

Molecular Detection of Mycobacterium tuberculosis in Urinary Bladder FFPET Samples Could be a Proposed Specific Entity Of Bladder Carcinoma.

كشف جزيئي عن عصيات السل في نماذج سرطان المثانة الشمعية المثبتة بالفورمالين وإمكانية اعتبارها مؤثر خاص لسرطان المثانة.

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الخلاصة

خلفية البحث: سرطان المثانة هو أحد الأمراض السرطانية الإنسانية الأكثر شيوعاً في العراق والعالم. ويعتبر التدخين، الجنس، العمر، والتعرض المهني إلى الأمين العطري من بين عوامل الخطورة الأبرز لهذا المرض، يضاف إلى هذه العوامل عاملاً الالتهاب لفترة طويلة والإصابات المرضية المزمنة واللذان قد يلعبان دور كبير في نشوء سرطان المثانة.

الارتباط بين الالتهاب الجرثومي وسرطان يُعدّون أقوى خصوصاً في النوع المزمن من العدوى، ذكرت العديد من الدراسات دور الإصابة المزمنة بـ *Escherichia coli* وارتباطها بسرطان المثانة لكن لم نجد دراسة تذكر دور الإصابة بعصيات السل المزمن وعلاقتها بسرطان المثانة. في الدراسة الحالية عملنا على عينات نسيج المثانة المحفوظة في الفورمالين لمرضى عراقيين وقد شملت العينات عينات سرطان المثانة وعينات لإصابات مرضية غير سرطانية، أجريت الدراسة الحالية للكشف عن عصيات السل في الإنسان باستعمال تقنية تفاعل أنزيم البلمرة المتسلسل.

الهدف: الكشف عن دور عصيات السل وتأثيرها في سرطان المثانة.

المنهجية: الدراسة الحالية عملت على 75 نموذج من الأنسجة المثبتة بالفورمالين للمثانة خمسون عينة من العينات كانت لمرضى سرطان المثانة وخمس وعشرون عينة مثانة لإصابات غير سرطانية مختلفة جمعت العينات من عدة مختبرات خاصة في محافظة النجف. 84 % من عينات سرطان المثانة كانت المجموعة السائدة فيها من الذكور والفئة العمرية كانت 61-80 سنة (60 %) لكلا الجنس. حالات انتشار من مستوى الدرجة واطي كانت الأكثر شيوعاً وقد سجلت (64 %). جزء كبير من الدراسة تم إجراءه في مركز البحوث الطبية في كلية الطب / جامعة أيوا / الولايات المتحدة الأمريكية. تم استخلاص الحامض النووي من العينات وقد تم تقييمه كمياً ونوعياً بواسطة جهاز النانودروب وباستخدام جين البيتا كلوبين على التوالي.

النتائج: تم استعمال جين البيتا كلوبين في التقييم النوعي للحامض النووي، أظهرت نتائج تفاعل البلمرة المتسلسل (PCR) بأن 80 % من العينات كانت إيجابية والعديد من العينات أظهرت وجود نسخ متعددة. الكشف عن بكتيريا السل الرئوية تم باستعمال تفاعل -PCR,IS6110 33 % من النتائج كانت إيجابية بالنسبة لمرضى سرطان المثانة بينما العينات الغير سرطانية كانت إيجابية بنسبة 4 % من العينات، باختلاف إحصائي هام $P > 0.01$. تم تشخيص النوع الدقيق للبكتيريا باستعمال ITS-PCR، التصنيف النوعي تم باستعمال مناطق تفاعل متسلسل داخلية منسوخة وقد استعمل في هذا الاختبار العينات التي أظهرت نتيجة إيجابية باختبار تفاعل -PCR,IS6110 (55.56 %) من الحالات أظهرت نتيجة إيجابية.

الاستنتاجات: تم الكشف عن وجود نسبة عالية من عصيات السل في سرطان المثانة مقارنة بحالات إصابات المثانة الغير سرطانية. MTBC - لها ارتباط مؤثر باستحداث سرطان المثانة وهذا المقترح يحتاج إلى دراسة أكثر.

التوصيات: اعتماد التقنيات الحديثة في التشخيص الجزيئي للكشف عن علاقة عصيات السل وتحديد العلاقة بين الإصابة المزمنة بعصيات السل وتأثيرها في استحداث سرطان المثانة.

Abstract

Bladder cancer is one of the most common human cancers in Iraq and the world. While smoking, age, gender, and occupational exposure to aromatic amines are the most prominent among the risk factors identified, long term Inflammation and chronic infection may largely play some role in urinary bladder cancer (UBC) development.

The association between bacterial inflammation and cancer appears to be stronger especially in chronic type of infection, many studies mentioned the role of chronic *Escherichia coli* (*E. coli*) infection and UBC but there was no study mentioned the role of chronic *Mycobacterium tuberculosis* (MTB) infection in association with UBC. There are many cellular and immune responses that occur during chronic Mycobacterial infection such as irritation, long term inflammatory cytokine production and DNA damaging due to bacterial metabolic products are some factors that may give chance for UB neoplastic changes. In the present study we worked on urinary bladder cancer and noncancerous Formalin-Fixed Paraffin Embedded (FFPE) Tissue specimens of Iraqi patients, the current study used polymerase chain reaction (PCR) for detection of *Mycobacterium tuberculosis* genome.

Objectives: investigation for the role of *Mycobacterium tuberculosis* and its association with urinary bladder cancer.

Methods: The current study used urinary bladder cancer (UBC) formalin-fixed paraffin embedded tissues (FFPET) specimens of Iraqi patients collected from several private histopathology labs in AL-Najaf Governorate, the specimens were 50 samples of UBC patients and 25 different noncancerous pathological bladder samples. 84% of UBC cases were male and the predominant age group was 61-80 years (60%) for both gender. low grade cases were the most common which recorded (64%) and they were mostly TaN0M0 staging (42%). Large part of the study was performed at the Medical Education Research Facility (MERF)/Carver college of medicine/University of Iowa/United States of America. Samples of DNA were extracted and DNA quantitation was performed using NanoDrop DNA quality was evaluated using human β -globin gene amplification.

Results: human β -globin gene amplification used in DNA quality evaluation, PCR results showed that 80% of samples were β -globin positive and many samples showed appearance of multiple copies. Detection of *Mycobacterium tuberculosis* complex (MTBC) DNA using IS6110-PCR amplification showed 33% of UBC samples with positive results and 4% of noncancerous samples showed positive result, with high significant difference $P < 0.01$. Identification of MTB subspecies was performed using Internal Transcribed Spacer Regions-PCR (ITS-PCR), 55.56% of UBC IS6110-PCR positive cases were ITS-PCR positive.

Conclusions: A high percentage of MTBC and MTB detected among patients with UBC in comparison to noncancerous cases, suggesting that *MTBC-associated bladder carcinoma* is a proposed specific entity of bladder cancer which need to be more clarified.

Recommendations: Modern techniques should be used for MTBC and MTB detection in laboratories and further study for the relationship between chronic MTB infection and UBC.

Keywords: UBC: urinary bladder cancer, MTB: *Mycobacterium tuberculosis*.

INTRODUCTION

Urinary Bladder Cancer (UBC) can be divided into different types, including transitional cell carcinoma, squamous cell carcinoma and adenocarcinoma. Bladder cancer was the 9th most common cancer globally in 2008 in which it was the 7th most common cancer among males and 17th most common malignancy in females worldwide⁽¹⁾. UBC is the third most common cancer among Iraq patients.⁽²⁾ Risk factors including smoking, male sex and old age are the most common risk factors and accounts for approximately half of all UBCs, Occupational exposure to aromatic amines and polycyclic aromatic hydrocarbons are other important risk factors⁽³⁾. However, chronic infections of the human urinary bladder as well as, is an important risk factor in the pathogenesis of bladder cancer⁽⁴⁾. It has been noticed that preneoplastic lesions of humans and rodents bladder included a variety of types of proliferative cystitis, which are caused by infection⁽⁵⁾. It is well known that chronic urinary bladder *Schistosomiasis* is associated with UBC, specially squamous cell carcinoma (SCC)⁽⁶⁾ chronic inflammation and irritation in the urinary bladder due to deposition of *Schistomes* at the site of inflammation was found to be associated with the SCC initiation.⁽⁷⁾

It has been found that presence of endogenous bacterial infections (cystitis) and some intestinal opportunistic bacterial infection would metabolically activate the bladder procarcinogens⁽⁸⁾. Although, the connection between bacterial infection and UB carcinogenesis is still with some controversy this might be due to absence of clear agreement on the molecular mechanisms by which bacteria might induce carcinogenesis. However, some studies hypothesized that, Bacterial toxins and secondary metabolites formed by chronic bacterial infection might predispose to carcinogenesis in addition, Chronic inflammation resulted from persistent bacterial infections may lead to tissue neoplasia.^(8,9) The oldest and well known cause of chronic infection is *Mycobacterium tuberculosis* (MTB), during the course of Mycobacterial infection within the host, Nitric Oxide Species NOSs is produced by both macrophages and Mycobacterial production of nitrate reductase that can lead to DNA damage and mutations in urothelial cells.⁽¹⁰⁾

The theory by which tuberculosis promote cancer consider that Inflammation and fibrosis are an important factors in carcinogenesis; although many factors contributing in this

process, but tumor necrosis factor α (TNF- α) is critical and can promote tumor-cell survival through the induction of genes encoding nuclear factor- κ B-dependent antiapoptotic molecules.⁽¹¹⁾

AIM OF THE STUDY

The current study seeks to investigate the possibility of potential association of MTB as risk factors for urinary bladder cancer among Iraqi patients. Understanding Mycobacterial-induced carcinogenesis might enable us to prevent and cure some forms of urinary bladder cancers.

MATERIALS AND METHODS

Patients :

Seventy five formalin fixed paraffin embedded urinary bladder tissues were collected from patients with minimum age 17 years and the maximum age was 99 years. 50 samples were for UBC and 25 sample were for different pathological noncancerous cases.

DNA extraction:

Using sterile microtome blades (Leica Microtome RM2135) each tissue blocks were sectioned into pieces with 40 μ m thickness and preserved inside 2 μ l eppendroff tube in order to be used for DNA extraction. Qiagen DNeasy Blood & Tissue mini kits (50 tests each) were used to extract genomic DNA from FFPE tissue. The extraction procedure was performed according to the manufacturer instructions.

DNA quantity measurement was done before starting the PCR, to calculate the total amount of DNA sample that should be used in each PCR process, Measuring of DNA quantity was done using NanoDrop system which was consisted of NanoDrop machine (Thermo Scientific NanoDrop 1000 Spectrophotometer) and its software.

The quantity of sample's DNA used in PCR process was calculated according to the formula $(C_i) \cdot (V_i) = (C_f) \cdot (V_f)$.

C_i = initial concentration

C_f = final concentration

V_i = initial volume

V_f = final volume

“Initial” refers to the concentration/volume of the “stock” reagents and “final” refers to the desired concentration/volume.

Evaluation of samples DNA quality:

The DNA quality was evaluated by amplification of a 260-bp fragment of β -globin gene by using GH20 & PCO4 primers. To assess DNA integrity, Beta-globin (β -globin) amplification was chosen as it is a very conservative housekeeping gene and can be used as an indicator⁽¹²⁾, the gene was PCR amplified in each sample using the following GH20 & PCO4 primers sequences:

GH20 (GAAGAGCCAAGGACAGGTAC). **PCO4** (CAACTTCATCCACGTTTACC).

All oligonucleotides used in the study were synthesized by Integrated DNA Technology (IDT, USA, Iowa). The amplification process was done according to the following protocol.

PCR was carried out using *Taq* DNA polymerase (Qiagen GmbH, Hilden, Germany) and PCR buffer. Genomic DNA (10 μ L) was amplified in a total volume of β -globin PCR reaction of 50 μ L; β -Globin was considered positive if 260 bp bands visualized using electrophoresis with Tris-Boric Acid-EDTA (TBE) buffer, a nil DNA reaction was used as a negative control for all PCR reactions.

The β -globin gene was amplified using BIO-RAD thermocycler, the following PCR program was used for amplification: 95°C-5 minutes, 95°C -15 seconds, 55°C-30 seconds, 72°C-30 seconds, Go to 2 repeat 35 cycles, 72°C-10 minutes, Hold at 4 °C and End.

The Detection assay was carried out using 2% TBE agarose gel electrophoresis and Ethidium Bromide (EtBr) staining was used for amplicon detection, 12µl was loaded in each gel well (consisted of 10µl PCR product+2µl 5X loading dye). 100bp ladder was used for detection of positive bands, any bands with 260bp molecular size was considered β-globin positive, bands were imaged using BIO-RAD Gel Doc®EZ imager.

Preparation of all PCR buffers and reagents in this study were done according to http://www.protocol-online.org/prot/Molecular_Biology.

Molecular detection For the Mycobacterium insertion sequence IS6110.

All UB-DNA samples were tested for the presence of *Mycobacterium tuberculosis* complex by the amplification of a 123 bp fragment of the insertion sequence *IS6110*. For detection of presence and absence of *IS6110* of MTBC, PCR was performed by amplification of 50µl PCR reaction and using of BIO-RAD thermocycler, primers used for amplification were mentioned by several studies⁽¹³⁾⁽¹⁴⁾. All oligonucleotides used in this step were synthesized by IDT, USA, Iowa. Primers sequences were :

IS6110F (CTCGTCCAGCGCCGCTTCGG).*IS6110R*
(CCTGCGAGCGTAGGCGTCGG).

Reagents used in the amplification process were the same one used in the amplification of β-globin gene. The following thermocycling program was used for DNA amplification: 95°C-5 minutes, 94°C-1 minute, 68°C-1 minute, 72°C-1 minute, Go to 2 repeat 45 cycles, 72°C-10 minutes, Hold at 4 °C and End.

The Detection assay was carried out using 1.5% agarose gel electrophoresis and EtBr staining for amplicon detection, Tris-Boric Acid-EDTA (TBE) buffer was used for electrophoresis, 12µl was loaded in each gel well (consisted of 10µl PCR product +2µl 5X loading dye). 100bp ladder was used for detection of positive bands, any bands with 123bp molecular size was considered *IS6110* positive, bands were imaged using BIO-RAD Gel Doc®EZ imager.

Molecular identification of *M.tuberculosis* subspecies.

Genus and Species-Specific PCR Primers were used in this step, primers sets for checking the Internal Transcribed Spacer Regions (ITS) for Conservative and polymorphic ITS sequences of *M. tuberculosis* (MTB) complex the primers sequences were.

TBF(5'-TGGTGGGGCGTAGGCCGTGA-3')**TBR**(5'-CACTCGGACTTGTTCCAGGT-3')

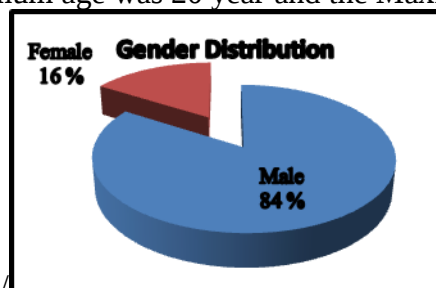
Species-specific primers were designed according to Park, *et al.*, 2000⁽¹⁴⁾. All oligonucleotides used in this experiment were synthesized by (IDT, USA, Iowa). The DNA samples were amplified using same reagents mentioned previously. ITS-PCR reaction was started with a 'hot start' followed by the following program; 94°C- 5 min, 94°C-1 min, 60°C-1 min, 72°C-1 min, Go to step 2 repeat 30 cycles, 72°C-10 min , hold at 4°C and End.

All *IS6110* positive samples were tested again with the Genus- and species-specific primers to confirm whether they were of *Mycobacterium tb.* or not. Negative control run was consisted of primers with sterile water and same PCR reagents mentioned previously. Amplified products were electrophoresed in 1.5% agarose gel and TBE buffer, gel was stained with ethidium bromide.

The ITS sequence has two conserved regions and several polymorphic regions. PCR with the *M. tuberculosis* complex-specific primers, TBF and TBR, was expected to amplify an approximately 121-bp fragment only in the *M. tuberculosis* strains appearance of 121bp bands in Gel electrophoresis were considered to be positive. Retesting of the same samples was performed to detect the actual size by using hyperlink ladder with molecular band size starting with 100bp and then 50bp, 100bp and 100bps. Respectively.

RESULTS:

Minimum age for all patients included in this study was 17 years and Maximum age was 99 years, the mean age was 60.43 and standard deviation was 14.44, the predominant age group for both malignant and noncancerous (NCA) pathological conditions for males and females was between 61 and 80 years old. UBC patient's average age was 63.52 the Minimum age was 20 year and the Maximum age was 99 year figure (1).



Figure(1): Gender distribution of UBC patients.

Evaluation of DNA Quantity:

DNA extraction process revealed different concentrations of DNA for each sample. The results showed a significant difference between concentrations in which, the minimum initial concentration was 1.8ng/ml and the maximum one recorded 336.8 ng/ml. with Mean 99.58 and Standard deviation 96.05. Fifteen (20%) of the samples were having a very low DNA concentrations and required to add 40µl of the sample alone to reach to the final volume (50µl) of the PCR volume.

Table (1): number and percentage of β -globin gene positive and negative amplification.

β -globin amplification	NCA		UBC		Total	
	No.	%	No.	%	No.	%
Positive	18	72	42	84	60	80
Negative	7	28	8	16	15	20
Total	25	100	50	100	75	100

$$X^2 = 1.5, P = 0.2207 (1 \text{ df})$$

Evaluation of DNA Quality:

sixty (80%) samples were positive for β -globin amplification and fifteen 20% of the samples were negative there was a non-significant difference between negative and positive cases $P = 0.2207$ shown in table (1).

Table (2): PCR results for detection of MycobacteriumIS6110 sequence among UBC and NCA samples.

Samples	IS6110 sequence		Total
	PositiveNo. (%)	NegativeNo. (%)	
UBC	17 (34%)	33(66%)	50(100%)
NCA	1(4%)	24(96%)	25(100%)
Total	18(24%)	57(76%)	75(100%)
$X^2 = 8.224$		$P = 0.004135 (1 \text{ df})$	

Molecular detection of Mycobacteriumtuberculosis complex insertion sequence IS6110:

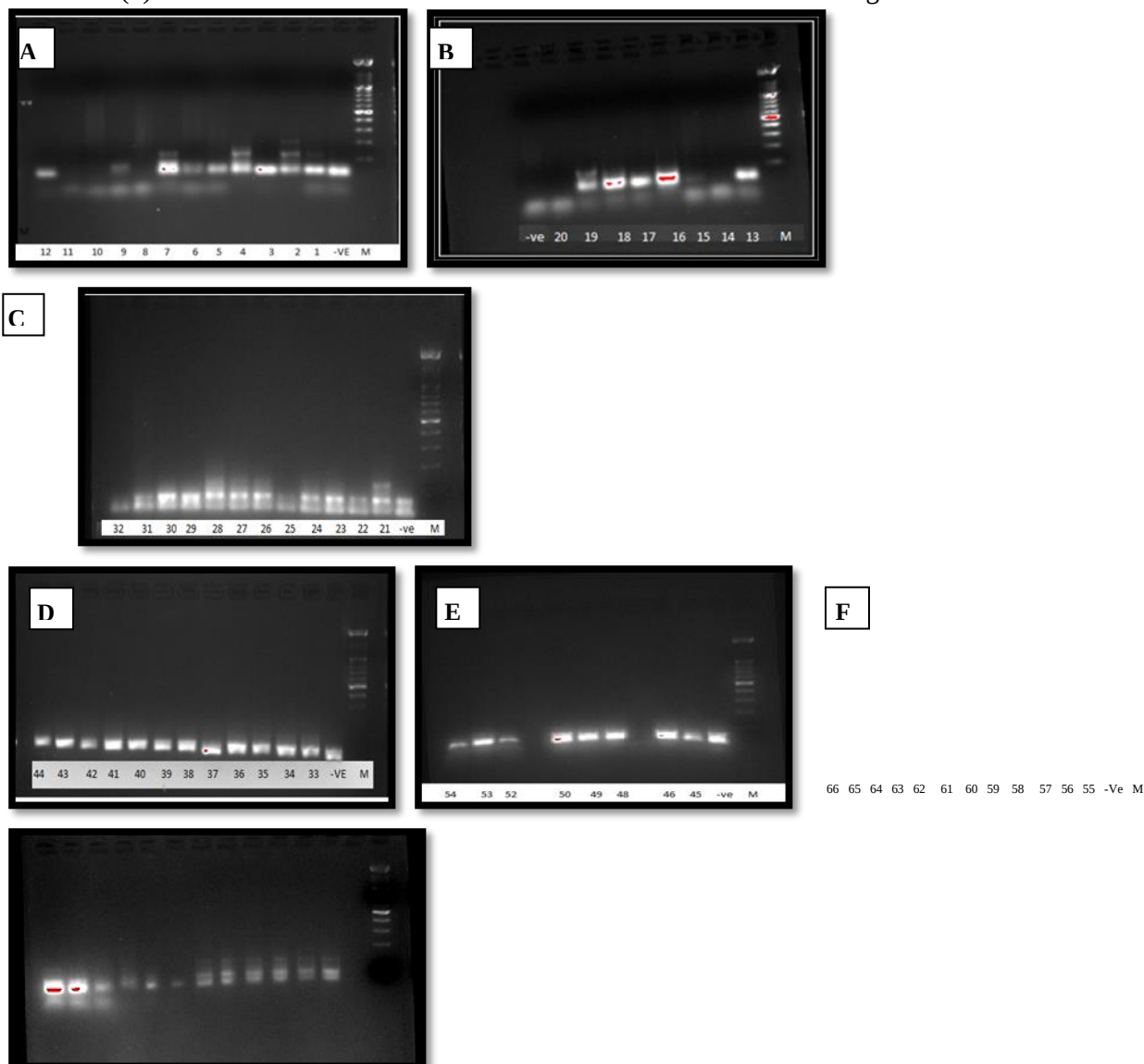
IS6110-PCR positive cases were 18(24%) and 17(33%) of them were for UBC samples and only one case 4% of NCA samples showed positive result, difference between malignant and NCA cases was highly significant P -value <0.01 table (2) and figure (2/A-G).

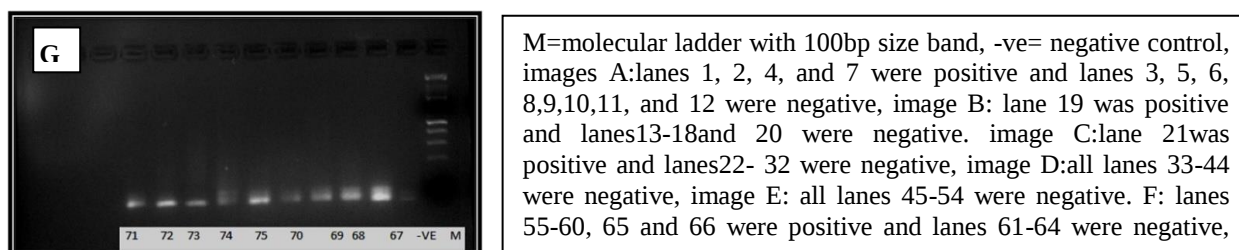
Table(3): Number and Percentage of *M. tuberculosis* positive cases amplified using ITS-PCR for UBC and NCA patients.

Patients	MTB positive	MTB negative	Total
UBC	9 (18%)	41(82%)	50(100%)
NCA	1(4%)	24(96%)	25(100%)
$X^2 = 2.827$		$P = 0.0927 (1 \text{ df})$	

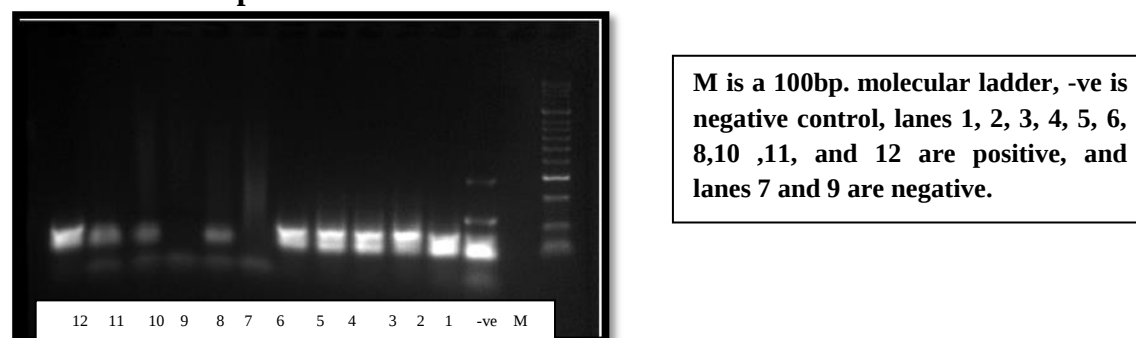
Detection of *Mycobacterium tuberculosis* subspecies:

According to the ITS-PCR retesting, results in Figure(3) revealed that *M. tuberculosis* species gave positive result in 10 patients out of 18 *IS6110* positive cases (55.56%), one case only gave polymorphic amplification (case no 1). Nine of these cases (90%) belongs to UBC patients forming percentage of 18% of the malignant cases (50 case) and one case only (4%) for NCA patient out of 25 case Table(3). P value was $P = 0.0927$ the difference was considered as non-significant.





Figure(2): PCR amplification of 123 bp region in IS6110 targeting PCR for *M. tuberculosis* complex.



Figure(3): Gel electrophoresis results for ITS-PCR retesting of IS6110 positive cases for detection of MTB species.

DISCUSSION:

Urinary Bladder cancer is a common malignancy, it is the 9th most common cancer worldwide⁽¹⁵⁾, the results showed that the majority of cases were of male group for both UBC and noncancerous patients, in which 77.33% samples out of 75 samples were from males, and UBC samples were 84% out of 50 case of UBC the male: female ratio recoded 5.25:1.0 these findings are higher than that published by Stenzl *et al.*, (2012)⁽¹⁶⁾ in their EAU guideline updating, they estimated that male: female ratio is of 3.8:1.0 and higher than that reported by the American Bladder Cancer Society, (2010)⁽¹⁷⁾ who reported that in the general population a man has a 1 in 27 and a woman a 1 in 84 chance of getting bladder cancer in their lifetime, this equates to a 3% chance in men and a 1% chance in women. This high percentage of UBC recorded among the study group can be attributed to many reasons including socioeconomic reasons (e.g. smoking), environmental reasons and due to exposure to a wide range of potentially carcinogenic substances such as weapons residue at war time.

Differences in the gender prevalence of bladder cancer can be due to many factors in addition to exposure to chemicals and tobacco smoking⁽¹⁸⁾ a study suggested that the differences in oestrogen and androgen levels between men and women could be responsible for some of the difference in the gender prevalence of bladder cancer⁽¹⁾.

UBC predominantly occur among old age patients, Olsson (2012)⁽¹⁹⁾ results showed, that patients in his study have a median age of 73 years at diagnosis, and 17% of them were female and these results are in agreement with the current study results, patients descriptive data showed that the predominant age group for UBC patients was 61-80 years followed by the 41-60 years regardless the gender. These results were in agreement with Matthew and colleagues results in 2008⁽²⁰⁾ they mentioned that late age (85 years or older) peak Incidence of UBC, and the percent of those patients with UBC is increasing with time much more rapidly than that fraction of the population or patients with other cancers. Ploeg, *et al.*, 2009⁽²¹⁾ also reported that more than 90% of UBC cases occur in people older than 55, and 50% of cases occur in people older than 73 years. In the other hand, the study results disagreed with a

Lebanese Case-Control Study conducted by Kobeissi *et al.*, 2013⁽⁶⁾ in which they reported that the average age was 67.1 years among Lebanese patients, our study recorded a lower age average (60.43 years), presence of 4-patients with age below 20 years decreased the age average in the study group.

Spectrophotometer NanoDrop was used to measure DNA concentration there was a large difference between minimum and the maximum concentrations. This difference can be attributed to many factors one of these is that the FFPE tissues used in the study were stored for 2-3 years and many of these samples were TURBT containing small pieces of tissues, these factors lead to decrease DNA yielding, most cases that gave high DNA concentration were for cystectomy patients. Using of NanoDrop system gave an idea about the real DNA concentration, other advantage of measuring initial DNA concentration is to make quality control of nucleic acid samples that lead to significant savings in time and money. During fixation DNA fragmentation may occur, and covalent bonds by DNA-proteins can be formed. Rate of DNA fragmentation depend on many factors including size of fragment, time of sample storage and fixation which is very important. Amplification of a 270 bp fragments can be done after a thirty-days of formalin fixation, while amplification of a 1.300 bp cannot be performed after a fixation period longer than 24 hours. human β -globin gene short fragments (270 bp) can be amplified after 15-year storage. In contrast, success rate of PCR amplification of fragments more than 500 bp rapidly decreases after 1 to 2 years of storage⁽²²⁾.

DNA quality of FFPE tissues were checked by amplification of β -globin primers PC04 and GH20, 80% of samples were positive and 20% of the samples were negative. there was a non-significant difference between positive and negative cases. Negative β -globin cases were mostly either because of low initial DNA concentration due to small tissue slices after cutting the FFPE by microtome, or for less extent the old FFPE tissue with bad storage that may lead to DNA degradation. a study suggested that using GH20 and PC04 probes give a guaranteed positivity of this segment⁽²³⁾.

PCR results showed appearance of more than one copy for some of the amplified β -globin gene and these are in agreement with Grimholt, *et al.*, 2014⁽²⁴⁾ and Sobah and Michael, 2001⁽²⁵⁾ results they worked on human globin gene locus and found that replication was initiated at more than one 5' site of β -globin gene PC04 and GH20 amplify two different regions in the same gene making Multiple copies appearance possible.

Depending on previous and recent studies that confirmed the role of *Mycobacterium* in different tumors types^(26, 10), the current study was conducted to confirm the role of chronic tissue inflammation with *Mycobacterium tuberculosis* complex genus in general and more specifically *Mycobacterium tuberculosis* subspecies in the process of UB carcinogenesis. As far as we know there is no other study documented the isolation of *Mycobacterium* DNA from archived FFPE tissue of UBC.

There were different techniques used for detection of MTBC in tissue samples but the most common, faster method and able to identify dead microorganisms was the PCR technique. Depending on the studies of Zink and Erlich, 2004⁽¹³⁾ and Cataloluk, *et al.*, 2003⁽²⁷⁾ the current study used IS6110-PCR test for detecting the insertion sequence IS6110 in the samples. The IS6110 is a 1191-bp repetitive insertion sequence that is usually present 6-20 times in the MTBC genome although as few as one copy has been observed⁽²⁸⁾. this sequence has been used frequently for detection and for molecular epidemiological studies of the MTBC group and in general, this sequence was approved to be present among Iraqi MTBC as shown in the studies of AL-JEBORY⁽²⁹⁾, 2006 and Ali, *et al.*, 2014⁽³⁰⁾.

The prevalence of MTBC among patient's urinary bladder FFPE tissue was 34% of malignant samples and only one case 4% of noncancerous samples depending on IS6110-PCR retesting results. The results came with a significant difference between malignant and noncancerous cases P-value <0.01.

MTBC share some virulence steps with microbial oncogens that help them to predispose the precancerous state or being a risk factor for tissue carcinogenesis e.g. persistency, chronic irritation, ROI production and being intracellular that may give chance for genetic interaction either with cellular genome or environment⁽²⁶⁾.

the results confirmed the presence of *IS6110* among MTBC isolates, this result was in agreement with the results of AL-JEBORY⁽²⁹⁾, 2006 and Ali, *et al.*, 2014⁽³⁰⁾ in which their data provided an important baseline information on the genetic diversity of *M. tuberculosis* in Iraq. Although, all those studies were conducted on *Pulmonary tuberculosis* (PTB) sputum samples and bovine milk samples and not on UBC.⁽³¹⁾

The use of DNA amplification for detection of *M. tuberculosis* in formalin fixed paraffin embedded tissue samples is a useful method for patients in whom diagnosis depends on tissue examination, rather than detection of *M. tuberculosis* in body secretion^(32,33).

However, despite that MTBC genomes are highly conserved, but comparative sequence analysis studies of the 275-bp internal transcribed spacer (ITS) region, showed that these regions are highly polymorphic region and it separates the 16S rRNA and the 23S rRNA, leading to complete conservation between MTBC members(Park, *et al.*, 2000)⁽¹⁴⁾. According to the above facts, our study used ITS primers specifically designed to amplify *M. tuberculosis* ITS regions, the retesting results showed that positive results appeared in 10 patients out of 18 *IS6110* positive cases (55.56%). Nine of these cases (90%) belongs to UBC patients forming percentage of 18% of the malignant cases and one case only (4%) for noncancerous patient. There was a non-significant difference between malignant and noncancerous cases this might be due to sample size.

As far as we know there was no previous study used ITS regions to detect MTB among UBC tissue samples to compare our results with them, but many studies confirmed the usefulness of using ITS- PCR for detection of MTB in FFPE tissues like Zink and erlich, 2004⁽¹³⁾ and HOSEK, *et al.*, 2006⁽³⁴⁾.

IS6110-PCR and ITS-PCR positive results were (18 and 10 respectively) ITS-PCR negative results may belong to species of MTBC other than MTB due to the presence of *IS6110* sequence in all species belonging to the MTBC. Although Richard, *et al.*, 2003⁽³⁵⁾ mentioned that the commonly used MTBC-defining element *IS6110* has been shown to be absent from some *M. tuberculosis* strains and in the other hand it is present in low percentage in *Mycobacterium* other than MTBC (MOTT) the possibility of being the patients infected with MOTT is also probable and this issue requires further investigation.

CONCLUSIONS

The results support the hypothesis that MTBC especially MTB strain can contribute to urinary bladder cancer development, particularly in chronic infection and/or inflammations suggesting that *MTBC-associated bladder carcinoma* is a proposed specific entity of bladder cancer which need to be more clarified. *IS6110*-PCR and ITS-PCR amplification method are feasible and reliable in diagnosing MTBC and MTB respectively in UBC FFPE tissue samples.

RECOMMENDATIONS:

- 1) Screening test should be performed on previously diagnosed UBC cases to test the presence of MTBC infection.
- 2) Using of *IS6110*-PCR and ITS-PCR are reliable and other methods of MTBC molecular techniques, such as spligotyping is required to aid diagnosis. In addition to Acid fast staining and routine methods used in UBC diagnosis.

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