solution to rule out any contamination during the cystoscopy. Cultures of these materials were negative.

Another important urogenital tract pathogen is *Corynebacterium urealyticum. Corynebacterium urealyticum* is a slow growing opportunistic pathogen that can be isolated up to 30% in samples of patients with long hospital stay, older, and immunosuppressant [17]. *Corynebacterium urealyticum* is the leading cause of encrusted cystitis [17]. In our study, we identified *C. mucifaciens* in both urine and biopsy cultures of two patients. To the best of our knowledge, *C. mucifaciens* has not been reported as a causative agent in patients with IC/BPS. Corynebacteria are slowly growing so longer; incubation periods might be required to identify this pathogen in patients with chronic urinary infections and IC/BPS.

Our study cohort was mainly women, and in this unique population, endometriosis should be kept in mind as an important differential diagnosis and a possible co-existence. Endometriosis is a well-known gynecological disorder which is seen in 1–7% of female population and it is observed in 70% of women with chronic pelvic pain [18]. A recent systematic review reported 48% co-existence of endometriosis and IC/BPS in women with chronic pelvic pain and this was defined as "Evil-Twin Syndrome" [19]. Both etiologies and pathogenesis of endometriosis and IC/BPS are unclear. Inflammation is mainly blamed for the pelvic pain symptoms and progressions [20]. We excluded participants with a history of endometriosis, suspected endometriosis cases in history and gynecologic exam and who has dysmenorrhea.

The main limitation of this study was that we were not able to identify L-form bacteria after 0.22 µm pore-size filtration. This finding may be explained by their feature of stable/non-stable form transformation. We also think that 16S rRNA sequencing or 0.45 µm pore-size filters may approve the existence of same microorganisms those we had identified (Table 2) on different day culture passages before 0.22 µm pore-filter phase. During the Rt-PCR analysis, we only looked for an existence of any STD pathogen. As an STD pathogen, the existence of *Ureaplasma* was considered as a urethral contamination.

Another possible limitation is the microorganisms in Table 2 which could be re-evaluated by Rt-PCR/16S r RNA sequencing. Future studies may highlight this issue.

Conclusion

PG or LEM like media which consisted of osmolar modifications may identify possible pathogens in patients with IC/BPS. Extraordinary mediums along with longer incubation periods can be promising in the near future, especially in a specific subset of patients describing symptomatic relief by some kind of antibiotics despite negative conventional culture results.

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Compliance with ethical standards

Ethical approval This research involves human participants and informed consent was taken from each participant.

Conflict of interest The authors declare no conflict of interest.

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