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RESEARCH ARTICLE

PHENOTYPIC AND MOLECULARCHARACTERIZATION OF PLASMID -MEDIATED AMPC β-LACTAMASES AMONG GRAMNEGATIVE CLINICAL ISOLATES.

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Abstract

There are currently no standardized phenotypic methods for the screening and detection of plasmid -mediated AmpC enzymes.

Aim: to evaluate two phenotypic methods (AmpC E test and cefoxitin – cloxacillin double disc synergy) to detect AmpC enzymes in Escherichia coli, Klebsiella spp., and Proteus mirabilis using multiplex PCR as gold standard method.

Materials and methods: total of 1500 gram negative isolates were screened for potential plasmid-mediated AmpC enzymes by Cefoxitindisc.AmpC E test and cefoxitin –cloxacillin double disc synergy tests were used to confirm detection of plasmid-mediated AmpC enzymes.The genotypic identification was done using multiplex PCR.

Results: The potential Amp C-producing isolates among all the studied isolates were only 4.7 % (70/1500) by cefoxitin disc. Among the cefoxitin resistant isolates,22.9 % and 24.3 % confirmed to be P-AmpC by cefoxitin-cloxacillin double disc synergy test and AmpC-Etest, respectively. Plasmid encoded AmpC genes were detected by PCR in 27% of cefoxitin resistant isolates. The most prevalent AmpC gene family was CIT and MOX.The sensitivity of AmpC E test and cefoxitin –cloxacillin double disc synergy were 81.3 % and100 % respectively and the specificity were 92.3% and 95.9%.

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Introduction:-

Plasmid-mediated AmpC β-lactamases(PAmpC) have been found worldwide but are less common than extended-spectrum β-lactamases (ESBLs), These enzymes have been detected invarious species lacking inducible AmpC genes, including Klebsiella spp., Proteus mirabilis, Salmonella enterica and Shigella spp. PAmpC C have been also reported in clinical strains of *Escherichia* coli **(Jacoby, 2009).**

PAmpC beta-lactamases are class C or group I cephalosporinases that confer resistance to a wide variety of β-lactam antibiotics including penicillins, expanded-spectrum cephalosporins (with the exception of cefepime and cefpirome), cephamycins, monobactams, and beta-lactam inhibitors. In contrast to expanded-spectrum beta-lactamases (ESBLs), AmpC beta-lactamases are inhibited by boronic acid and cloxacillin ***(Tan et al, 2009).***
Currently there are no CLSI guidelines for pAmpC detection. Reduced susceptibility to cefoxitin in the Enterobacteriaceae may be an indicator of AmpC activity; however cefoxitin resistance may be mediated by mechanisms other than AmpC such as porin channel mutation Hence it should be confirmed by other tests ***(Ananthan and Subha, 2005).***

Some phenotypic tests are available to confirm detection of PAmpC like Cefoxitin Hodge, three-dimensional method, AmpC disk test and inhibitor based methods have been performed. However, these phenotypic tests are not

able to differentiate between chromosomal AmpC genes and AmpC genes that are carried on plasmids ***(Tan et al., 2009).***The multiplex PCR is gold standard" for plasmid-mediated AmpC β-lactamase detectionand wasused to differentiate the six plasmid-mediated *AmpC* specific families (MOX, CIT, DHA, EBC, FOX and ACC-1)***(Perez-Perez and Hanson, 2002).***

Materials and methods:-

Bacterial Isolates:-

A total of 1500 nonduplicate clinical isolates that lack or minimally expressing *chro*mosomally encoded AmpC beta-lactamases; including *E. coli*=768, K. *Pneumoniae* = 662 and *Proteus mirabilis*=42) recovered from the microbiology lab of Zagazig University hospital during the time period from July 2014 to February 2015.The clinical isolates were collected from different clinical samples (Pus, sputum, blood, urine, CSF and other body fluids). All the isolates were identified by MALI-TOF mass spectrometry.

Screening for AmpC Production:-

The clinical isolates were screened for cefoxitin (30 µg) insusceptibility as an indicator for AmpC production by Kirby Bauer disc diffusion test disc. The inhibition zone sizes were interpreted according to CLSI guidelines. Isolates with inhibition zone diameter ≤18 mm were considered potential Amp C producers.

Phenotypic Tests for Detection of AmpCβ-lactamases:-

All the screen positive isolates were subjected to two confirmatory phenotypic tests (AmpC E test and cefoxitin – cloxacillin double disc synergy). Klebsiella Pneumonia ATCC- 1144™ (Microbiologics, MediMark, Europe) was used as Positive control strain for PAmpC.

The Etest AmpC (BioMérieux SA, France):-

E test was performed according to the manufacturer's instructions. The test principle comprises a strip impregnated with a concentration gradient of cefotetan on one half of the strip and cefotetan with cloxacillin on the other half of the strip. MICs of cefotetan alone and cefotetan with cloxacillin were determined as recommended by the manufacturer. Ratios of cefotetan versus cefotetan/cloxacillin of ≥8 were considered positive for AmpC beta-lactamase production.

The cefoxitin-cloxacillin double disc synergy test (CC-DDS) (HI Media Laboratories Pvt.Ltd):-

This test is based on the inhibitory effect of cloxacillin on AmpC using disks containing either 30 µg of cefoxitin or 30 µg of cefoxitin plus 200 µg of cloxacillin. A difference in the cefoxitin-cloxacillin inhibition zones minus the cefoxitin alone zones of ≥4 mm was considered indicative for AmpC production.

Detection of bla PampC by multiplex PCR:-

Bacterial DNA was extracted using PureLink Genomic® DNA Mini kit-Invitrogen-life technologies according to the manufacturer's instructions. Genes encoding PMABLs were amplified using the primers described by Perez-Perez &Hanson (2002).All primers were synthesized and supplied by Invitrogen-life technologies.

PCR was performed using the following conditions: initialdenaturation step at 95˚C for 5 min followed by 30 cycles of Denaturation at 94˚C for 45sec, Annealing at 62˚Cfor 45 sec, Extension at 72˚C for 1 min, with final extension at 72˚C for 5 min. PCR products were visualized on a 2 % agarose gel stained with ethidium bromide.

Statistical analysis:-

All data were coded, checked, entered and analysed using SPSS(statistical package for social science) software version 18;Performance of phenotypic tests is assessed by sensitivity, specificity, positive predictive value, negative predictive value and accuracy.

Results:-

Out of the included 1500 Gram negative clinical isolates, only 70 (4.7%) were positive by cefoxitin screening test, including 21 (30%) E.coli, *46 (65*.7%) Klebsiella *pneumoniae* and3(4.3%)Proteus *mirabilis*. The majority of The 70 Cefoxitin-resistant strains were isolated from blood and sputum (30% and 28.6% respectively).

Among 70 cefoxitin resistant strains, AmpC phenotype was confirmed in these isolates by cefoxitin-cloxacillin double disc synergy test and AmpC-Etest in22.9 % (16 / 70) and 24.3 % (17 / 70) respectively.

Comparison of confirmation assays for AmpC production:-

The performance of Etest AmpC and the cefoxitin-cloxacillin CC-DDS method were compared as phenotypic confirmation tests using multiplex PCR as the gold standard method. The results of the Etest AmpC were inconclusive in 3 isolates where MICs exceeded the scale of the test for cefotetan alone and cefotetan in combination with cloxacillin. With the CC-DDS, 7 inconclusive results were observed where no inhibition zone was present for cefoxitin alone and in combination with cloxacillin. Isolates with inconclusive results were not included in the calculation of performance parameters.

Table (1): Validity of CC-DDS test in comparison to multiplex PCR as Gold standard:

	PCR		Total	Kappa	P
CC:	**+ve**	**–ve**			
+ve	14	2	**16**	**0.73**	**<0.001****
-ve	0	47	**47**		
Total	**14**	**49**	**63**		
Validity	**Sensitivity: 100%**		**Specificity: 95.9%**		
	PVP: 87.5%		**PVN: 100%**		
Accuracy	**96.8%**				

Table (2): Validity of E-test Amp Ctest in comparison to PCR as Gold standard:

	PCR		Total	Kappa	P
E test:	**+ve**	**–ve**			
+ve	13	4	**17**	**0.63**	**<0.001****
-ve	3	47	**50**		
Total	**16**	**51**	**67**		
Validity	**Sensitivity:81.3%**		**Specificity: 92.3%**		
	PVP: 76.5%		**PVN: 94%**		
Accuracy	**89.6%**				

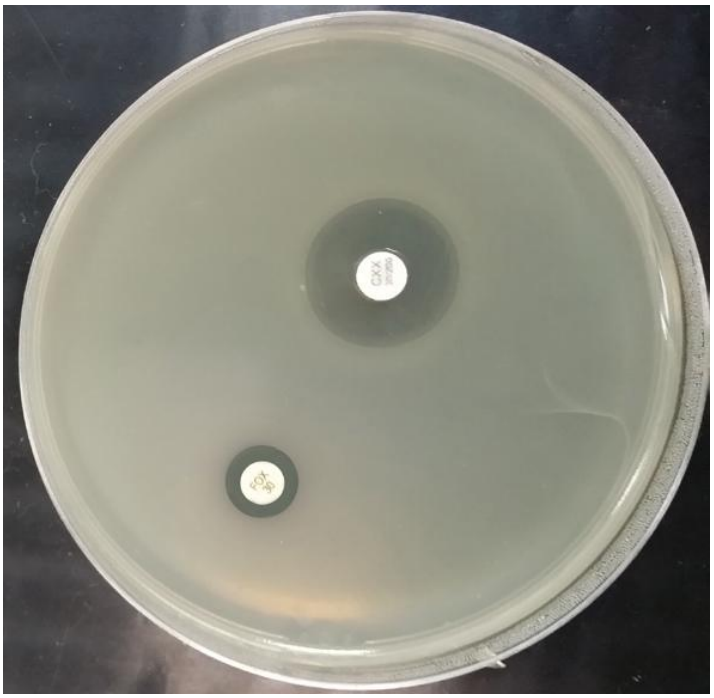

Figure (1): Positive CC-DDS test (Difference between inhibition zone diameter between FOX and CXX (cefoxitin+ cloxacillin)> 4 mm).

Figure (2): Positive E-test AmpC (CN (cefotetan)/CNI (cefotetan +cloxacillin) :> 32/ 0.5 µg/mL).

Figure (3): Negative E-test AmpC (CN (cefotetan)/CNI (cefotetan +cloxacillin) 0.5/ 0.5 µg/mL

Of The 70 Cefoxitin-resistant isolates, 19 (27 %) isolates were confirmed as being plasmid-mediated AmpC β-lactamase producers by the multiplex PCR. The distributions of the detected genes were as follows: 10 genes belonged to CIT family, 6 genes belonged to the MOX family and 3 belonged to DHA family.No genes belonging to the FOX, ACC or EBC families were detected in all isolates.

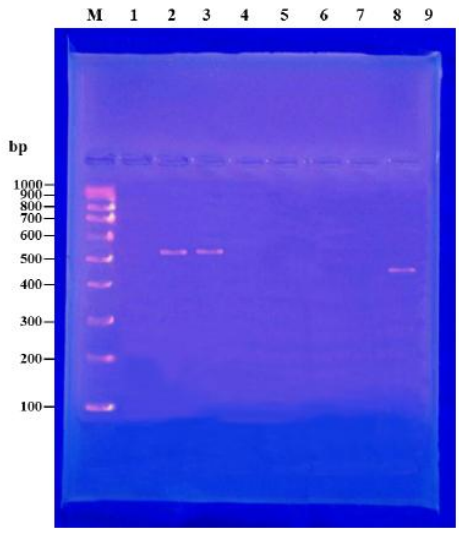

Figure (3): Multiplex PCR for detection of PAmpC beta lactamases genes of PAmpC beta lactamases genes: Lane1: Molecular mass marker (100bp DNA ladder), Lane 2: Negative water control, Lane3: Negative K. Pneumoniae, Lane4: Negative E.coli. Lane 5:K.pneumonaie with 462bp CIT gene, Lane 6, 7, 8: Negative K.pneumonae, Lane 9: K.pneumonae with 405bp DHA gene.

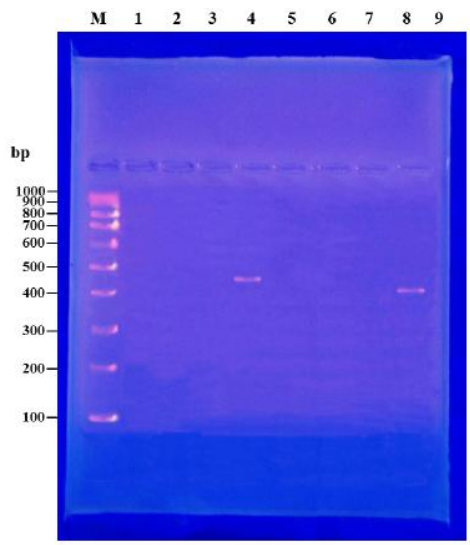

Fig (4): Multiplex PCR for detection of PAmpC beta lactamases genes: Lane1: Molecular mass marker (100bp DNA ladder), Lane 2: Negative water control, Lane3: Negative K. Pneumoniae, Lane4: Negative E.coli. Lane 5:K.pneumonaie with 462bp CIT gene, Lane 6, 7, 8: Negative K.pneumonae, Lane 9: K.pneumonae with 405bp DHA gene.

From the distribution of different P AmpC β-lactamase genes among the clinical isolates (Table3), it was observed that enzymes from the AmpC group were predominantly present in *k.pneumonae* isolates (28, 6%) followed by E.coli (23.9%)

Table (3): Distribution of ampC genes within study isolate.

Organism	PAmpC negative	CIT positive	DHA positive	MOX Positive	PAmpC genotypes N (%)
KP (n=46)	35	5	2	4	11(23.9)
E.coli (n=21)	15	4	1	1	6(28.6%)
P.M (n=3)	1	1	0	1	
Total (n=70)	51	10	3	6	27%

Discussion:-

In this study 4.7% of the screened Gram negative isolates were Cefoxitin-resistant, 16(22.9%) and 17(24.3%) of which produced AmpC as detected by cefoxitin-cloxacillin double disc synergy test and AmpC-Etest, respectively. The reason for this discrepancy is presence of mechanisms other than plasmid-mediated AmpC involved in cefoxitin resistance such as porin mutation or efflux pump ***(Tan et al, 2009).***

Wassef et al., 2014 reported similar cefoxitin prevalence rate (5.8%) but it was lower than **Helmy and Wasfy, 2014** reported higher prevalence rate 18.2%. difference of type and sample size of screened populationcould contribute in this variation between the 2 studies.

In the present study CC-DDS showed higher sensitivity and specificity than Etest AmpC (100% vs. 81.3%) and(95.9%. vs. 92.3%)..The result was agreed with ***Polsfuss et al,2011*** they have documented that CC-DDS had higher sensitivity than Etest AmpC (97.2% vs.77.4%) respectively, and the specificity was 100% for both methods. The higher specificity in the later study could be explained: firstly the later study assessed validity of E test to detect AmpC activity including plasmid-mediated AmpC beta-lactamases and chromosomal AmpC hyperproduction in E.coli and that decrease incidence of false negative results.

In the present study 19 of 70 (27%) cefoxitin resistant isolates were confirmed to possess PAmpC gene by multiplex PCR. The result showed agreement with Egyptian studies previously conducted at Cairo University hospital **(Wassef et al., 2014**) and Theodor Bilharz Research Institute (TBRI) (**Fam et al., 2013)** where PAmpC prevalence 26%, and 28.3% respectively.

In the present study, multiplex PCR revealed that CIT as the most predominant gene (52.6%) followed by MOX (31.6%) and DHA (15.8%). CIT-type enzymes appear to be prevalent in China ***(Li et al, 2008***), India***(Shanthi et al, 2012),*** Turkey ***(Yilmaz* et al., 2013**), Tunisia (***Chérif et al., 2016***). In Egypt, these results also were in agreement with studies carried in Cairo by **Helmy and Wasfy, 2014**, **Fam et al., 2013**and **Hosny and Kashif 2012** in which CIT showed the highest prevalence rate, it was detected in 86.9%, 76.5% and 60% respectively. In other study conducted by **Wassef et al. 2014**, the FOX family showed the highest prevalence rate.

Conclusion:-

Isolates of E. coli, K. pneumoniae, and Proteus mirabilis showed the occurrence of plasmid mediated AmpC β-lactamase which is alarmingbecause of probability of dissemination of these plasmid mediated resistance genes within the hospital.The multiplex PCR revealed that CIT and MOX are the most predominant genes.Regarding phenotypic confirmatory tests, CC-DDS showed higher sensitivity and specificity than Etest AmpC, besides E-test AmpC is costly compared to CC-DDS to be used by clinical laboratories for routine PAmpC screening procedureTherefore, CC-DDS is a more suitable routine confirmatory procedure for early detection of PAmpC-producing bacteria.

References:-

1. ***Ananthan S and Subha A (2005):*** Cefoxitin resistance mediated by loss of a porin in clinical strains of *Klebsiella pneumoniae* and *Escherichia coli*. Indian J Med Microbiol; 23:20-3.
2. ***Chérif T, Saidani M, Decré D, Boutiba-Ben Boubaker I, Arlet G. (2016):*** Co-occurrence of multiple AmpC β-lactamases in *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis* in Tunisia. Antimicrob Agents Chemother 60:44–51.
3. ***Fam N, Gamal D, El Said M, et al (2013):*** Detection of plasmid-mediated AmpC beta-lactamases in clinically significant bacterial isolates in a research institute hospital in Egypt. *Life Science Journal*; 10 (2):2294–2304.
4. ***Helmy M and Reham Wasfi (2014):*** Phenotypic and Molecular Characterization of Plasmid Mediated AmpC β-Lactamases among Escherichia coli, Klebsiella spp., and Proteus mirabilis Isolated from Urinary Tract Infections in Egyptian Hospitals.BioMed Research International, Article ID 171548, 8 pages.
5. ***Hosny AE-DMS and Kashif MT (2012)***: A study on occurrence of plasmid mediated AmpC ß-lactamases among gram negative clinical isolates and evaluation of different methods used for their detection. *Journal of Applied Sciences Research*; 8(4):2280–2285.
6. ***Jacoby AG (2009):*** AMP C β-lactamases. Clin.Microbiol. Rev.; 22(1):161-182.
7. ***Li Y, Li Q, Du Y, Jiang X, Tang J, et al (2008):*** Prevalence of plasmid-mediated AmpC beta-lactamases in a Chinese university hospital from 2003 to 2005: first report of CMY-2-Type AmpC beta-lactamase resistance in China. *J. Clin. Microbiol.*46, 1317–1321.
8. ***Perez-Perez FJ and Hanson ND (2002):*** Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR. J Clin Microbiol; 40: 2153-62.
9. ***Polsfuss S, Guido VB, Jacqueline GVM, Erik CBMH (2011)***: Practical Approach for reliable detection of Amp C beta-lactamase producingEnterobacteriaceae. *Clin Microbiol* 2011; 49:2798-803.-***Wassef M, Behiry I M, Younan M, El Guindy N, Mostafa S, and Abada E (2014):*** "Genotypic identification of AmpC *β*-lactamases Production in gram-negative *Bacilli* isolates," *Jundishapur Journal of Microbiology*, vol. 7, no. 1,Article ID e8856.
10. ***Shanthi M, Sekar U, Arunagiri K and Sekar B (2012***): Detection of Amp C genes encoding for beta-lactamases in Escherichia coli and Klebsiella pneumoniae.Indian J Med Microbiol. 2012 Jul-Sep; 30(3):290-5.
11. ***Tan T Y, Ng L S, He J, Koh T H, and Hsu L Y (2009***): Evaluation of screening methods to detect plasmid-mediated AmpC in Escherichia coli, Klebsiella pneumoniae, and Proteus mirabilis. Antimicrob.Agents Chemother. 53:146–1447.
12. ***Yilmaz N O, Agus N, Bozcal E, Oner O, Uzel A (2013):*** Detection of plasmid –mediated AmpC β-lactamase in E.coli and *Klebsiella pneumoniae*. Indian J Med Microbiol [serial online] 2013 [cited 2016 Feb 23]; 31:53-9.