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RESEARCH ARTICLE

EVALUATION OF EXTENDED-SPECTRUM BETA-LACTAMASES IN GRAM NEGATIVE RODS ISOLATED FROM TIGRIS RIVER IN BAGHDAD CITY.

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Abstract

Gram-negative bacilli producing extended-spectrum beta-lactamase (ESBL) comprise an important interest in human medicine today. It was verified that pathogenic bacteria may efficiently inactivate the newer third-generation Cephalosporins (such as cefotaxime, ceftazidime, and ceftriaxone). This study was designed to determine the prevalence of extended-spectrum β-lactamases (ESBLs) among gram-negative isolates obtained from river water specimens with double disc diffusion synergy test. Ten fresh river water specimens were collected from upstream, midstream and downstream of Tigers River in Baghdad City. Additional thirty five municipal drinking water specimens were collected from various public water supplies in Baghdad City. Microorganisms isolated from all specimens were identified at species level according to standard microbiology methods. Isolates resistant to cefotaxime were tested for ESBL production by double disc synergy test. Fresh water specimens detected (7%) ESBL-positive environmental isolates, whereas municipal drinking water specimens recorded (20%) ESBL-positive environmental isolates. *E. coli*, *Prot. Mirabilis*, and *Ent. cloacae* were the most prevalent ESBL-positive isolates in environmental water specimens. Our study therefore, does not only declare the presence of ESBL producing *Enterobacteriaceae* but also confirm that they are multi-drug resistant. More research work is required using molecular techniques to specifically find out the genes responsible for this grave resistance.

Introduction:-

The production of extended-spectrum beta-lactamase (ESBL) is one of the major antimicrobial resistance mechanisms in Enterobacteriaceae, and the increasing number of ESBL-producing Enterobacteriaceae isolated from water environments has posed a serious threat to the public health [1].

Bacterial resistance to beta-lactams may be mediated by enzymatic destruction of the antibiotics, altered antibiotic target, or decreased intracellular uptake of the drug. All three pathways play an important role in clinically relevant antibacterial resistance, but bacterial destruction of beta-lactams by producing beta-lactamase is by far the most common method of resistance. Gram-negative bacteria, including Enterobacteriaceae, P. aeruginosa, and Acinetobacter spp. produce dozens of different beta-lactamase types that mediate resistance to one or more of the betas-lactams antibiotics [2]. It has been well known for many years that several genera of Gram-negative bacteria possess naturally occurring, chromosomally mediated β-lactamases, which are thought to have evolved from penicillin-binding proteins as a result of the selective pressure exerted by soil organisms producing β-lactams [3]. The first plasmid-mediated β-lactamase isolated from Gram-negative bacteria, which hydrolyzed penicillin and penicillin derivatives, such as ampicillin, carbenicillin, Piperacillin and first-generation Cephalosporins, was TEM-1, described in the early 1960s [4]. This enzyme was found in a blood culture isolate of Escherichia coli from a

Greek patient named Temoniera, hence the designation TEM. As the latter enzyme was plasmid and transposon mediated, it spread easily to many different species of Gram-negative bacteria [5]. Subsequently, SHV-1 β-lactamase, chromosomally encoded in the majority of Klebsiella pneumoniae and plasmid mediated in E. coli, was reported [6]. It was just 2 years after the introduction of oximino-β-lactams, such as cefotaxime, ceftazidime and ceftriaxone, and the oximino-monobactam, aztreonam, which were specifically designed to resist the hydrolytic action of β-lactamases, that extended-spectrum β-lactamases (ESBLs) were first isolated in Germany in 1983 from K. pneumoniae strains [7]. Since then, several outbreaks have been reported in a number of European countries and the USA, and the problem has reached endemic dimensions in several places worldwide. The selective pressure of the use and overuse of the oximino-cephalosporins, which are widely prescribed for the treatment of serious Gram-negative nosocomial infections, has led to the isolation of a large number of ESBL enzymes worldwide, mainly from different genera of the Enterobacteriaceae [8].

The widespread use of beta-lactam antimicrobial agents as first-line therapy for the treatment of serious infections has led to the development of various resistances that have compromised the use of some agents. In certain countries, the lack of local or national surveillance programs limits the ability to detect these resistant strains and prevent their dissemination. Data regarding evaluation of extended-spectrum β-lactamases (ESBLs) from Iraq are very scarce, although substantial studies have been carried out for the investigation of antimicrobial resistance in Gram-negative bacteria. This study was designed to determine the prevalence of extended-spectrum β-lactamases (ESBLs) among gram-negative isolates obtained from environmental specimens with double disc diffusion synergy test.

Materials and Methods:-

This study was carried out during the period from June 2015 until October 2015.
Environmental specimens were collected from the following locations:

- Ten fresh river water specimens were collected from upstream, midstream and downstream of Tigers River in Baghdad City.
- Thirty five municipal drinking water specimens were collected from various public water supplies in Baghdad City

A preliminary test for coliforms was performed by inoculating a sample of water into tubes of lactose broth containing Durham tubes. After 24 hours of incubation, the tubes were examined for the presence of acid and gas as an indication of lactose fermentation. Other than coliforms, few organisms found in water can ferment lactose rapidly with production of gas. Gaseous fermentation of lactose within 24 to 48 hours provides presumptive evidence of the presence of coliforms. Confirmation of this result should be attempt to preclude other organisms that indicate lactose positivity.

The confirmed test was done by plating a sample of the positive lactose broth culture onto a differential agar medium. Eosin methylene blue (EMB) agar was used for this purpose. Microorganisms isolated from all specimens were identified at species level according to standard microbiology methods, that's including: direct microscopic examination of gram stained preparations, biochemical profiling test, and antimicrobial susceptibility methods. The API 20 E system (bioMérieux Inc., France) represented a type of kit for rapid identification of bacterial isolates. In addition, identification of bacterial isolates detected in environmental water specimens (fresh and municipal water specimens) were done by Mini API ID 32 E system for the identification of Enterobacteriaceae and other non-fastidious Gram-negative rods, bioMérieux – France.

The antimicrobial susceptibility test was performed according to Kirby-Bauer (disk diffusion) technique using Muller-Hinton agar and different single antimicrobial discs supplied commercially. Results were read according to the National Committee for Clinical Laboratory Standards guidelines (NCCLS). Any organism that was resistant to any of the 2^{nd} and 3^{rd} generation Cephalosporins (ceftazidime, ceftriaxone cefotaxime) were tested for ESBL production using the double disc synergy test (DDST). Several plates of Mueller- Hinton agar were prepared and 30µg discs of ceftazidime and cefotaxime were placed 15 mm center to center from an amoxicillin – clavulanic acid disc (20:10µg) (Oxoid UK). Inoculated media were incubated for 18 – 24 hours at 37^{0} C. Enhanced zone of inhibition between any of the beta- lactam discs and the center disc (amoxiclin/clavulanic acid) was recorded [6]. Isolates of Gram-negative bacteria with resistance or with decreased susceptibility (intermediate by NCCLS criteria) to third generation cephalosporins were screened for ESBL production by using double disc synergy test (DDST). The following antimicrobial agents and break point diameters are indicators of ESBL production; ceftazidime

(<22mm), cefpoxidime (<22mm), and aztreonam (<27mm). Reduced zones around discs of cefotaxime (<22mm) or ceftrioxone (<25mm) may also indicate ESBLs but are less sensitive indicators [8].

Results:-

Frequency of faecal coliform isolates in environmental water specimens from various locations at Baghdad City is shown in table 1. It was clearly demonstrated that 10 (22%) environmental isolates were detected in fresh water specimens, whereas 35 (78%) isolates were detected in municipal drinking water. Out of the total, E. coli isolates were 4 (9%) in fresh water specimens, and 5 (11%) isolates were detected in municipal drinking water specimens, whereas Ent. cloacae isolates were 3 (7%) in fresh water specimens, and 9 (20%) isolates were detected in municipal drinking water specimens.

Table 1: Frequency of faecal coliform isolates in environmental water specimens from various locations at Baghdad City.

Isolates	Fresh water	Municipal water
	No. (%)	No. (%)
E. coli	4 (9%)	5 (11%)
Kleb. pneumoniae	0	3 (9%)
Kleb. oxytoca	0	1 (3%)
Prot. Mirabilis	2 (2%)	9 (20%)
Ent. cloacae	3 (7%)	9 (20%)
Ent. sakazaki	0	1 (3%)
Pantia spp.	1 (10%)	4 (11%)
Citr. koseri	0	1 (3%)
P. aeruginosa	0	1 (3%)
P. luteola	0	0
Acineto. Baumani	0	0
Ochrobact. Anthropi	0	0
Total	**10 (22%)**	**35 (78%)**

In table 2, frequency of ESBL-producing environmental water isolates in water specimens is shown. Fresh water specimens detected 3(7%) ESBL-positive environmental isolates, whereas municipal drinking water specimens recorded 9 (20%) ESBL-positive environmental isolates. E. coli, Prot. Mirabilis, and Ent. cloacae were the most prevalent ESBL-positive isolates in fresh water specimens, whereas Ent. cloacae and Prot. Mirabilis were the most prevalent ESBL-positive isolates in municipal drinking water specimens.

Table 2: Frequency of ESBL-producing environmental water isolates in water specimens.

Isolates	Fresh water		Municipal water	
	ESBL +ve	ESBL –ve	ESBL +ve	ESBL –ve
	No. (%)	No. (%)	No. (%)	No. (%)
E. coli	1 (2%)	3 (7%)	1 (2%)	4 (9%)
Kleb. pneumoniae	0	0	0	3 (7%)
Kleb. oxytoca	0	0	0	1 (2%)
Prot. Mirabilis	1 (2%)	1 (2%)	3 (7%)	6 (13%)
Ent. cloacae	1 (2%)	2 (4%)	4 (9%)	5 (11%)
Citr. freundii	0	0	0	1 (2%)
Burkh. cepacia	0	0	0	1 (2%)
P. aeruginosa	0	0	1 (2%)	0
Acineto. Baumani	0	0	0	1 (2%)
Pantia spp.	0	1(2%)	0	4 (9%)
Total	3 (7%)	7 (16%)	9 (20%)	26 (57%)

Table 3 shows antibiotic susceptibility of ESBL producers and non-producers to various non-ß-lactam antibiotics in environmental isolates. The most resistant isolates to various antimicrobial agents were E. coli. It was clearly demonstrated that the antimicrobial agent to which most ESBL positive environmental isolates still sensitive was imipenem, amikacin, and piperacillin.

Table 3: Antibiotic susceptibility of ESBL producers and non-producers to various non-ß-lactam antibiotics in environmental isolates.

Antibiotics	**ESBL +ve, n =12 (27%)**	**ESBL –ve, n = 33 (73%)**
	Susceptible No. (%)	**Susceptible No. (%)**
Amikacin	11(92%)	31(94%)
Cephotaxime	0	24(69%)
Imipenem	12 (100%)	35(100%)
Nitrofurantoin	2 (17%)	30(86%)
Norfloxacin	3 (25%)	22(63%)
Piperacillin	7 (58%)	28(80%)
Ciprofloxacin	0	0
Gentamicin	0	0
Cephalothin	0	0
Ceftazidime	1(8.3%)	25(71%)
Ceftriaxone	1(8.3%)	24(69%)
Co-trimoxazole	4 (33%)	23(66%)
Cefepime	3(25%)	28(80%)

In table 4, E. coli, Prot. Mirabilis and Ent. cloacae were shown to be the most prevalent ESBL-producing environmental isolates that expressed multi-drug resistance (MDR) to certain antimicrobial agents.

Table 4: Frequency of ESBL-producing environmental water isolates that expressed multi-drug resistance (MDR) to certain antibiotics.

Environmental water Isolates	**MDR +ve**		**ESBL +ve**	
	No.	**%**	**No.**	**%**
E. coli	2	17%	2	17%
Kleb. pneumoniae	0	0	0	0
Kleb. oxytoca	0	0	0	0
Prot. Mirabilis	4	35%	4	35%
Ent. cloacae	5	42%	5	42%
Citr. freundii	0	0	0	0
Citr. koseri	1	8%	1	8%
Burkh. cepacia	0	0	0	0
P. aeruginosa	0	0	0	0
P. luteola	0	0	0	0
Acineto. Baumani	0	0	0	0
Ochrobact. Anthropi	0	0	0	0
Total	12	100%	12	100%

Fig.1: Double Disk Synergy Test shows ESBL positive E. coli.

Discussion:-

The prevalence of ESBLs among members of Enterobacteriaceae constitutes a serious threat to the current beta-lactam therapy, leading to treatment failure and consequent escalation of costs. There is an urgent need to emphasize rational use of drugs to minimize the misuse of available antimicrobials [9]. Our results shows that organisms concealing ESBL enzymes are multi-drug resistant and may have substantial therapy challenges. Organisms may easily transfer ESBL-containing plasmids to other organisms. The overall result of the present study shows that while the prevalence of ESBL producing isolates in Iraq is currently not very high, it may escalate and cause a very serious public health problem, if not checked in good time. Most clinicians in our hospitals may have no information regarding ESBL-producing organisms because of little knowledge for this health problem, therefore no preventive methods will be adopted to control its spread. This unawareness may lead to destructive consequences. Results of sensitivity tests in our study revealed that ESBL-producing isolates were more susceptible to certain members of floroquinolone and aminoglycoside antibiotics than other agents. Consequently, this result may indicate the importance of these antibiotics as a drug of choice for presently treatment of certain human infections caused by ESBL- E. coli and Klebsiella pneumoniae in our locations.

The double-disk synergy test was not routinely used in our clinical microbiology laboratories. The occurrence and spread of ESBL-producing members of the family Enterobacteriaceae may be higher than suspected since some of ESBL-producers are not detectable routinely. In our study, fresh water specimens detected 3(7%) ESBL-positive environmental isolates, whereas municipal drinking water specimens recorded 9 (20%) ESBL-positive environmental isolates. Sanitation measurements and procedures should be confirmed strictly in all production lines of drinking water for human consumption because any defect in this activity may lead to introduction of offensive organisms with serious hazards to water consumers.

World Health Organization standard stated that all water for human consumption should be totally free of coliforms but our study shows that this water produced and distributed do not met the required standard and as such not fit for human consumption. An outbreak of disease from drinking water has been reported worldwide, it was estimated that water-borne disease might account for one-third of the intestinal infections globally [11]. Another study reported that poor sanitation and hygiene were responsible for 40% of all deaths and 5.7 % of the total disease burden occurring worldwide [12].

The present study revealed that there is poor sanitary standard of municipal drinking water in Baghdad City, the isolation of coliforms indicates inadequate treatment of water or post treatment contamination and also could be contaminated from normal flora of personnel involve in the production because some of this coliforms are present as normal flora of the body. Three different enteric organisms were isolated in low numbers from all the municipal water although but this is very serious because of their high resistance to most of the antibiotics tested which are common antibiotics used in treating infections caused by such organisms. This will pose a serious therapeutic problem when such antibiotics used for treating infections caused by these organisms. This study supported the

results from other researchers in case of multi-drug resistance from water as reported in different parts of the world [13].

Antibiotic resistance has been reported in different parts of the world where E .coli was found to be resistant to all fluoroquinolones, aminoglycoside and some beta lactam antibiotics [14]. The detection of 9(20%) coliforms expressed ESBL enzyme should be considered as a signal of an urgent need for proper sanitary method of production and inspection of all water producing municipal stations to enforce that water production is according to WHO standards. This will help in eradicating the existence of these resistance organisms that could lead to a very serious public health problem in the near future. ESBL producing organisms are known worldwide to harbor multi-drug resistance genes in plasmids, which confer resistance to wide range of antibiotics. Non- ESBL producing organisms are known to be more susceptible to antimicrobial agents than ESBL producers because they lack the mutation that has occurred in the active serine site of ESBL producing organisms but in our results organisms found to be non-ESBL are also as resistant as ESBL producers. This suggests for more research work in this area using molecular technique to specifically find out the genes responsible for this grave resistance. Antibiotic resistance could be transferred from non-pathogenic bacteria to pathogenic ones by transfer of resistance genes in closely related species such as members of the Enterobacteriaceae family. To the best of our knowledge, studies on ESBL producing organisms from water is scarce and this is so far the first study that evaluated the presence of ESBL producing organism from water in Baghdad City and our findings call for urgent surveillance of presence of resistance organisms in water and for effective monitoring by regulatory bodies to make sure that water manufactured and distributed for drinking in Baghdad City conforms with WHO standard.

Conclusions:-

The following upshots could be concluded from the present study:

1. While the prevalence of ESBL producing pathogenic isolates in Iraq is currently not very high, it may increase rapidly and may lead to a serious health problem, if not tested appropriately.
2. The result of our study shows that ESBL producing isolates are multi-drug resistant and thus, may be difficult for management.
3. The detection of 9 (20%) ESBL producing Coliforms should pay attention for appropriate sanitation method of all water producing municipal stations to stress that water production is according to WHO standards.

Recommendations:-

More research work is required using molecular techniques to specifically find out the genes responsible for this grave resistance.

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