ISOLATION AND MOLECULAR STUDY OF SOME BACTERIAL URINARY TRACT INFECTIONS OF SHEEP IN BASRAH PROVINCE

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ABSTRACT

This study was aimed isolation and molecular detection of some causative agents of urinary tract infection (cystitis and pyelonephritis). Out of 108 tested urine samples (56 from females and 52 from males); 60 samples (55.55%) have infected with *Escherichia coli* and *Klebsiella pneumoniae* were 36 (64.2%) females and 24 (46.1%) males. The sixty infected samples contain from 56 *E. coli* and (4) *K. pneumoniae*, this samples identified by Vitek 2 (44 isolates *E. coli* and 2 isolates *K. pneumoniae*) were subjected to DNA extraction. A total of 44 *E. coli* isolates detected to *FimH* and *pai* genes. 44/44 (100%) were positive for presence of *FimH* gene, and 20/44 (45.45%) were positive for presence of *pai* gene. The two isolates of *K. pneumoniae* which detection of *Ecpa* gene and given positive result to this gene 100%.

Keywords: Isolation of some bacterial, Urinary tract infection of Sheep

عزل ودراسة جزيئية لبعض المسببات الجرثومية لالتهاب المسالك البولية في الاغنام في محافظة البصرة

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المستخلص

صممت هذه الدراسة لعزل وتشخيص جزيئي لبعض مسببات التهاب المجاري البولية المتضمن التهاب المثانة والتهاب الكلية (الاشريكيا القولونية، الكلبسيلا الرئوية) في الاغنام. من خلال جمع 108 عينة إدرار أغنام منها 56 عينة من النعاج و 52 عينة من الاكباش، كانت المصابة 60 عينه وبنسبة 55.55 % معزولة من الأغنام المجزورة، 36 (64.2%) منها من الإناث و 24(46.1%) من الذكور. العينات المصابة تضمنت 56 عزلة للاشريكيا القولونية وأربع عزلات للكلبسيلا الرئوية، جميع العزلات خضعت لاختبار الفايتك، الذي اظهر بدوره وجود 44 عزل للاشريكيا القولونية وعزلتان للكلبسيلا الرئوية، جميع هذه العزلات تم استخلاص منها الحامض النووي واجري عليها اختبار تفاعل البلمرة المستمر. أظهرت النتائج وجود جين *FimH* بنسبة 100% كون 44 عزلة للاشريكيا القولونية أظهرت نتائج موجبة، بينما اظهر جين *pai* نسبة 45.5% لنفس العزلات حيث ظهرت 20 عزلة بنتيجة موجبة من 44 عزلة. فيما اظهر جين *Ecpa* نتائج موجبة لعزلتي الكلبسيلا الرئوية وبنسبة 100%.

الكلمات المفتاحية: عزل المسببات الجرثومية، التهاب المسالك البولية في الاغنام

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INTRODUCTION

Urinary system is one of the most important system in the animal's body, this function includes removal of toxic waste from the body and regulation of the components of body fluids, as well as control the hormonal secretion which promote the bone marrow to red blood cells formation (15). Urinary tract infection (UTI) means the colonization and infections by one or more urinary tract parts (32). The sources of UTI are by emanating bacteria which come from gastrointestinal tract causing colonizing of the external genitalia, invasion of the bladder and urethra against the flow of urine (6). UTI also cause damage of vascular of urinary bladder then decrease the function of kidney competence, and disturbances the excretion of end products metabolic (9). Urinary system of sheep has been less commonly affections compared with other species of ruminant, the important bacteria infected are *Corynebacterium pseudotuberculosis*, *C. renale*, *E. coli*, *K. pneumoniae*, *Actinomyces pyogens*, *Staphylococcus aureus*, and *Proteus* (4,27). The urine moves to the urinary bladder through the ureter come from the kidneys. To helps urinary system from bacterial invasion, it is found the valve to prevents the come back the urine to the ureter from the bladder, if happened invasion, may be opportunistic germs caused by the normal flora. This type of infection happened from the ureter to the kidneys (35). Pyelonephritis is the inflammation of all parts of kidney. It is occasionally affected the sheep while behold firstly a bovine disease, (27). A few reports of bacterial sheep urinary system infections with *E. coli* and other gram-negative coccobacillus (18). *Escherichia coli*, as well as *K. pneumoniae* are from the family of enterobacteriacea, gram negative bacteria, aerobic and anaerobic growing (14). In our country, a few researches have been executed for the very important system. The significant economic losses cause by this system by quantitative and qualitative reduction of animal productivity. This study was designed for isolation and molecular detection of some causative agents of urinary tract infection (cystitis and pyelonephritis) in sheep's.

MATERIALS AND METHODS

One hundred and eight samples were collected during the period of April to December 2019 (56 from female and 52 from male suspected with UTI) were collected from Al-Basrah abattoir in Basrah province, and after collected from sheep, the case history from the sheep owner, frequent attempts to urinate, discomfort, a slight fever, anorexia, polyuria and sometimes colic. Checking the animal before slaughtering. Then collected urine and tissue samples for microbiological, molecular and histopathological analyses.

Methods of collection

Tied the urinary bladders to retain the urine, by polyethylene bag wrapped and transported to laboratory by cold recipient. Sterile syringes and needles were using, after cleaning the puncture sites with water then alcohol 70%, urine was taken from the urinary bladders (10). To isolate *E. coli* and *K. pneumoniae*, gram stain is done (10,11). Poured the urine samples into capped sterile centrifuge tubes, then centrifuged for 10min at 2,000g, 1ml approximately of the sediment of urinary were added to 10 ml Tryptone soy broth, discarded the supernatant solution, incubated for 24hrs at 37ºC. Pre-enriched with trypton soy broth, then showing a loop-full turbidity of culture, streaked MacConkey plates and Eosin methylene blue, then incubated at 37ºC for 24 hours (9). By naked eye concerning their shape, colour and size of the colonies. Then the gram staining was done, and Vitek 2 confirmation. The antibiotic sensitivity test was carried out according to (20). In this study eight antibiotics were used, penicillin, chloramphenicol, gentamycin, streptomycin, ciprofloxacin, erythromycin, trimethoprim and tetracycline.

Molecular study

DNA extraction: By using bacterial DNA extraction kit (DNA extraction mini kit, Promega / USA) following the instructions of manufacturers. The concentration and purity of extracted DNA have determined using NanoDrop spectrophotometer (Optizen, Korea) at 260 nm and 280 nm and stored at -20°C (30).

Polymerase chain reaction

By using performing PCR technique, DNA of bacterial was amplified by used (Go Taq

Green Master mix(M7122), Promega/USA). Three primers pairs were designed to identify important bacterial organisms including *E. coli* two primers, those are *fimH* gene (F: 5'-GCCAAACGAGTTATTACCCTGTT -3' and R: 5'- CCTTGATAAACAAAAGTCACGCC -3') and *Pai* gene (F: 5'-TAGCTCAGACGCCAGGATTTTCCCTG -3' and R: 5'-CCTGGCGCCTGCGGGCTGACTATCAGG-3') (25) and *Klebsiella Ecpa* gene (F: 5'-AATGGTTCACCGGGACATCATGTCC -3' and R: 5'-AAGGATGAAATATCGCCGACATCC -3') (8). The amount used in this PCR, green master mix 25 µl, F primer 2 µl, R primer 2 µl, DNA template 10 µl and nuclease-free water 11 µl. The annealing temperature for fimH and Pai primers was 60C˚, while Ecpa primer was 62C˚. Detected the PCR product on agarose gel (1%) stained with ethidium bromide by used two ladders, 1,500 bp ladder (Promega/USA) and 1,500 bp ladder (Bioneer, Korea). Initial denaturation for PCR, 5min at 95°C. followed by 30 cycles of 95°C for 45sec, 58°C for 45sec, 72°C for 45sec. The reaction was then at 72°C for 6min, and cooled down 4°C for 5min. Detected the PCR product by agarose gel stained. PCR product then sent to Macrogene (Korea) company for sequencing. By using Parbi-Doua and NCBI BLAST programs, the sequences were edited and aligned.

Macroscopically and microscopically examination of kidneys and urinary bladders of sheep's

After slaughtered animals, limited the size of both kidneys and consistency, as well as urinary bladder for macroscopically examination. Then taken kidney and urinary bladder of infected slaughtered and healthy animal to histopathological examination according to (21).

RERSULTS AND DISCUSSION

The result of our study started by limited urinating as a very important sings, increased gradual with fluctuating temperature, loos of appetite, poor body condition, all these sings which represented from the case history according to the results out of the 108 tested urine samples; 60 samples 55.5% infected from suspected UTI of sheep (Table 1), all these sings which represented from the case history (28). The result of this study agreed to Petrovski in the cause of urinary tract infection are the bacteria and characterized by fever, colic and pyuria phenomenon and/or haematuria (24).

Table 1. Number and percentage of infected samples isolated from urine sheep

No. of Samples	No. of Infected samples	%	Sex	No. of Samples	No. of Infected samples	%
108	60	55.55	Female	56	36	64.2
			Male	52	24	46.1
Total			Both	108	60	55.55

The sixty infected sample was divided to 56 (52.8%) *E. coli* and 4 (3.7%) *K. pneumoniae*. This sample identified confirmative by Vitek 2, the result of Vitek 2 in this study are 46 isolates, divided to 44/56 (78.5%) *E. coli* and 2/4 (50%) *K. pneumoniae*. One of the most commonly pathogen isolated from pyelonephritis is *C. renal* and *E. coli*. In current study, the result accepted with Nikvand et al (23) who recorded that *E. coli* more important causative agent 21% of urine than *K. pneumoniae* was 5.3%. The differences of infected percentage between male and female because the female animals more susceptible to infected than male by urinary tract infection because many reasons like trauma of urethra, short urethra, effects of hormonal and reproductive system infection (26,34). The percentage of this study were disagreeing with the study of Fatihu et al (10) that reported the rate of infected female 6.3% and in male 16.7%. In our study, the Vitek 2 results for confirmation bacteria accepted with used Vitek 2 technology to identify *E. coli* (31).

Molecular identification by PCR assay

A total of 46 isolate samples which identified by Vitek 2 (44 isolates *E. coli* and 2 isolates *K. pneumonia*e) used for DNA extraction, the PCR used for confirmed *E. coli* by *FimH* and *pai* genes. 44/44 (100%) gave the positive result of *FimH* gene (Figure 1), and 20/44 (45.45%) gave the positive presence of *pai* gene (Figure 2). In the other hand, the present

study recorded 2/2 (100%) gave a positive results of *K. pneumoniae* isolates of *Ecpa* gene (Figure 3). The PCR results of 44 isolates of *E. coli* shown positive result with percentage rate 100% of the *FimH* gene presence, this result agrees with the results of Abdullah and Mustafa (2); Garofalo et al (12) that recorded the most prevalent virulence gene are *FimH* gene with percentage of the isolates 100%, as well as in Japan, recorded 99.4% of isolates have *FimH* gene (19). The main factor of virulence is a fimbria, which is very important in the adhesion to receptors of cells host, and protect bacteria from host response (7). The very important stage to development UTI due to the bacteria adherence to urinary epithelial cells, this allows bacteria to resistant and action flushing of the urine flow and bladder emptying, all this process stimulate the bacteria and activates to increase probability to staying in the urinary tract of the host (22). *FimH* gene is a gene responsible of fimbria, this give indicate the big problem because all this sample have this gene, which added the virulence of bacteria to adherence to urinary epithelial cells. The present study showed the positive result of *pai* gene in *E. coli* 20/44, the percentage rate 45.45% for presence of *pai* gene, the result has some deferent with the reported of Anad (1) in Iraq because he reported 57.1% of *E. coli* isolates by *pai* gene. From two sample of *K. pneumoniae* isolates which were identified by Vitek2, the result of PCR assay for detection *Ecpa* genes 2/2 (100%) were positive for presence gene. Our result agreed with Cruz-Córdova et al (8), who reported that 100% of the *K. pneumoniae* isolates have *Ecpa* gene, as well as Yassein reported 96% of clinical *K. pneumoniae* isolates (33). But the percentage of our result has higher than reported by Alcántar-Curiel et al (5) reach to 96%. The percentage changing of the of *Ecpa* gene because the *K. Pneumoniae* fimbriae nature played important role in the bacteria adherence to epithelial cells, it is regarded as a pathogenic an virulent agent which have related to the pathogenesis of *K. pneumoniae* (29).

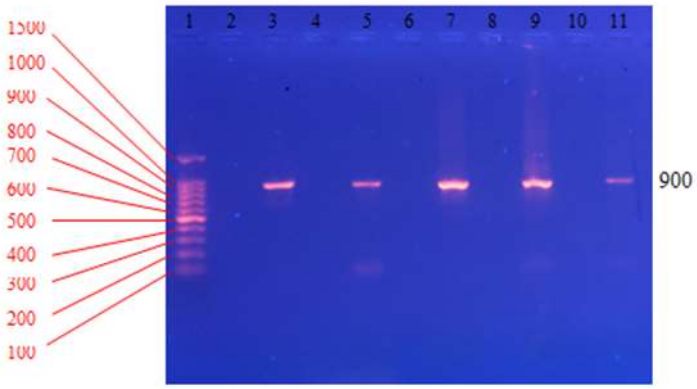

Figure 1. Agarose gel electrophoresis (1%) of PCR-amplified for *FimH* gene of *E. coli* isolates. Line. 1: DNA ladder (100bp). Lines. 3,5,7,9,11: *FimH* gene ≈ 900 bp; Line 2,4,6,8,10: Negative control

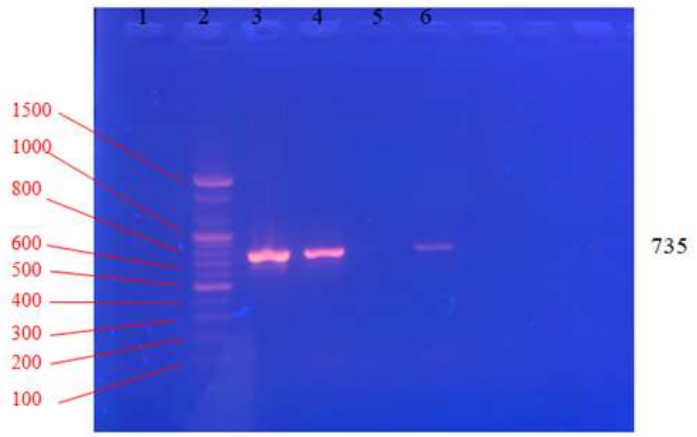

Figure 2. Agarose gel electrophoresis (1%) of PCR-amplified for *pai* gene of *E. coli* isolates. Line. 2: DNA ladder. Lines. 3,4,6: *pai* gene ≈ 735 bp; Line 5: Negative control

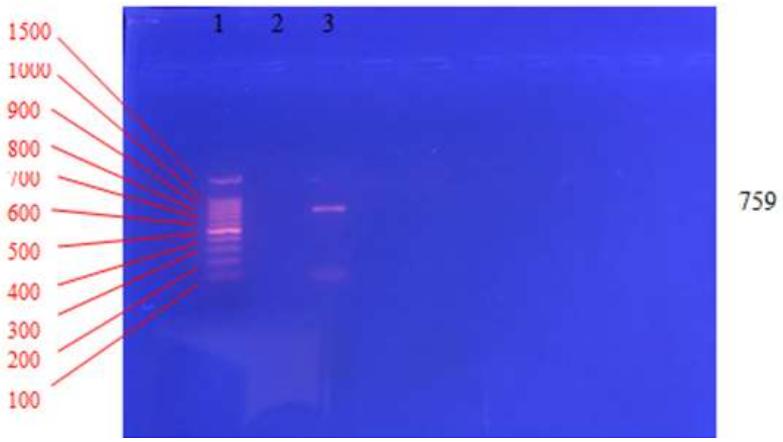

Figure 3. Agarose gel electrophoresis (1%) of PCR-amplified for *Ecpa* gene of *K. pneumoniae* isolates. Line. 1: DNA ladder. Line. 3: *Ecpa* gene ≈ 759 bp; Line 2: Negative control

Antibiotic sensitivity test

Table (2) showed the result of forty-four isolates of *E. coli* identified by PCR were tested for the susceptibility to eight antibiotics by using method of disc diffusion. The result showed 100% resistance to tetracycline and erythromycin, 56.8% for penicillin but showed 100% sensitive to streptomycin and gentamycin, 90.9%; 75% sensitive to ciprofloxacin and trimethoprim respectively and 59% for chloramphenicol. Table (3) showed the result of two isolates of *K. pneumoniae* identified by PCR were tested for the susceptibility to eight antibiotics by using method of disc diffusion. The result showed 100% resistance to penicillin , erythromycin and tetracycline, and the result shoed 100% sensitive to streptomycin, gentamycin, ciprofloxacin and trimethoprim, while 50% sensitive to chloramphenicol. All the antibiotic sensitivity test matched to Abdullah and Mustafa (2) research because the same area of sampling and same conditions but different animals. The present study matches with Soud in *E. coli* resistance to erythromycin which record 97%, as well as our study agreed with Soud in sensitive to gentamycin and streptomycin (31), but disagree with Islam et al their recorded erythromycin resistant to *E. coli* are 73.3% and same percentage susceptible to tetracycline (16).

Table 2. Antibiotic sensitivity test of 8 different antibiotics against 44 *E. coli* isolates

Antimicrobial agent	Concentration	Susceptible		Intermediate		Resistance	
		No.	%	No.	%	No.	%
Chloramphenicol (C)	10mcg	26	59	2	4.54	16	36.36
Ciprofloxacin (CIP)	5mcg	40	75	1	2.7	3	6.8
Erythromycin (E)	15mcg	-	-	-	-	44	100
Gentamycin (CN)	10mcg	44	100	-	-	-	-
Penicillin (P)	10mcg	18	40.9	1	2.7	25	56.8
Streptomycin (S)	10mcg	44	100	-	-	-	-
Tetracycline (TE)	30mcg	-	-	-	-	44	100
Trimethoprim (TR)	5mcg	33	75	2	4.54	3	20.45

Table 3. Antibiotic sensitivity test of 8 different antibiotics against 2 *K. pneumonia* isolates

Antimicrobial agent	Concentration	Susceptible		Intermediate		Resistance	
		No.	%	No.	%	No.	%
Chloramphenicol (C)	10mcg	1	50	1	50	-	-
Ciprofloxacin (CIP)	5mcg	2	100	-	-	-	-
Erythromycin (E)	15mcg	-	-	-	-	2	100
Gentamycin (CN)	10mcg	2	100	-	-	-	-
Penicillin (P)	10mcg	-	-	-	-	2	100
Streptomycin (S)	10mcg	2	100	-	-	-	-
Tetracycline (TE)	30mcg	-	-	-	-	2	100
Trimethoprim (TR)	5mcg	2	100	-	-	-	-

Sequencing and blast Analysis

The BLAST analysis performed through NCBI software to determine identity of *E. coli* and *K. pneumoniae* isolates in this study compared our reported with complete sequencing of genes *Pai*, *FimH* and *Ecpa*. Three straines for *pai* gene, two straines for *FimH* gene and only one strane for *Ecpa* gene are listed in table 4. This table represented the accession number which recived from NCBI.

Table 4. Accession number and identity of *Pai*, *FimH* and *Ecpa* genes sequence

Type of gene	Query	Subject	Identity (%)
***Pai* gene**	**MN180244**	**CP042969.1**	**98.92%**
***Pai* gene**	**MN180245**	**AP019675.1**	**99.41%**
***Pai* gene**	**MN180246**	**CP042250.1**	**99.56%**
***FimH* gene**	**MN180236**	**CP041749.1**	**98.95%**
***FimH* gene**	**MN180239**	**CP041678.1**	**98.85%**
***Ecpa* gene**	**MN180230**	**LR607348.1**	**99.58%**

Histopathological result

The present study recoded high enlargement in both kidneys size, the consistency is softening and pale. While the urinary bladder was distended with cloudy urine of alkaline pH of 8. Some causes the enlargement of kidney appeared in right kidney and found patchy congestion, and haemorrhage. The result of histopathological study represented in figures 4 and 5. Figure (4A) showed the normal kidney of sheep, this figure showed normal glomeruli and normal renal proximal tubules while figure (4B) showed infected kidney of sheep, there was atrophy of glomeruli, necrosis of renal proximal tubules and infiltration of inflammatory cells. Figure (5A) showed normal urinary bladder of sheep, with normal epithelium, and normal sub mucosa while figures (5B) showed infected urinary bladder of sheep, with hyperplasia of epithelium, infiltration of inflammatory cells and thickening of sub mucosa.The current study got close to Hajikolaei et al (13) and Ismail (17) who reported some similar changes, those studies in cow and buffalo showed congestion of blood vessels, hyperplasia of epithelium and thickening of kidneys. The result agreed with Abdullah and Ismail (3) who diagnosed pyelonephritis from urinary bladder of infected cow, hyperplasia and thickening of epithelium, and in the same study reverted that was hyperplasia and thickening of epithelium and blood vessels congestion in buffalo.

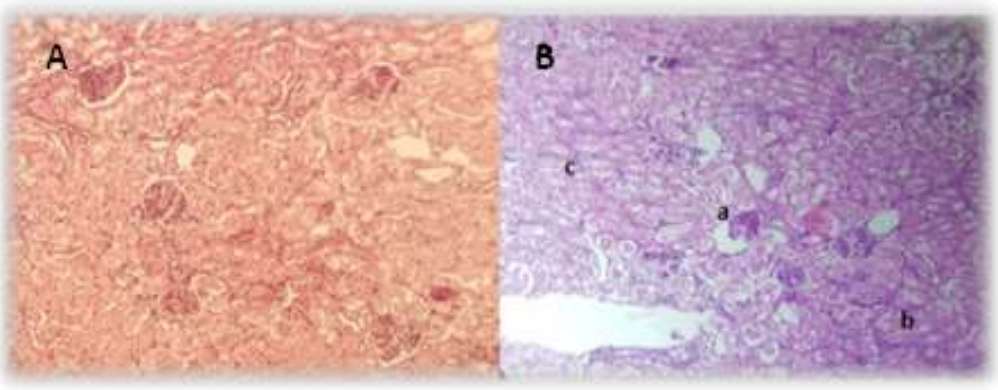

Figure 4. (A) Normal kidney of sheep: Normal glomeruli and normal renal proximal tubules. (B) Infected kidney of sheep: (a) Atrophy of glomeruli, (b) necrosis of renal proximal tubules and (c) infiltration of inflammatory cells. (H&E) (40X)

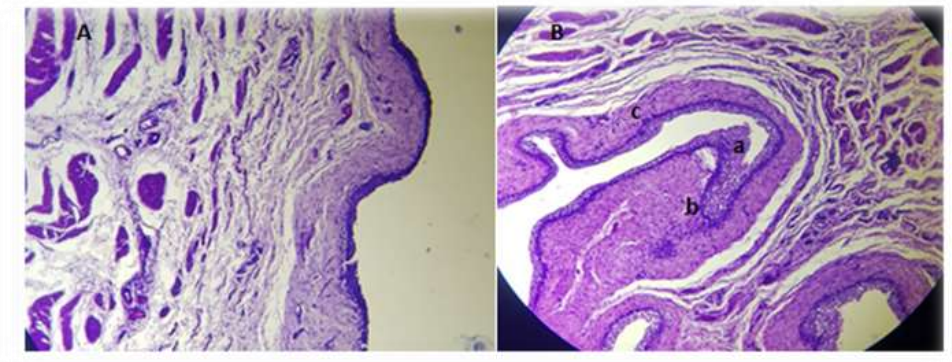

Figure 5. (A) Normal urinary bladder of sheep: normal epithelium and normal sub mucosa. (B) Infected urinary bladder of sheep: (a) Hyperplasia of epithelium, (b) Infiltration of inflammatory cells and (c) Thickening of sub mucosa. (H&E) (40X)

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