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Immunonohistochemical Detection of Gram Positive Bacterial LTA associated with Urinary Tract Infection among Urinary Bladder Cancer patients.

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Abstract
Urinary Tract Infections (UTIs), and their complications, cause serious health problems, which affect millions of peoples every year, Many researchers isolated pathogenes causing UTIs and some studies investigated the role of such infections as a risk factor for Urinary Bladder Cancer (UBC). Although, such studies confirmed that UTIs are mainly caused by gram negative bacteria with 60-70% of them caused by *Escherichia coli* (*E.coli*), and for less extent group B *Streptococcus* and *Staphylococcus* species. However, few of them focused on detection of some important gram positive bacteria associated with UTIs specially among immunocompramised patients including UBC patients. In our study we tried to investigate the association and the severty of these infections among forty eight UBC patients and twenty control group of non-UBC patients. In the current study IHC technique was used for detection of *S. pneumoniae, L. monocytogenes* (all serotypes), *B. cereus* and *B. subtilis* within the tissue using specific antibody targeting gram positive lipotichoic acid. Immunohistochemistry test showed 39.71% positive results, 55.56% of them were for UBC and 51.85% of them were for male. sixty percent of those patients showed sever infection with more than 6 microscopical fields with ≥10 bacterial cells/one field. (30.56%) of UBC cases showed appearance of diplococci bacteria, Gram positive cocci recorded 41.67% and Gram positive bacilli cells were the third rate in our study 27.78% . In conclusion, using of IHC technique in diagnosing bacteria associated with UTI is very usefull method and can reflect the true picture for the infection within the tissue comparing to other techniques. *S. pneumoniae, L. monocytogenes* (all serotypes), *B. cereus* and *B. subtilis* should be considered as UTI causal agents, it is important to be aware of this unlisted emerging pathogens specifically because they are a way from their specific site of infection and it can be more virulent. Those are important gram positive pathogens and have a significant virulance through being in contact with human being life. The high recurrence rates of UTIs and increasing antimicrobial resistance among uropathogens require further detection for some pathogens that have not been considered as a causal agents for reducing the threaten and the greatly increasing economic burden of these infections.

Keywords: Urinary tract infection, immnunohistochimestry, *Listeria*, *Streptococcus pneumoniae*

1. Introduction

Urinary tract infections (UTIs) are among the most common infectious diseases occurring in the community or healthcare centers. Complicated UTIs may occur in all sexes and age groups and are frequently associated with immunosuppression and pregnancy (1).

Gram-negative bacteria are the main UTI causal bacteria including *E.coli* 60-70%, *Klebsiella* 10%, *Proteus* 5–10%, and *Pseudomonas* 2–5% (2). Gram positive bacteria were isolated less frequently than gram negative and group B *Streptococcus* and *Staphylococcus* species were the main Gram positive bacteria isolated from UTIs and Urinary Bladder Cancer (UBC)pateints (1,3,4). However, voided urine was used in most studies to isolate UTI causing bacteria (5,6). Despite the fact that, urine culture contamination is not completely avoidable and there are several precautionary measures should be taken before and after urine collection (7). However, it considered simple and noninvassive (8).

Although, numerous studies worked on detection of UTIs causing organisims(1,2,3,4,5), but till now little is known about the role of some other important gram positive bacteria. In our study we tried to investigate the association of some bacteria that have been not considered as a UTIs causative agents including (*Listeria monocytogenes* (all serotypes), *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecium*, *Bacillus cereu* and *Bacillus subtilis*) and measuring the severity of infections among UBC cases using Transe Urothelial Resection of Bladder Tissues (TURBT) samples. *Listeria monocytogenes* which is a facultative intracellular gram-positive bacterium, and causes human listeriosis (9). The severe and deadly forms of *Lm* infection mostly occurs in immunocompromised patients (10,11, 12). *Enterococcus faecium* is a significant bacterial pathogen, which is able to cause hospital acquired infections, It is the second most common pathogen isolated from hospital associated urinary tract and wound infections especialy among immunocompromised patients (13). *Streptococcus pneumoniae* is a gram positive diplococci shape bacteria that play a role in septicemia, pneumonia, and meningitis (14).

2. Materials and Methods

2.1. Patients

Samples involved in the current study were collected using TURBT, 68 patient's Formalin Fixed Paraffin-Embedded Tissues (FFPT) blocks were collected from different histopathological laboratories at AL-Najaf Governorate. UBC patient's age was 50-80 years while the control group age was between 35-62 years old. Immunohistochemistry technique have been used for

detection of infection within the tissue and to predect the severity of infection.

2.2. Samples Preparation

The study was performed at the Medical and Educational Research Facility (MERF) building, Urology Department, Professor Yi Lou Immunotherapy Laboratory, Carver College of Medicine, United States of America. Two slides were prepared for each FFPE tissue sample using Lieca microtom, tissues with 4µm thickness were laid over a Fisherbrand™ Superfrost™ Plus Microscope Slides, tissues were cut using sterile microtome blades. To confirm Histopathological diagnosis, One slide was stained with Hematoxylin and Eosin stain, and the second slide was used for immunohistochemistry staining with Alkaline phosphatase universal AK-5200 (Vectastain® ABC kit).

2.3. Gram positive Lipotichoic acid antibody

Abcam Mouse monoclonal antibody, isotype IgG1 targeting Lipoteichoic Acid (LTA) of Gram positive bacteria was used as a primary antibody in the current study. It reacts specifically to *Listeria monocytogenes* (all serotypes), *Streptococcus pneumoniae, Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecium, Bacillus cereus, Bacillus subtilis* and group *B Streptococcus* (weak), it does not react with *Clostridium perfringens*. The immunogen of this antibody is an intact *Listeria monocytogenes* antigene. The antibody storage buffer preservative content is 0.1% Sodium Azide, pH 7.2.

Mouse monoclonal IgG1(Abcam) targeting mouse ovalalbumin was used as a primary antibody for testing the control slide and detection of nonspecific binding.

2.4. Immunohistochemistry procedure:

IHC technique was carried out using vector laboratory manufacturer instructions, Deparaffinization and rehydration was done using DACKO auto-staining machine. Ag Epitope retrieval was done by cooking of slides for 15 minutes in Na-citrate buffer (10 mM , pH6.5)(vector) in microwave (Pelco Biowave). Blocking of endogenous peroxidase activity was done with 1% H2O2 (1.5 ml of 30% stock Sigma H-1009 in 50ml Phosphate Buffer Saline(PBS)) for 20 minutes, then slides were soaked in PBS for 5 minutes. Blocking of non–specific antigens was done by incubation of slides in vector blocking serum for 20 minutes (1drop of horse serum in 5 ml PBS), then excess serum was blotted from sections. The primary Abs was diluted (1:50) and slides were covered with the primary antibody and incubated for 1 hour, slides were soaked in PBS for 5 minutes at room temperature. The control slide was incubated with mouse monoclonal nonspecific IgG1(abcam) antibody for checking of false positive or nonspecific binding. The conjugated secondary Ab was added to slides and incubated for 30 minutes at room temperature, then soaked in PBS for 5 minutes. ABC reagent (2 drops of reagents A & B in 10 ml of PBS) was added to slides and incubated for 30 minutes at room temperature, slides were soaked in PBS for 5 minutes, then rinsed under cold tap water for 5 minutes. Slides were dipped in counter stain Hematoxylin (Fisher CS401-D) for 20 secondes, and rinsed in tap water until water came out clear. Slides were rehydrated through alcohol gradient started at 30 % ethanol up to 100% ethanol (2 minutes each), then soaked in xyline 3times for 5 minutes. Slides were mounted with premount (Fisher SP15-100) and pressed with coverslip and let to dry, finally, slides were examined and imaged using Olympus BX51 Microscope.

2.5. Calculation of severity of infection:

To calculate severty of infection all slides were divided into 6-fields, Each field containing >10 bacterial cells were considered as a positive field. Severty of infection was divided into four levels according to number of fields in which sever infection contained ≥6 fields, moderate infection= 3-5 fields, mild infection ≤ 2 fields and 0 fields =no infection.

2.6. Bacteria outlining:

According to bacterial morphologies recognised in each microscopic field, bacterial cells were divided into Gram positive cocci, Gram positive bacilli and diplococci (*Streptococcus pneumoniae*).

2.7. Statistics :

Chi-square test was used to calculate P value , P<0.05 and P<0.001 were consedered as significant result. Fisher's odds ratio calculator was used to calculate odds ratio, risk ratio and Confidence Intervals.

3. Results

3.1. Patients descriptive data:

Pateint's history sheets were collected to verify patient's descriptive data (Table 1).

Table (1) Total Patients Number and Gender.

Patients	Male No. (%)	Female No. (%)	Total
UBC	41 (85.42%)	7 (14.58%)	48(100%)
Control	13 (65%)	7(35%)	20(100%)
Total	54(79.41%)	14(20.59%)	68(100%)
P=0.06151, Risk Ratio= 0.4167 and Odds Ratio = 0.3171			

Male was the predomenant gender for both UBC and control groups (79.41%), High percentage (86.41%) of UBC pateints were suffering from low grade non-invasive urinary bladder carcinoma. There were a nonsignifiant difference P> 0.05 between age groups.

3.2. Immunohistochemical staining results:

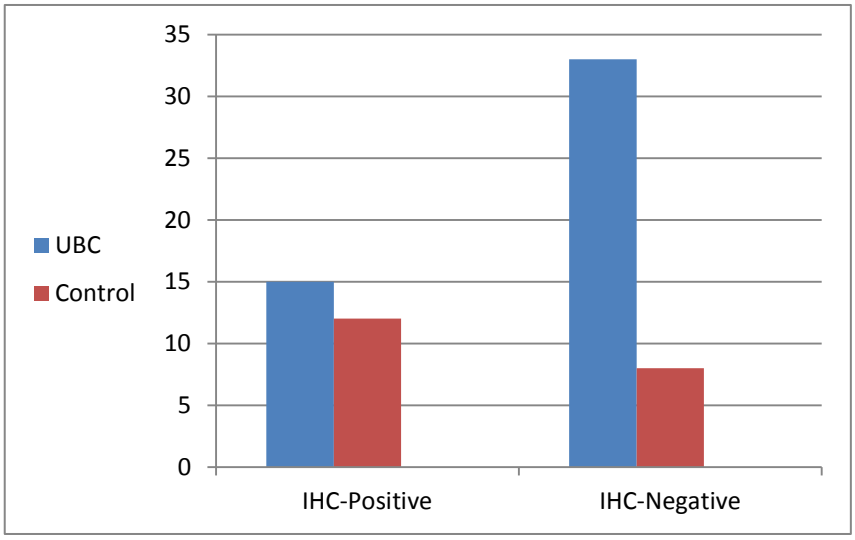

Figure 1: Immunohistochimestry Positive results among Urinary bladder cancer and control groups patients P=0.02726, Risk Ratio= 1.7188 and Odds Ratio = 3.3).

Urinary bladder tissue samples showed appearance of IHC positive results among 27 (39.71%) of pateints with a significant difference P<0.05 (figure1).

Table (2) Immunohistochemistry Staining results according to gender.

Patients	Gender	IHC results				
		Positive	%	Negative	%	Total
UBC	Male	14	51.85	27	65.85	41
	Female	1	3.71	6	14.63	7
Control	Male	8	29.63	5	12.19	13
	Female	4	14.81	3	7.31	7
Total		27	39.71	41	60.29	68(100%)
Chi-square = 5.8961			P=0.11677			

High percentage 51.85% of positive cases were for UBC infected male in comparison to control group male who showed a lower percentage (29.63%). Female group were the lowest in both UBC and control group (3.7 and 14.81%) respectively, results were a non sgnificant and P>0.05 (Table 2).

3.3. Severity of infection:

Severty of infection was calculated according to the criteria mentioned in 2.5. The results showed that,there were no mild infection (0%) among UBC patients and most infections were sever (60%), followed by moderate infection (40%), while the control group showed a lower percentage of sever infection 8.33% and mild and moderate infection were the predomenant (33.34 and 58.33%) respectively. The results were highly significant and P<0.05 (table 3).

Table (3) Severty of Infection among UBC and control patients.

Patients	Severty of infection			
	Mild	Moderate	Sever	Total
UBC	0(0%)	6 (40%)	9 (60%)	15(55.56%)
Control	4 (33.34%)	7 (58.33%)	1 (8.33%)	12(44.44%)
Total	4(14.81%)	13(48.15%)	10(37.03%)	27(100%)
Chi-square= 10.2704		P-value =0.005886		

3.4. Bacteria outlining:

Microscopical examination of bacterial cell morphology showed appearance of mixed infections and presence of more than one type of bacterial cells within all samples (table 4) (figure 2). Cocci bacteria were the commonest among both UBC and control group (41.67 and 40%) respectively the rates of bacilli and diplococci bacteria were low in both groups (27.78%,20%, 30.56% and 35%) respectively.

Table (4) Bacterial morphology within UBC and control group tissues.

Patients	Bacterial morphology			
	Cocci	Bacilli	Diplococci	Total
UBC	15 (41.67%)	10 (27.78%)	11 (30.56%)	36(100)
Control	13 (40%)	7 (20%)	4 (35%)	24(100)
Total	28	17	15	60
Df=2,Chi-square= 1.6		P-value =0.449		

All bacterial types were almost in the same rate within both UBC and control group with a non-significant difference between them. Many samples showed the presence of intracellular infected urothelial cells mostly with bacilli shaped bacteria and extracellular infection with cocci, bacilli and diplococci bacteria. No positive reactivity was found during control slide examination (figure 3).

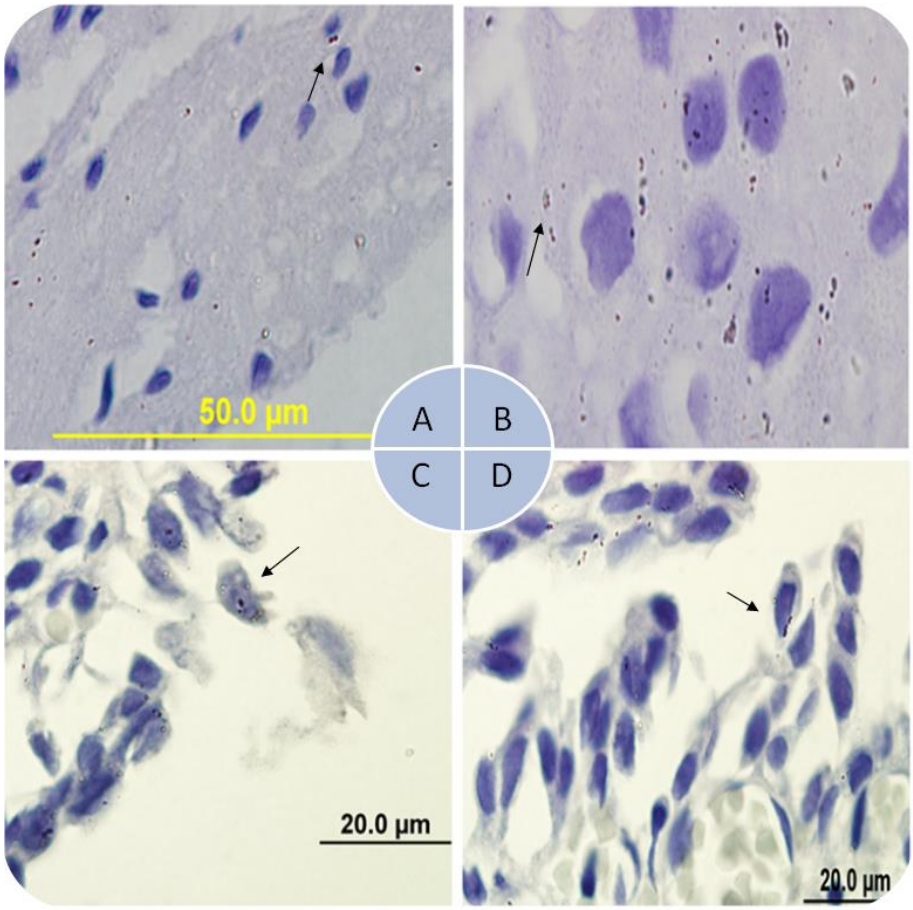

Figure 2: different types of Gram positive infections recognised among UBC patients, A- Gram positive diplicocci bacteria, B- mixed infection with predominant diplococci, C- cocci infection and D- Extracellular and intracellular Bacilli infection.

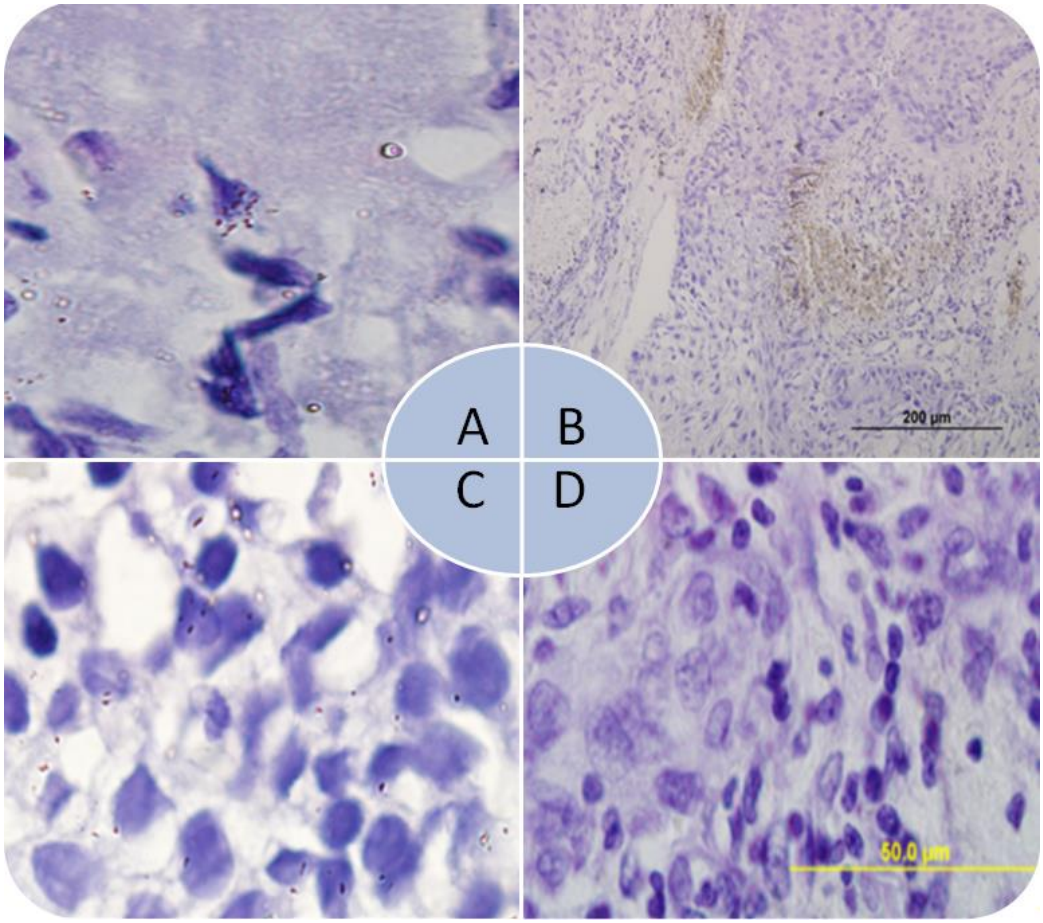

Figure 3: Different forms of gram positive infection with one negative control slide, A -Extracellular bacilli cells, B - 6-feilds UBC-IHC positive infection, C-Intracellular Bacilli cells, and D- Negative control.

4.Discusion

Urinary Tract Infections, and their complications, cause serious health problems, which affect millions of peoples every year, UTIs are the second most common type of infection in the body. In men, they are not as common as in women yet they can be very serious when they do occur (15). Studies confirmed the role of gram negative bacteria specially *E. coli* in UTI pathogenesis and even it was considered as a cause of neoplasia and may lead to urinary bladder carcinoma (16,17,18,19). Others considered that, UTI may be a consequence of UBC rather than a cause of the disease. However, it may have a later-stage effect in bladder carcinogenesis by promoting initiated tumors (20, 21). The role of some important gram positive bacteria in association with UTIs specially among UBC patients is unclear for some extent. In our study we tried to investigate the association and the severty of UTIs caused by some important gram positive bacteria among forty eight UBC patients and twenty control. In the current study IHC technique was used to detect bacterial LTA within the tissue, most studies used clean-catch midstream (CCMS) technique to collect urine (4,7) but midstream urine samples donot always able to give the true picture of UTI causing pathogens due to contamination of urine during voiding and are of no significance. In addition urine being an excellent supportive medium for growth of most bacteria and can be immediately contaminated in laboratory unless refrigerated or preserved (21). The use of voided urine in diagnosing UTI causing agents is not always suitable especially in Iraq due to low level of health care education among patients and unawareness of CCMS. IHC-results were 39.71% positive (figure 1 and table 2), 55.56% of them were for UBC patient's this rate of gram positive infection is simillar to other studies who showed that gram negative infection formed seventy-seven percent of the positive cultures and 23% grew gram-positive bacteria (4,22). Although, there were a nonsignificant difference between the study groups according to gender $P>0.05$, but, the UBC infected male recorded the highest rate of infection 51.85% followed by men from control group and this is an expected result because the total number of men in this study is higher than the female number (table 2). UTI is common in older men, especially nursing home residents and it is always regarded as a complicated infection among UBC infected and non infected men with increase in incedance that is caused by a range of factors including pyelonephritis and prostatitis, a neurogenic bladder, urologic abnormalities (e.g.,obstructive uropathy due to prostatic hypertrophy), kidney stones, chronic renal failure, diabetes mellitus, weakened immunity and about 43-56% of all UTI is associated with the use of indwelling urinary catheters (23,24). All the above factors explain severity of infection among UBC patients (table 3) in

which, high rate of UBC patients (60%) showed sever infection with more than 6 microscopical fields filled with ≥10 bacterial cells/one field. In addition, most cases were having mixed infection of gram positive bacteria and almost all cases having an almost equal rate of cocci and diplococci infection (table 4). Although, published reports of UTI associated with *S. pneumoniae* are scarce, and it is not generally considered as an agent of UTI in either adults or children(14, 15). But among our study group (30.56%) of UBC cases showed apearance of diplococci bacteria (figure 1-Aand B) which is a characteristic cellular morphology for *S. pneumoniae* (25). Zefiryn and collegues 2013 used multiplex polymerase chain reaction in the diagnosis of microorganisms isolated from urine of patients treated in cancer hospital and they couldn't detect *S. pneumoniae* (26). In a study for the incidence of *Streptococcal* diseases among elderly adults they found that the incidence of Group B *Streptococci* (GBS) disease is lower than that of *S. pneumoniae* infection, but GBS accounts for a greater proportion of the pathogen-specific total disease burden among elderly persons than either *S. pneumoniae* or group A *Streptococcus* (GAS) infections. Each of these 3 pathogens has a high case-fatality rate among elderly patients with invasive infection and *S. pneumoniae* was having the highest Case-fatality rate among elderly persons, deaths per 100,000 population =9.1so it requires paying more attention for diagnosis (24).

Gram positive cocci bacteria recorded 41.67% of the IHC results among UBC patients and 40% among control group and this is a high percentage in comparison to the rate of other study condudcted in Erbil/Iraq who isolated only *S. aureus* (14.29%) and *S.epidermidis* (7.14%) this variation in the rate can be atributed to the fact that gram positive pathogens can be missed when using voided urine culture (4) and this is one advantage for using IHC for detection of gram positive bacteria. The primary antibody in our study is specifically reacting with *S. aureus, S. epidermidis* and *E. faecium* LTA, these are cocci bacteria and they are an important pathogens, requiring anti-microbial therapy. *S. aureus* and *E. faecium* strain's are able to cause sever hospital acquired infections and are becoming increasingly resistant to various antibiotics and require serious therapeutic management. These are the second most common organisms recovered from hospital associated urinary tract and wound infections and the third most common cause of hospital associated bacteraemia specifically methicillin-resistant *S. aureus*, methicillin-resistant coagulase-negative *Staphylococci*, vancomycin-resistant *enterococci* (13,27).

Gram positive bacilli cells were the third rate in our study (27.78%) of the positive results among UBC patients. The primary antibody in this study is specifically reacting with the LTA of *L. monocytogenes* (all serotypes), *B. cereus* and *B. subtilis* which are gram positive bacilli shaped bacteria. As far as we know, there were no other IHC study recorded the isolation of such organisms or considered them as a UTI causing agents. However, our study is the first study that confirm association of Gram positive bacilli bacteria with UTI infection (figure 1-D and figure 2-A and-C). Two types of bacilli infection were found, intracellular and extracellular, for our study we cannot confirm *L. monocytogenes* infection and it requires further molecular diagnosis for species identity confirmation. The results of this study in our opinion is very important and it gives clear picture for the gram positive infection among UBC patients that should be payed more attention.

In conclusion, using of IHC technique in diagnosing of bacteria associated with UTI is very usefull method and can reflect the true picture for the infection. *S. pneumoniae, L. monocytogenes* (all serotypes), *B. cereus* and *B. subtilis* should be considered as UTI causal agents. It is important to be aware of this unlisted emerging pathogens because they are a way from their specific site of infection and it can be more virulent. Culture and Gram stain alone can be inconvenient for diagnosis of UTI causal pathogens because intracellular organisims are diffecult to be isolated using urine while dead gram positive bacteria appear red and can be false diagnosed as a gram negative specially for gram positive bacilli cells.

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