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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing *Escherichia coli* in the Calgary Health Region: emergence of CTX-M-15-producing isolates. *Antimicrob. Agents Chemother.* 51: 1281-1286.

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ARTICLES

Extended-Spectrum-Beta-Lactamase Producing Uropathogenic *Escherichia Coli* Infection In Dhaka, Bangladesh

Saiful Islam, Abdullah Yusuf, Shaheed Ara Begum, Arshedi Sattar, Afzal Hossain and Sushmita Roy

Use Of Real Time Polymerase Chain Reaction (PCR) And Histopathological Changes For Detection Of The *Toxoplasma Gondii* Parasite In Male Rats (Experimental Study)

Ghiadaa Abass Jassem

Full Length Research Paper

Extended-spectrum-beta-lactamase producing uropathogenic *Escherichia coli* infection in Dhaka, Bangladesh

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Extended-spectrum-beta-lactamase (ESBL) producing *Escherichia coli* that cause urinary tract infection (UTI) is a burning issue. This study was carried out to detect extended spectrum beta lactamase producing *E. coli* isolated from patients presented with UTI. This cross sectional study was conducted in the Department of Microbiology at Dhaka Medical College, Dhaka from January to December 2005, a period of one year. Clinically diagnosed cases of infected (UTI) patients were included in this study. The clean catch mid-stream (CCMU) technique was employed to collect urine sample. Microscopical examination of urine was done and pus cell ≥ 5 /HPF was included in the study. Urine samples were inoculated into blood agar and MacConkey agar media. All the organisms were identified by their colony morphology, staining character, pigments production, haemolysis, motility and other relevant biochemical tests as per standard methods. Antibiogram for all bacterial isolates were done by disc diffusion method of modified Kirby-Bauer technique using Mueller Hinton agar plates. Detection of ESBL producers was performed by double disc diffusion test. Phenotypic confirmatory test was done by E test. A total of 250 samples of urine were collected and within this, 103 (41.2%) samples were shown in positive culture. Out of 103 positive urine samples, majority were *E. coli* (67.0%) followed by *Klebsiella* species (19.4%), *Pseudomonas* species (7.8%) and *Proteus* species (5.8%). Out of 69 *E. coli* isolates, ESBL producers were found in 22 (31.9%) urine samples. The difference between the rate of isolation of *E. coli* with ESBL and other than *E. coli* with ESBL is statistically significant ($p=0.0001$). *E. coli* strains showed 100.0% resistance to amoxicillin, aztreonam, cefotaxim, ceftazidime, ceftriaxone and cephadrine. However, more than 80.0% resistant was observed in cotrimoxazole, amikacin and nalidixic acid. Nitrofurantoin and mecillinam were more than 50.0% resistant. All strains were sensitive to imipenem. A considerable number of ESBL producing *E. coli* was detected from UTI cases, indicating it as the major challenge for future antibiotic therapy.

Key words: Extended-spectrum-beta-lactamase (ESBL), bacterial agents, urinary tract infection, antimicrobial susceptibility.

INTRODUCTION

Drug resistance is a burning problem in the field of medicine (Altoparlak et al., 2004). There are many ways to mediate resistance, among them beta-lactamases play a major role in developing resistance against Gram negative organisms (Siro, 1995). During the last three decades many betalactam drugs have been used against the hydrolytic action of betalactamases for the treatment of Gram negative bacterial infections. Microorganisms are gradually developing resistance to these beta-lactam antibiotics by producing betalactamases (Altoparlak et al., 2004). Due to increased spectrum of activity of betalactamases especially against the oxyimino cephalosporins, they are called extended spectrum betalactamases (ESBLs) (Siro, 1995).

ESBL was first reported in 1983 from Germany in isolates of *Klebsiella pneumoniae* (Shukla et al., 2004). Gradually, many more ESBLs have been described (Bradford, 2001). ESBL producing bacteria are *Escherichia coli*, *Klebsiella* spp., *Salmonella* spp., *Morganella morganii*, *Proteus mirabilis*, *Serratia marcescens*, *Pseudomonas aeruginosa* and many more. Frequency of ESBL production is high among *E. coli* and *Klebsiella* species (Nathisuwan et al., 2001). This has created a worldwide problem resulting in treatment failure. ESBLs are enzymes that mediate resistance to extended spectrum (3rd generation) cephalosporins like ceftazidime, ceftriaxone, cefotaxime and monobactams like aztreonam but do not affect 2nd generation cephalosporins such as cephamecin (CDC, 1999).

In United States, occurrence of ESBL production in Enterobacteriaceae ranges from 0 to 25.0% depending on institutions (Bradford, 2001). In Europe, the prevalence is 23.0-25.0% for *Klebsiella* spp. and 5.4% for *E. coli* (Foury and Araj, 2003). In Asia, the proportion of ESBL production in *E. coli* varies from 4.8% in Korea to 8.5% in Taiwan and up to 12.0% in Hongkong (Bradford, 2001). In Singapore, 16.1% *E. coli* are ESBL producers (Chlebicki and Oh, 2004). In India, 58.1% *E. coli* are ESBL producers (Anathakrishnan et al., 2000). In Bangladesh, 26.9-43.2% *E. coli* are ESBL producers (Rahman et al., 2004; Alim, 2005).

This study has been designed to isolate ESBL producing *E. coli* from urine samples by double disc diffusion method and to confirm them by E-test ESBL method. Susceptibility pattern of ESBL producing *E. coli* were also observed.

METHODOLOGY

This cross sectional study was conducted in the Department of Microbiology at Dhaka Medical College, Dhaka from January to

December, 2005, a period of one year. Clinically diagnosed cases of infected (UTI) patients were included in this study.

Urine collection

The clean catch mid-stream (CCMU) technique was employed to collect urine sample. Urine was collected into two sterile containers of which one for microscopy and another for culture (Cheesbrough, 2000). In the case of catheterized patients, urine was collected from the catheter by sterile disposable syringe after proper cleaning of the catheter. Approximately 20 ml of urine was collected aseptically (Cheesbrough, 2000). The containers were properly labeled with patient's name and ID number. The specimens were then transported to the laboratory as quickly as possible.

Microscopical examination

Urine was transferred into a clean and dry 15 ml centrifuge tube and was centrifuged at 1000 rpm for 5 min. The supernatant was discarded and one drop of sediment was taken into a clean glass slide, a cover slip was placed over it and then examined for pus cells under light microscope using 10 and 40x objectives. Samples with pus cell ≥ 5 /HPF were included in the study (Colle et al., 1996).

Culture of urine

Urine samples were inoculated into Blood agar and Mac Conkey agar media by calibrated loop technique (Colle et al., 1996; Hoeprich, 1960).

Isolation and identification of organisms

All the organisms were identified by their colony morphology, staining character, pigments production, haemolysis, motility and other relevant biochemical tests as per standard methods (Cheesbrough, 2000; Colle et al., 1996).

Antimicrobial susceptibility test (Bauer et al., 1966)

Antibiogram for all bacterial isolates were done by disc diffusion method of modified Kirby-Bauer technique using Mueller Hinton agar plates and commercially available antimicrobial disc (Oxoid Ltd. UK). For *E. coli*, the following discs were used amoxicillin (Amx), co-trimoxazole (SXT), gentamicin (CN), amikacin(AK), nalidexic acid (Na), nitrofurantoin (Nf), netilmycin (NET), ciprofloxacin (CIP), cephradine (CL), ceftriaxone (CRO), ceftazidime (CAZ), imipenem (I) and aztreonam (ATM).

For ESBL producers

To see the susceptibility against quinolones, fluoroquinolones and cephamycin, the following discs were used: ciprofloxacin (CIP), gatifloxacin (GTX), levofloxacin (LEV), ofloxacin (OFX), sparfloxacin (Sp), lornefloxacin (LOM), pefloxacin (PEF) and cephamycin (CF).

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Quality control

Discs from each batch were first standardized by testing against reference strain of *E. coli* ATCC-25922 and zones of inhibition were compared with standard value (Cheesbrough, 2000; Jeong et al., 2004).

Inoculum standardization

With a sterile wire loop, three isolated colonies were transferred to screw-capped tube containing 4 ml of sterile normal saline and standardized with 0.5 Mac Farland standards by adding more organisms or more saline, the solution approximately corresponds to 1.5×10^8 organisms/ml (Cheesbrough, 2000).

Inoculation of test plate and disc placement

Before use, the Mueller-Hinton agar plates were dried in an incubator at 37°C for 30 min. Within 15 min after standardization of inoculums, a sterile cotton swab was immersed into the bacterial suspension. The excess broth was removed by retaining the swab with firm pressure against the inner side of the tube above fluid level. The swab was then streaked evenly on the surface of the plate in 3 different planes by rotating the plate approximately 60° each time to get a uniform distribution of inoculum. The inoculum was allowed to dry for 10-15 min at room temperature keeping the lid closed. The discs were then placed on the inoculum surface by a disposable niddle 15 mm away from the edge of the Petridish and having 20-25 mm gap between the discs. The plates were incubated at 37°C for 24 h (Cheesbrough, 2000).

Reading of the sensitivity tests

After over right incubation, each plate was examined and diameter of the complete zones of inhibition was measured in mm with the help of scale placed under surface of the Petridish. Zones of inhibition were measured in two directions of right angles to each other through the center of each disc and the average of the two readings was taken and compared with the standard (Washington, 1985).

Interpretation of zone

The zone of inhibition in growth produced by each antimicrobial agent on the test organisms was compared with that produced on the control organisms (CLSI, 2007).

Detection of ESBL producers by double disc diffusion test (Jarlier et al., 1988)

Synergy between a disc of augmentin (amoxicillin and clavulamic acid) and 3rd generation cephalosporins was detected as clavulanic acid in augmentin inhibits β -lactamases around 3rd generation cephalosporin disc in agar plate.

Procedure

Mueller-Hinton agar plates were prepared and inoculated with standardized inoculum (compared with 0.5 McFarland standards) by sterile cotton swab. Augmentin disc (20 μ g amoxicillin+10 μ g clavulanic acid) was placed in the center of the plate. 3rd generation cephalosporins (ceftazidime, ceftriaxone, cefotaxime) and aztreonam

discs were placed 20-30 mm apart (center to center) from the augmentin disc. The plate was observed after over night incubation at 37°C (Linscott and Brown, 2005).

Interpretation

Inhibition around the 3rd generation cephalosporins or aztreonam disc were increased towards the augmentin disc or neither disc were inhibitory alone but bacterial growth was inhibited where two antibiotics diffuse together, interpreted as ESBL positive (Shukla et al., 2004).

Quality control

E. coli ATCC-25922 were used as negative control and *K. pneumoniae* ATCC 700603 were used as positive control collected from BSMMU (Jeong et al., 2004).

Phenotypic confirmatory test (Chaudhary and Aggarwal, 2004)

E test ESBL

Principle: E-test ESBL strip consists of a thin, inert and non-porous plastic carrier (5x60 mm). One side of the strip is calibrated with MIC reading scales in μ g/ml while the reverse surface carries two predefined exponential gradients. TZ code for ceftazidime (0.5-32 μ g/ml) gradient and TZL for ceftazidime (0.064-4 μ g/ml) plus 4 μ g/ml clavulanic acid. The test is set up like a standard E test MIC procedure, two inhibition ellipses in opposing alignment appears at the end of the strip. The presence of ESBL was confirmed by the appearance of a phantom zone or deformation of the TZ ellipse or when TZ MIC was reduced by >3 log (8 times) dilutions in the presence of clavulanic acid.

Reagents: E-test ESBL strips containing (0.5-32 μ g/ml) ceftazidim alone and (0.064-4 μ g/ml) ceftazidim plus (4 μ g/ml) clavulanic acid were collected from AB BIODISK, Solna, Sweden.

Procedure: Inoculum was prepared and standardized with 0.5 McFarland standard and streaked by sterile swab over the entire agar surface of Mueller Hinton agar plates three times, rotating the plate approximately 60° each time to ensure even distribution of inoculum and kept for 15 min to absorb excess moisture.

Application of E test ESBL strip: With a forcep the ESBL strip was gripped at areas labeled TZ or TZL and placed at the middle of the inoculated plate in such a manner that the whole length of the strip was in complete contact with the agar surface, so that no bubble appeared under the strip and incubation was done at 35°C for 16-18 h.

Reading and interpretation (AB BIODISK, Solna, Sweden): After over night incubation, the plates were examined to see the values. TZ and TZL MIC values were recorded where the respective inhibition ellipses intersect the strip. Growth along the entire gradient, that is no inhibition ellipse indicates that the MIC was greater than the highest value on the reading scale. An inhibition ellipse below the gradient indicates a MIC less than the lowest value on the scale. When mutant colonies are present in the inhibition ellipse, MIC value was read where the colonies were completely inhibited. ESBL production was determined by a \geq two

Table 1. Rate of isolation of bacteria from urine samples (n=250)

Isolated bacteria	Frequency	Percentage
Growth of bacteria	103	41.2
No Growth	147	58.8
Total	250	100.0

Table 2. Isolation rate of different bacterial species from urine samples (n=250).

Isolated bacteria	Frequency	Percentage
<i>Esch. coli</i>	69	67.0
<i>Klebsiella</i> species	20	19.4
<i>Pseudomonas aeruginosa</i>	8	7.8
<i>Proteus</i> species	6	5.8
Total	103	100.0

fold concentration decrease in the MIC of TZ in presence of clavulanic acid (Linscott and Brown, 2005). Occasionally a 'rounded' zone (phantom zone) was seen below the TZL gradients while no ellipse was seen around the TZ end. The TZ inhibition ellipse may also be deformed at the tapering end. Presence of a phantom zone or ellipse deformation also indicates ESBL production due to synergy between TZ and clavulanic acid diffusing across from the TZL sections.

Interpretation: ESBL was positive if $TZ \geq 1$ and $TZ / TZL \geq 8$ or phantom zone or deformation of TZ ellipse and ESBL producer and resistant to all penicillins, cephalosporins and aztreonam (NCCLS M 100-S series). ESBL was negative if $TZ < 1$ or $TZ / TZL < 8$. ESBL non-producer and report actual MIC of all relevant drugs as determined by an MIC method. ESBL was non-determinable (ND) if $TZ > 32$ or $TZL > 4$. ESBL non-determinable and report actual MIC of all relevant drugs as determined by an MIC method. If ESBL is suspected, this was confirmed by CLSI methods and genotyping.

Quality control: *E. coli* ATCC 25922 as negative control and *K. pneumonia* ATCC 700603 as positive control collected from BSMMU were used.

RESULTS

A total of 250 samples of urine were collected and within this, 103 (41.2%) samples were shown in positive culture (Table 1).

Out of 103 positive urine samples majority were *E. coli* (67.0%) followed by *Klebsiella* species (19.4%), *Pseudomonas* species (7.8%) and *Proteus* species (5.8%) (Table 2). Out of 69 *E. coli* isolates, ESBL producers were found in 22 (31.9%) urine samples. The difference between the rate of isolation of *E. coli* with ESBL and other than *E. coli* with ESBL is statistically significant ($p=0.0001$) (Table 2).

E. coli strains showed 100.0% resistance to amoxicillin, aztreonam, cefotaxim, ceftazidime, ceftriaxone and cephadrine. However, more than 80.0% resistant was observed in cotrimoxazole, amikacin and nalidixic acid. Nitrofurantoin and mecillinam were more than 50.0% resistant. All strains were sensitive to imipenem (Figure 1).

DISCUSSION

ESBLs are the enzymes produced by a variety of organisms like enterobacteriaceae as well as *P. aeruginosa* (Bradford, 2001). Failure to detect these enzymes has contributed to their uncontrolled spread and therapeutic failure (Thomson, 2001). In this study, ESBL producing *E. coli* was isolated and the sensitivity pattern was recorded. Regarding that issue, a total number of 250 urine samples were analyzed and among these 103 (41.2%) samples yielded bacterial growth. A study was conducted at Bangabandhu Sheikh Mujib Medical University by Mostaqim (2007) who found 69.4% bacteria from various samples which is not consistent with the present study. The reason of low bacterial growth in this study may be use of urine sample only; on the other hand, culture of different samples was performed by the previous author. In addition to that, majority of the patients with UTI usually take the antibiotic from the pharmacy before coming to hospital and this irrational use of antibiotic may hinder the growth of bacteria from urine (Table 3).

In this study, majority of isolated bacteria were *E. coli* (67.0%) followed by *Klebsiella* species (19.4%), *Pseudomonas* species (7.8%) and *Proteus* species (5.8%). In the present study, *E. coli* were the predominant

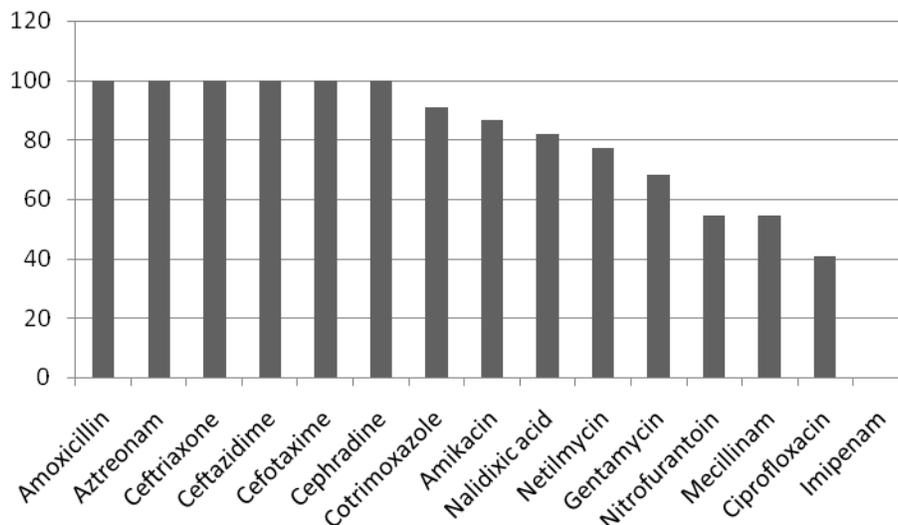


Figure 1. Antimicrobial drug resistance among the ESBL producing *E. coli*.

Table 3. Rate of isolation of ESBL among *Escherechia coli* isolates (n=69).

Isolated bacteria	ESBL producer		Total	p value
	Positive	Negative		
<i>Escherechia coli</i>	22(31.9)	47(68.1)	69(100.0)	
Other bacteria	9(25.0)	25(75.0)	34(100.0)	0.0001
Total	31(30.1)	72(69.9)	103(100.0)	

*Chi-square test has been performed. *Figure within parenthesis indicates percentage.

bacteria isolated from urine samples. From this result, it has been cleared that *E. coli* are the most prevalent bacteria isolated from urine. Similar result was reported by Mansour et al. (2009) and stated that *E. coli* is still the most common cause of UTI. Ferry et al (1988) published that *E. coli* were the most common isolated bacteria from the urine samples of the patients presented with UTI. This result also corresponds with the result obtained by other investigator (Jakobsen et al., 2012). Some have shown, however, that the percentage of *E. coli* is slowly declining, being replaced by other members of the Enterobacteriaceae and enterococci (Weber et al., 1997).

Out of all *E. coli* isolates, ESBL producers were found in 31.9% urine samples. The difference between the rate of isolation of *E. coli* with ESBL and other than *E. coli* with ESBL is statistically significant ($p=0.0001$). Mostaqim (2007) found 56.7% *E. coli* and among them, 34.1% were ESBL producers which correlate with the findings of the present study. Again, Rahaman et al. (2004) reported that *E. coli* was the predominant organism in urine and among them majority were ESBL producers which are similar to this present study. In another study, Alim (2005) found that 26.9% *E. coli* isolates from urine were ESBL

producers which are more or less similar to the present result. In Bangladesh Rahaman et al (2004b) found that 43.20% *E. coli* were ESBL producer which is higher than the present study. The explanation regarding high rate is that they are isolated from pus samples.

In this study, sensitivity pattern of ESBL producing *E. coli* was done against different antibiotics and has shown that *E. coli* strains have 100.0% resistance to amoxicillin, aztreonam, cefotaxim, ceftazidime, ceftriaxone and cephadrine (Table 4). However, more than 80.0% resistant was observed in cotrimoxazole, amikacin and nalidixic acid. Nitrofurantoin and mecillinam were more than 50.0% resistant. All strains were sensitive to imipenem. Higher resistance to other antibiotics like cephadrine, cotrimoxazole, gentamycin, amikacin against ESBL producing *E. coli* was observed in this study which indicates that ESBL producing organisms are multidrug resistant and genes that code for ESBL are linked to other resistance genes (Ahmed and Salam, 2002). ESBL strains were 100% sensitive to imipenem. According to CDC, ESBL are defined as enzymes which hydrolyze 3rd generation cephalosporins and aztreonam but sensitive to cephamycin and imipenem (CDC, 1999). Nakamura

Table 4. Antimicrobial susceptibility profiles of ESBL *E. coli* and non ESBL *E. coli* isolates.

Antibiotic name	ESBL <i>E. coli</i> (n=22)	Non ESBL <i>E. coli</i> (n=47)	OR (95% CI)	P value
Amoxicillin	22 (100.0%)	38 (80.8%)	1.24 (1.08-1.40)	0.049
Aztreonam	22 (100.0%)	26 (55.3%)	1.81 (1.40-2.34)	0.0001
Ceftriaxone	22 (100.0%)	28 (59.6%)	1.68 (1.33-2.12)	0.0001
Ceftazidime	22 (100.0%)	29 (61.7%)	1.62 (1.29-2.03)	0.0001
Cefotaxime	22 (100.0%)	30 (63.8%)	1.57 (1.26-1.94)	0.001
Cephadrine	22 (100.0%)	42 (89.4%)	1.12 (1.12-1.23)	0.169
Cotrimoxazole	19 (86.4%)	33 (70.2%)	0.37 (0.095-1.46)	0.231
Amikacin	11 (50.0%)	41 (87.2%)	6.83 (2.06-22.61)	0.001
Nalidixic Acid	18 (81.8%)	28 (59.6%)	0.327 (0.096-1.12)	0.058
Netilmycin	16 (72.7%)	33 (70.2%)	0.884 (0.286-2.73)	0.830
Gentamycin	15 (68.2%)	24 (51.1%)	0.487 (0.168-1.41)	0.181
Nitrofurantoin	12 (54.5%)	17 (36.2%)	0.472 (0.169-1.32)	0.150
Mecillinum	12 (54.5%)	18 (38.3%)	0.517 (0.186-1.44)	0.205
Ciprofloxacin	8 (36.4%)	18 (38.3%)	1.09 (0.380-3.10)	0.887
Imipenem	0 (0.0%)	0 (0.0%)	NA	NA

and Komatsu (2005) found that 61.1% *E. coli* were ciprofloxacin resistant among the ESBL producers which is similar to the present study.

When ESBL producing organisms are confirmed by NCCLS guidelines, results should be reported as resistance to all penicillins, aztreonam and cephalosporins excluding cephamycin (CDC, 1999). Treatment of ESBL producing *E. coli* can be done by imipenem or cepharmycin. Imipenem is costly and not within the reach of the people of developing country like Bangladesh. But quinolone (ciprofloxacin) and fluoroquinolones (levofloxacin, gatifloxacin etc) are cheap, available and single dose drug which be may be used in the treatment against ESBL producing organisms. So early correct detection of ESBL producing *E. coli* by E test ESBL method and rational use of antibiotics can limit the spread of multidrug resistant pathogens (Medeiros, 1993).

Conclusion

A considerable number of ESBL producing *E. coli* from urine samples were isolated. Sensitivity was higher in the case of imipenem as these are expensive and dose inconvenient so may be used for reserve antibiotics. Awareness regarding proper identification of ESBL producing *E. coli* should be carried out for appropriate antibacterial agent against them can reduce the hospital stay and sufferings of the patients.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Use of real time polymerase chain reaction (PCR) and histopathological changes for detection of the *Toxoplasma gondii* parasite in male rats (experimental study)

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Toxoplasma gondii infection is wide spread in Iraq that is why this study was carried out to detect its presence in semen samples of infected male rats experimentally. Impression smears, real time polymerase chain reaction (PCR) and histopathological changes were used for detection of *T. gondii*. The current study included the isolation of local strains of *T. gondii* from placenta of women that experienced abortion that had toxoplasmosis history, 0.3 ml of suspension contained 100 tissue cyst per rat injected interperetionally in 40 rats. Eight weeks post inoculation, rats were sacrificed then dissected and epididymis was immediately removed. Impression smear were made from semen stained with eosin-nigrosin as initial diagnosis of infection confirmed by the presence of cysts of parasite. This confirmation is dependent on the molecular diagnosis by real time PCR which successfully detected the parasite in 90.3% of rats inoculated with aborted placenta suspension. Some histological changes in testicular tissue were collapse and shrinkage in somniferous tubules with multinucleated giant cells with vacuolar degeneration of lydig cell. These finding suggest that *T. gondii* infection cause a temporary impairment and insufficiency in male reproductive activity with probability of transmission of the parasite in semen to females or to other animals such as cats.

Key words: Testes, placenta, semen, real time polymerase chain reaction (PCR), impression, interperitonally, reproductive, male.

INTRODUCTION

Toxoplasma gondii is a protozoan parasite that is widespread globally and infect man and animals (Shi-guo et al., 2006). Most infections in humans are asymptomatic but at times the parasite can produce devastating disease, toxoplasmosis ranks high on the list of diseases

which lead to death in patients with acquired immunodeficiency syndrome (AIDS) (Ahmad et al., 2012). In AIDS patients, any organ may be involved, including the testis, dermis and the spinal cord, while the brain is most organ frequently reported (Hill and Dubey, 2002).

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T. gondii is horizontally transmitted to humans by the accidental ingestion of oocysts in cat feces or by eating raw or undercooked meat containing cysts. *T. gondii* can be transmitted by solid organ transplants to the recipient and transfusion (Dubey and Jones, 2008).

T. gondii can be transmitted by semen, milk, saliva and eggs (Dubey and Lindsay, 2006). The disease primarily occurs in children and adults (including pregnant women) which is asymptomatic in most patients (Afshari et al., 2013). In immunocompromised patients, reactivation of latent disease can cause life-threatening encephalitis. After a short phase of acute toxoplasmosis, the infection proceeds into its latent phase when cysts are formed and this cyst survive for long period of host's life, mainly in neural and muscular tissues of infected subjects. In immunocompetent subjects, the latent phase of infection is considered symptomatic and harmless, increased accidents toxoplasmosis induced changes in the behavior of humans and rodents may be dramatically different even if they are induced by the same process, e.g. by production or induction of production of the same neurotransmitter in the host brain tissue (Doubey and Jones, 2008).

Rats are the best model for human toxoplasmosis investigation, whereas toxoplasmosis in human is similar to rats, both mammals (Afshari et al., 2013), Therefore, the result of the study may be applied for human toxoplasmosis (Abdoi et al., 2012)

The detection of *T. gondii* organisms in clinical specimens in mouse inoculation and then the detection of *T. gondii*-specific antibodies is sensitive and specific but time-consuming, taking up to 6 weeks to obtain a diagnosis. Currently, cell culture is the most practical method for the detection of *T. gondii* parasitemia, but this is also relatively slow and may lack sensitivity, PCR has been found to be a sensitive, specific and rapid method for the detection of *T. gondii* DNA in amniotic fluid, blood tissue samples and cerebrospinal fluid. Thus, a more efficient method is needed to provide rapid and quantitative results for the diagnosis of *T. gondii* infection. Several groups have reported the use of PCR to screen human samples easily. PCR is more appropriate, as there is no need for cultivation of the parasite to diagnoses *T. gondii* by PCR amplification of *T. gondii* DNA which is present in peripheral blood. PCR assay was designed for the simultaneous detection of *T. gondii* DNA from the cerebrospinal fluid, and PCR may be the investigation of choice for brain biopsy (Lin et al., 2000)

The objective of this study was to detect *T. gondii* in semen samples of rats which were experimentally infected by using suspension prepared from placenta of women that experienced abortion due to toxoplasmosis.

MATERIALS AND METHODS

Laboratory animals

Mature male rats (*Rattus norvegicus*) were reared in plastic cages

supplied with drinking water and food, and kept in animal house of the College of Veterinary Medicine of Al-qadisiya University. The animal house was supplied with ventilator fan and air conditioner in order to control the room temperature. Sterilized food and water were used for rats along the period of experiment.

Isolation of *T. gondii*

Parasite was isolated from placenta of women that experienced abortion with toxoplasmosis history. Placenta were cut into small pieces and mixed with an equal amount of normal saline and grinded by using mortar and pestle. This preparation (solution) was passed through a piece of gauze to avoid large particles and then centrifugation was done with 3000 rpm for 10 min. The supernatant was discarded and the sediment was suspended by normal saline and the process was repeated three times by washing the samples, 0.1 ml of compound 1,000 units of penicillin and streptomycin 100 mg was added to prevent contamination (Alkennay and Hassan, 2010). 0.3 ml suspension contains 100 tissues cyst of parasites used for experimental infection in 40 rats through intraperitoneal route, 10 rats without anything (as control). This method was used (AL-taie and Abdulla, 2008) for inoculation of the parasite from uncontaminated samples and it is a sensitive method .

Detection of parasite

After 8 weeks from the beginning of injecting the laboratory rats, the rats were anesthetized using 0.2 ml ketamine and 0.1 ml xylazine injected into intraperitoneal cavity by using a sterile syringe of 1 ml (Alves et al., 2011). Samples were collected from infected group and control group of rats to detect the parasite by using impression smear and to detect B1 gene by using qRT-PCR. After that, one testes was stored in formalin 10% for histopathological study.

Epididymis caudate for semen collection

The two epididymis were removed from each rat and immersed in 1 ml of warm physiological saline, then each epididymis caudate was minced using microsurgical scissor and kept at 37°C for later tests (Oyedeji et al., 2013).

Direct smears

Detection of *T. gondii* in experimentally inoculated rats by impression of smears which were obtained from semen was done. Smears were dried, fixed in methanol and stained with eosin nigrosin stain (Hill and Dubey, 2002).

Testicular histology

Samples collected were fixed in 10% formalin for 48-72h, and the material was processed by histopathological technique method as published by Salibay and Claveria (2006). 5 µm semi-serial sections were placed on slides and stained with hematoxylin and eosin (H&E) for microscopic examination.

Samples collection

50 samples of semen from rats inoculated with parasite were collected with control. The samples stored in a refrigerator until use for genomic DNA extraction.

Table 1. Real-time PCR TaqMan probe and primers.

Primer	Sequence
B1 gene	F TCCCCTCTGCTGGCGAAAAGT
Primers	R AGCGTTCGTGGTCAACTATCGATTG
B1 gene Probe	FAM-TCTGTGCAACTTTGGTGTATTTCGCAG-TAMRA

Table 2. The qPCR master mix.

qPCR master mix	Volume
Genomic DNA template	5 µL
B1 Forward primer (20pmol)	1 µL
B1 Reverse primer (20pmol)	1 µL
B1 TaqMan probe (25pmol)	2 µL
DEPC water	11 µL
Total volume	20 µL

Table 3. Thermocycler conditions.

qPCR step	Temperature	Time	Repeat cycle
Initial denaturation	95°C	5 min	1
Denaturation	95°C	15 s	
Annealing\extension	60°C	1 min	45
Detection (scan)			

Genomic DNA extraction

Genomic DNA was extracted from frozen semen samples of experimental rats by using (Genomic DNA mini Extraction kit. Geneaid. USA). 200 µL semen sample placed in 1.5 ml microcentrifuge and 20 µL proteinase K (10 mg/mL) was added for cell lysis. Then genomic DNA extracted according to kit instructions. The purified DNA was eluted in elution buffer provided with kit and store at -20°C, and the extracted DNA was checked by Nanodrop spectrophotometer.

Real-Time PCR

Real-Time PCR based TaqMan probe was performed for rapid detection of *T. gondii* according to method described by Mei-hui-lin *et al.* (2000). Real-Time PCR TaqMan probe and primers were used for amplification of conserved region B1 gene in *T. gondii*. These primers were provided by Bioneer Company, Korea as shown in Table 1.

The Real-Time PCR amplification reaction was done by using (AccuPower® DualStar™ qPCR PreMix Bioneer. Korea) and the qPCR master mix were prepared for each sample according to company instruction as shown in Table 2.

These qPCR master mix reaction components mentioned in Table 2 were added into AccuPower® DualStar™ qPCR PreMix tubes which contain Taq DNA polymerases, dNTPs, 10X buffer for TaqMan probe amplification. Then tubes were placed in Exispin vortex centrifuge at 3000 rpm for 3 min, after that transferred into MiniOpticon Real-Time PCR system and the thermocycler conditions shown in Table 3 was applied.

RESULTS AND DISCUSSION

After eight weeks of inoculating rats, there was impression smear of stained semen with eosin-nigrosin in the tissue cyst of *T. gondii* as initial detection of infection, the result revealed that the percentage of tissue cysts were 80% in different size and numerous bradyzoites (Figure 2A and B). This study is similar to that of AL-Kennany and Hassan (2010) who isolated the *T. gondii* in same way from placenta of aborted ewes and AL-Khaffaf and Abdullah (2005) who isolated the *T. gondii* bradyzoites from placenta of women that experienced abortion and confirmed by the appearance in different size and involved numerous number. Abdoli *et al.* (2012) referred that *T. gondii* parasite can infect the male genital tract. Dalimi and Abdoli (2013) reported presence of parasites in rats seminal fluid by using impression smear method. *T. gondii* can be found in different fluids and tissues of infected animals (Miller *et al.*, 2000). Among the reproductive complication caused by toxoplasmosis in aborted sheep, it is one of the most important causes of economic losses (Moreno *et al.*, 2012). The occurrence of infection forms in the semen of human and bulls (Scarpelli *et al.*, 2009), semen and milk of goat (Figueiredo *et al.*, 2001), semen of sheep (Lopes *et al.*, 2011) was through possible routes of transmission of disease by ventral rout with sours infection. The data

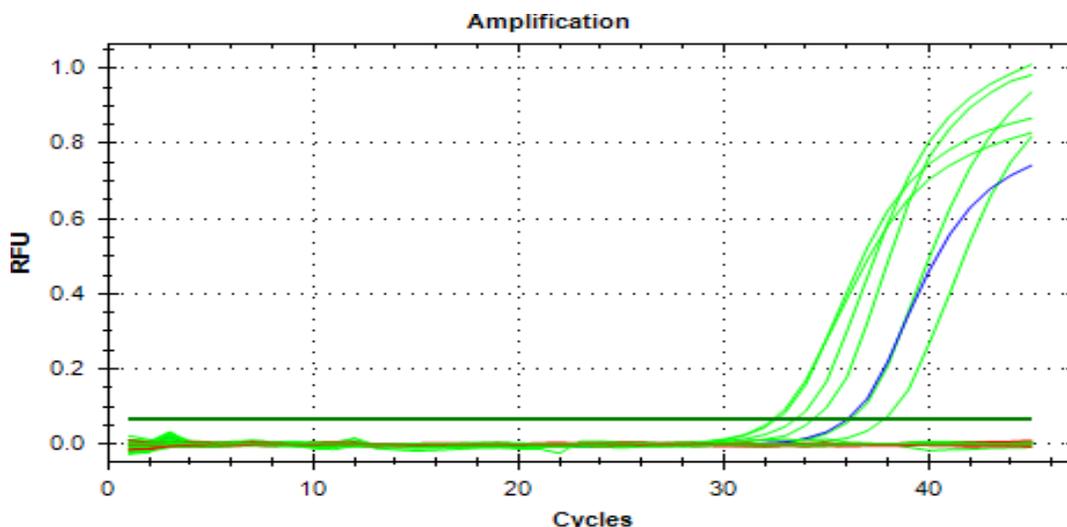


Figure 1. Real-time PCR amplification plot for B1 gene for the detection of *T. gondii* DNA in rat semen samples that show (green plot) positive result (Blue), positive control DNA *T. gondii* (Genekam .Germany), (red) negative result.

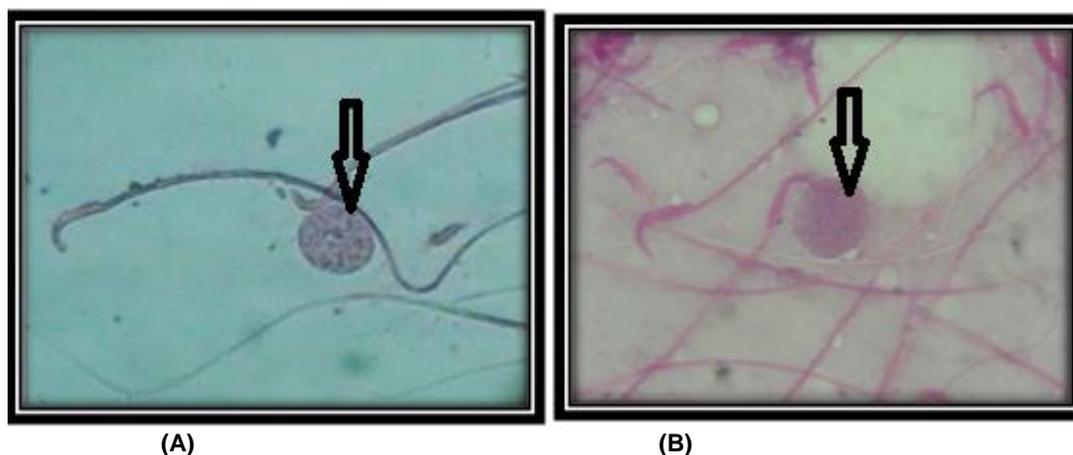


Figure 2. A and B: Toxoplasma cyst in the semen of infected rats stained with Eosin-Nigrosin.

obtained from limited studies performed in animal models reported presence of parasite in rat's brain (Terpsidis et al., 2009). While AL-Taie and Abdulla (2008) found *T. gondii* in brain, liver, heart, spleen, lymph nodes, kidney of the rats. The study was through possible routes of transmission of disease by ventral rout with sours infection.

The real-time PCR technique is very specific and sensitive method in detection of *T. gondii* when compared with direct impression smear test. The results of real-time PCR technique for semen showed the presence of parasite in high rate (90.3%) in rats inoculated with suspension of placenta (Figure 1). This is similar to the result of Shi-guo et al. (2006) who reported that the rate of infection was 100% in male rabbits. Also, this tech-

nique was used for detection of parasite in many samples of tissue and body fluid and in brain of sheep (Donovan et al., 2012). Mesquita et al. (2010) found *T. gondii* in blood and cerebrospinal fluid in AIDS patients while Amerizadeh et al. (2013) found *T. gondii* in serum samples and in amniotic fluid of pregnant women and in blood samples of newborns with congenital toxoplasmosis (48%) (Gunel et al., 2012).

Histologically *T. gondii* was detected as a cyst of parasite in some testicular tissue and parasites which was found in other section, there is sever congestion and hemorrhage in the somniferous tubules, presence of multinucleated giant cells, suppression of spermatogenesis vaculation of spematogonia with absence of spermatozoa, thickening in the interstitial tissue among

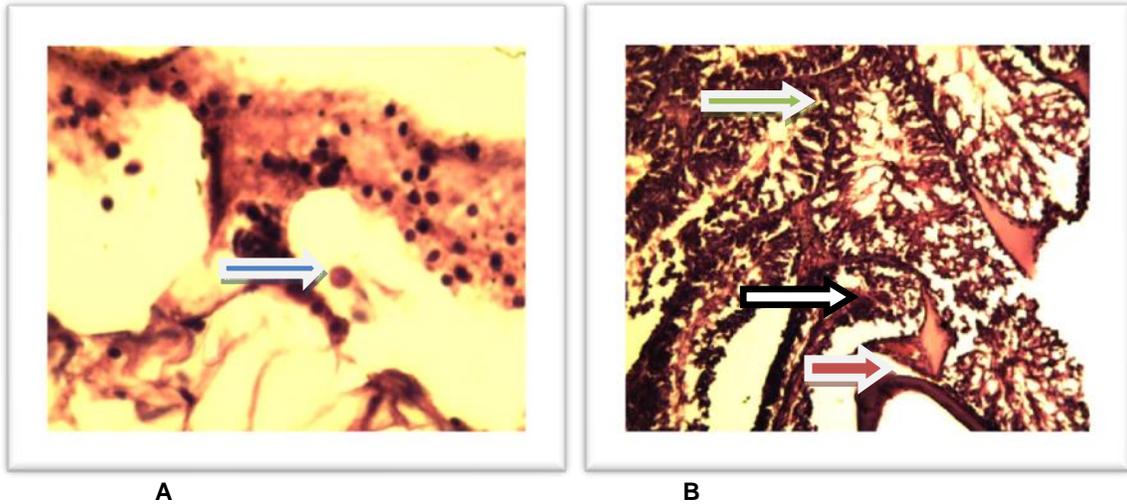


Figure 3. (A): *Toxoplasma gondii* cyst in rat testis (blue arrow). (B) Many histological changes of testicular (200X H&E) tissue in rat vacuolation of spermatogonia with absence of spermatozoa (green arrow), thickening in the interstitial tissue (white arrow), hemorrhage in the seminiferous tubules (red arrow) (H&E 200X).

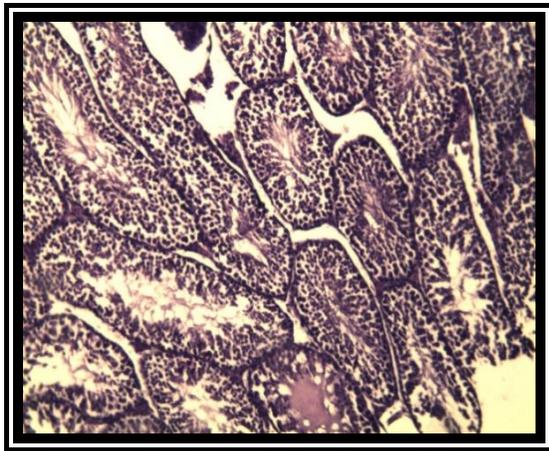


Figure 4. Histological section of rat testes. Control group. There are normal seminiferous tubules with normal spermatogenesis, normal spermatogonia and spermatid. 10X H&E.

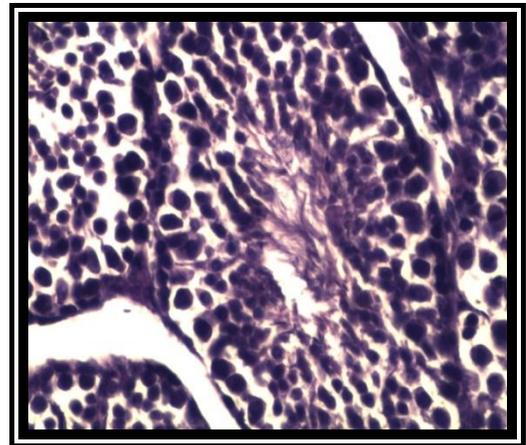


Figure 5. Histological section of rat testes. Control group. Higher magnification, there are normal seminiferous tubules with normal spermatogonia, normal primary and secondary spermatocytes with spermatid. 40X H&E.

somniferous tubules, many histological changes in testicular tissue collapse and shrinkage in somniferous tubules with multinucleated giant cell with vacuolar degeneration of lydig cells. These changes are not observed in the control rats, suggesting that these changes occur due to *T. gondii* infection (Figure 3A and B). Figures 4 and 5 show normal tissue and structure of spermatogenesis and somniferous tubules. Lopes et al. (2011) detected the parasite in parenchymal tissue of the male reproductive system of sheep by immunohistochemistry and the changes were multifollicular mononuclear interstitial inflammatory infiltrate and diffuse testicular degeneration with calcified foci, while Gheoca et al. (2009) reported rare case of *T. gondii* cyst in testicle

sample of small mammals species, *Apodemus flavicollis* (mammal). Terpsidis et al. (2009) and Abdoli et al. (2012) reported that no histological changes were detected in examined testes in their findings suggesting that *T. gondii* infection can cause temporary impairment of the reproductive system by insufficient male production with the parasite been transferred through semen of animals as different source of infection, this may occur with human toxoplasmosis.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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