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Occurrence of Plasmid-mediated ampC β-lactamases (PMABLs) Resistance Genes among E. coli O157:H7 Isolated from Bovine Diarrhea

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Abstract

A total samples (616) were collected from bovine (100 calf`s fecal samples; 216 calf`s internal organs; 100 cow`s fecal samples and 200 cow`s internal organs), the animals showed profuse diarrhea. The samples were submitted for the isolation and identification of E.coli O157:H7. The identified isolates were examined for their susceptibility to 16 antimicrobial agents. Multiplex PCR was used for the detection of plasmid-mediated ampC β-lactamases (MAABLs) in isolated strains.

E.coli O157:H7 isolated with rate of (2%) from the fecal sample of calves and cows. While only o.46% were isolated from internal organs of calves, meanwhile E.coli O157:H7 not isolated from cows samples. The isolates showed multidrug resistance ranged from 6%- 100%. AmpC plasmid mediated β lactamas genes were detected in all isolates of E.coli O157:H7, where 3 isolates showed presence CMY-7, BIL-1 at fragment 462bp, one isolate showed the presence of DHA-1, DHA-2 at fragment 405bp and one isolate showed MIR-1T and ACT-1 were detected at a of LAT-1 to LAT-4, CMY-2 to fragment 302bp.

PCR remains the gold standard for detection of plasmid AmpC β-lactamases. This study may represent the first report of variants of MIR-1Tand ACT-1 β-lactamase-producing E. coli in Egypt**.** Phenotypic methods alone may not reflect the true number of PMABLs producers. Genotypic methods need to be employed in national surveillance studies in Egypt.

Introduction

Plasmid-mediated ampC β-lactamases (PMABLs), originating from chromosomally-located ampC genes of different Gram-negative bacteria, have emerged since the 1980s. PMABLs production is one of the mechanisms of resistance to β-lactam antibiotics in Gram negative bacteria conferring resistance to a wide variety of β-lactam antibiotics including 7-α-methoxy cephalosporins (cefoxitin or cefotetan), oxyimino cephalosporins (cefotaxime, ceftazidime, ceftriaxone), monobactam (aztreonam) and are not inhibited by clavulanic acid (**Dahyot and Mammeri, 2011; Gupta et al., 2012 and Haldorsen, et al. 2008).**

Most plasmid-mediated AmpC genes are expressed constitutively even in the presence of a complete system for induction and the transfer of chromosomal genes

to plasmids allowed the expression of AmpC β-lactamases in Klebsiella spp.; E. coli.; P. mirabilis and Salmonella spp. **(Sabia et al. 2012)**. Resistance due to pampC enzymes is less common than Extended spectrum β-lactamases (ESBLs) production in most parts of the world, however they may be both harder to detect and broader in their spectrum of resistance activity and they are of special concern because self-transmissibility permits their spread among different bacteria **(Lee et al. 2009 and Manoharan et al. 2012).**

PMABLs can be divided into five structurally distinct clusters; the Citrobacter freundii cluster, represented by CMY-2, the Enterobacter spp. cluster with MIR-1 and ACT-1, the Morganella morganii group with DHA-1, the Hafnia alvei cluster represented by ACC-1, the Aeromonas spp. cluster with MOX-1 (also called CMY-1) and FOX-1 enzymes which constitute two distinct subgroups **(Mohamudha et al. 2012)**. The blaCMY-2 gene is the most prevalent and has been reported worldwide. Interestingly, blaCMY-2 is found in many different plasmid backgrounds, suggesting that it can be mobilized as a part of a smaller transferable fragment (**Haldorsen, et al. 2008** and **Qin, et al. 2008).** The aim of the present study is the isolation and identification of E.coli O157:H7 and detection of PMABLs among the isolated strains

Materials and Methods

A total samples (616) were collected from bovine, the type and number of samples were distributed in **Table (1).** Samples were collected according to **Galal, et al. (2013).**

Table (1): Types and Numbers of samples

Type of animals	Total number of animals	Type of samples	Total No. of samples
Calves	154	Fecal	100
		Internal organs	216
Cows	150	Fecal	100
		Internal organs	200
Total	204		616

Isolation and identification of E.coli O157:H7:

Samples were inoculated onto Sorbitol MacConkey agar (SMAC) ; chromogenic media and incubated for 18-24 hours at 35-37C. Sorbitol-negative colonies will appear colorless on SMAC was submitted for biochemical identification using API 20E and serotyping.

The isolates revealed motility; O157 suspected colonies did not ferment SMAC and its colonies were blue on chromogenic media while non 157 E.coli ferment SMAC

and colorless on chromogenic media**,** and identification byAPI 20E was observed **Fig. (1)**.

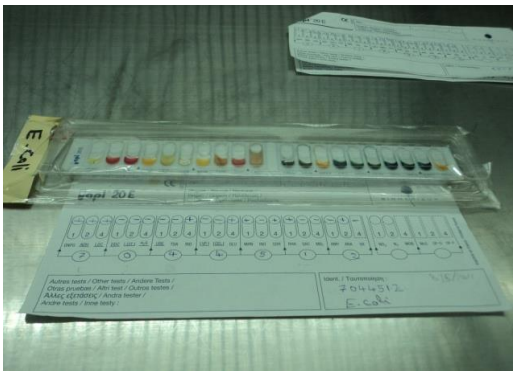

Fig. (1): E.coli isolate on API 20 E

Serotyping of O157:H7:

The determination of O antigen was carried out with heat-inactivated bacteria using the slide agglutination method according to the manufacturing procedure of Denka Seiken (Japan). The serotyping was performed in AHRI/ serology unit

Antimicrobial susceptibility test: according to **(CLSI 2012)**

Four or five typical colonies of similar morphological appearance were transferred using a sterile loop to a tube containing 5 ml of Muller-Hinton broth, incubated at 37ºC for 8 hours until its turbidity exceeds that of the standard McFarland tube No.0.5. A sterile cotton swab was dipped into the standardized bacterial suspension. The dried surface of a Muller-Hinton plates were streaked by the swab in 3 different planes by rotating the plate approximately 60º each time to ensure an even distribution of the inoculation. Plates were allowed to remain on a flat and level surface undistributed for 3 to 5 min., then the disks were applied with a fine pointed forceps on the inoculated plates, and pressed gently into the agar to ensure complete contact with the agar surface. The plates incubated in 37ºC for 24 hours. The sensitivity was measured by measuring the clear zone of inhibition around the disks and the interpretation was recorded in table according to (**CLSI 2007)**.

The used antimicrobial disc used are nalidixic acid (NA) 30µg; norfloxacin (NOR) 10 µg enrofloxacin (EFX) 10µg; ciprofloxacin (CI) 5 µg; amikacin (KA) 30 µg; gentamicin (CN) 10 µg; cefotaxime (CTX) 30µg; cephalothin (KF) 30 µg; ceftazidime (CAZ) 30 µg; ampicillin (AM) 10 µg; amoxacillin (AMO) 10 µg; amoxacillin + clavulinic acid (AMC) 20/10 µg; sulfa methaxozole trimethoprim (SXT) 1.25/23.75 µg; srythromycin (E) 10 µg; setracycline (TE) 30 µg; and chloramphenicol (C) 30 µg.

Multiplex PCR protocol

Extraction of plasmid DNA: according to (Birnboim and Doly 1979)

The tested E.coli O157:H7 isolates were grown on L-broth and incubated at37C for 18h. 0.5ml of culture was transmitted to a 1.5 eppendorf tube and centrifuged for 10000xg 15seconds. The supernatant was carefully removed; the pellets were thoroughly suspended in 100µl of solution I and incubated at 0°C for 30min. 200 µl of solution II was added and gently vortexed, where the suspension almost clear and slighltly viscous. The tube was maintained for 5min. at 0°C and then 150 µl of solution III was added and gently mixed by inversion for few seconds during which time a clot of DNA forms. The tube was maintained at 0°C for 60min. to allow most of protein, high molecular weight RNA and chromosomal DNA to precipitate. Centrifugation at 14000xg for 5min., four-tenth of the supernatant was removed to another centrifuge. Addition of cold ethanol and was held for 30min. at -20°C, the precipitate was collected by centrifugation at 14000xg r for 2min. The pellets were dissolved in 100µl of 0.1M sodium acetate/0.05M Tris-HCL (pH8.0) and reprecipitated with 2 volumes of cold thanol for 10min. at 20°C, the precipitate was dissolved in 40µl water. 10-20 µl was applied to an agarose gel for electrophoretic analysis.

Multiplex PCR protocol for detection of β-lactams resistance genes (Pérez-Pérez and Nancy 2002)

PCR was performed with a final volume of 50 µl in 0.5-ml thin-walled tubes. Each reaction contained 20 mM Tris-HCl (pH 8.4); 50 mM KCl; 0.2 mM each deoxynucleoside triphosphate; 1.5 mM MgCl2; 0.6µM primers CITMF, CITMR, DHAMF, and DHAMR; 0.5 µM primers, EBCMF, and EBCMR and 1.25 U of Taq DNA polymerase **(Table 2)**. Template DNA (2 µl) was added to 48 µl of the master mixture and then overlaid with mineral oil. The PCR program consisted of an initial denaturation step at 94°C for 3 min, followed by 25 cycles of DNA denaturation at 94°C for 30s, primer annealing at 64°C for 30s, and primer extension at 72°C for 1 min. After the last cycle, a final extension step at 72°C for 7 min was added. Five-microliter aliquots of PCR product were analyzed by gel electrophoresis with 2% agarose (Bio-Rad, Hercules, Calif.). Gels were stained with ethidium bromide at10µg/ml and visualized by UV transillumination by agarose gel electrophoresis: according to **Sambrook et al. (1989**), where electrophoresis grade agarose were prepared in 1X electrophoresis buffer (TBE) to reach the required concentration of 2%.

Table (2): Primer for β-lactams resistance genes: (Pérez-Pérez and Nancy 2002)

Target(s)	Primer	Sequence (5- to 3 -, as synthesized)	Expected amplicon size (bp)	Nucleotide position
LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	CITMF	TGG CCA GAA CTG ACA GGC AAA	462	478–498
	CITMR	TTT CTC CTG AAC GTG GCT GGC		939–919
DHA-1, DHA-2	DHAMF	AAC TTT CAC AGG TGT GCT GGG T	405	1244–1265
	DHAMR	CCG TAC GCA TAC TGG CTT TGC		1648–1628
MIR-1T ACT-1	EBCMF	TCG GTA AAG CCG ATG TTG CGG	302	1115–1135
	EBCMR	CTT CCA CTG CGG CTG CCA GTT		1416–1396

Results and discussion

Table (3) illustrated the isolation rate of E.coli O157:H7 from different samples, where E.coli O157:H7 isolated in the same rate (2%) from the fecal sample of calves and cows. While only o.46% were isolated from internal organs of calves. And samples of cows showed no E.coli O157:H7.

The rate of O157:H7 recorded in bovine fecal samples revealed the role of bovine in shedding E.coli O157 in feces. Cattle are considered the primary reservoir of E. coli O157:H7 that infect humans. Adult cattle and weaned calves that carry E. coli O157:H7 generally remain asymptomatic but shed the bacteria into the environment in their feces **(Wallace, 1999)**.

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It is an established fact that E. coli serves as a reservoir for the spread and dissemination of resistance phenotypes such as the dreaded ESBL and Carbapenemase resistance **(Akinduti et al.,2011)** this factors has also been recorded in extra intestinal E. coli infections, making treatment complicated and very expensive.

Table (4) illustrated the antimicrobial susceptibility test of E.coli O157:H7 against different antimicrobial agents, where the isolates showed multidrug resistance ranged from 6%- 100%.

Amoxicillin clavulanic acid, which used to be very active against β-lactamase producers, was not very active against our isolates, the fact that even the 3rd generation cephalosporin were not very active amongst the isolates. These results were nearly agreed with **Motayo, et al., (2012).**

AmpC plasmid mediated β lactamas genes were detected in all isolates of E.coli O157:H7, where 3 isolates showed presence of LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1 at fragment 462bp, one isolates showed the presence of DHA-1, DHA-2 at fragment 405bp and one isolate showed MIR-1T and ACT-1 were detected at a fragment 302bp. **(Fig.2)**

Genes encoding ESBL β-lactamases and carbapenemases are located on plasmids, while historically; genes encoding AmpC β-lactamases were located primarily on the chromosome of certain Enterobacteriaceae (**Livermore, and Brown, 2001**). Recently, however, ampC genes were documented to episomalize into plasmids and disseminate into various species of the family Enterobacteriaceae (**Vanwynsberghe, et al., 2007**). The plasmid borne ampC are often constitutively expressed and may confer high-level resistance to β-lactams (**Paterson, 2006)**

In the present study, the amplified portion of CMY-genes detected in this study includes CMY-2. These findings were consistent with previous studies confirming pAmpC β- lactamases of the CMY-2 type in E. coli as a major factor contributing to AmpC resistance phenotype **Mulvey et al., (2005)**; **Barwa et al., (2012**); **Woodford et al., (2007**) and **Soha and Lamiaa (2015)**.

The widespread distribution of plasmid CMY-2 among enterobacteriaceae could be attributed to specific transposon-like element ISEcp1 (**Chiu et al., 2004** and, **Su et al., 2006**]. The ISEcp1 has been presumed to be involved in the mobilization of blaCMY-2 from the Citrobacter freundii chromosome **(Su et al., 2006).** In reports from Canada, Europe, and the USA, CMY-2 was the most frequent plasmidic cephalosporinase in E.coli (**Jacoby, 2009).**

Also, O157:H7 showed the presence of DHA-1, DHA-2 at fragment 405bp. DHA-1 is a plasmid mediated AmpC β-lactamase that originated from the chromosomal AmpC gene of M. morganii **(Verdet, et al. 2000)**. It has been shown in study carried by **Pai et al. (2004)** and **Moland et al. (2008)** that mortality of patients infected with organisms that produce DHA-1 was higher than that of patients infected with organisms that produce CMY-1 and that raises concern for the spread of this inducible plasmid mediated AmpC β-lactamase. **Mai and Reham (2014)** recodred that plasmid encoded AmpC genes were detected by PCR in 88.46% of cefoxitin resistant isolates. The most prevalent AmpC gene family was CIT including

CMY-2, CMY-4, and two CMY-2 variants. The second prevalent gene was DHA-1 which was detected in E. coli and Klebsiella pneumonia. Also **Yilmaz et al. (2013)** used multiplex PCR and found that 24 pAmpC β-lactamase-positive isolates.

To our knowledge, this is the first report from Egypt identifying MIR-1T ACT-1 and in bacterial isolates. In Egypt as unfortunately, there is no Egyptian paper identifying MIR-1T ACT-1, where MIR-1T ACT-1 was detected in one isolate of E.coli O157:H7 at a fragment 302bp. ACT-1 and MIR-1 share 91.4% amino acid identity with each other but only 85 to 87% identity with most E. cloacae AmpC enzymes. However, the enzyme from E. cloacae strain GN7471 **(Kuga et al., 2000)** has 91.1% identity to ACT-1 and MIR-1, and an environmental strain of Enterobacter was 98% identical, so that origin from some Enterobacter species is likely. **(Philippon et al., 2002)**

It was concluded that dairy cattle are a reservoir of E.coli 0157:H7. Bovine feces are a potential vehicle for transmitting E. coli O157:H7 to cattle, food, and the environment. Appropriate handling of bovine feces is important to control the spread of this pathogen.

Further investigations of infected herds are necessary needed to understand the ecology of this organism in dairy and beef cattle, the mechanisms by which meat and milk become contaminated, and the potential for herd-based control measures to prevent this growing public health problem in Egypt.

It was concluded that CMY-type β-lactamase–producing E. coli strains are almost common

Presence of plasmid-encoded AmpC enzymes was noted in E.coli O157:H7 isolated from different samples revealed the misuse in the form of self medication may cause the spread of resistance, which will result in the prevalence of resistance against effective antibiotics.

PCR remains the gold standard for detection of plasmid AmpC β-lactamases. This study may represent the first report of variants of MIR-1Tand ACT-1 β-lactamase-producing E. coli in Egypt.

Phenotypic methods alone may not reflect the true number of PMABLs producers. Genotypic methods need to be employed in national surveillance studies in Egypt.

Table (3): Rate of E.coli O157:H7 in different samples

Type of samples	Total No. of samples	No. of O157:H7	%
Calf`s fecal sample	100	2	2
Calf`s internal organ	216	1	0.46
Cow`s fecal samples	100	2	2
Cow`s internal organs	200	0	0
Total	616	5	0.81

Table (4): Antibacterial susceptibility test using disc diffusion agar for E.coli O157:H7 (5 isolates)

Antibiotic disc	Conc. Of disc	Sensitive		Resistance	
		No.	%	No.	%
Quinolone					
Nalidixic acid (NA)	30µg	0	0	5	100
Fluroquiinolones					
Norofloxacin (NOR)	10 µg	1	20	4	80
Enrofloxacin (EFX)	10 µg	4	80	1	20
Ciprofloxacin (CI)	5 µg	0	0	5	100
Aminoglycosides					
Amikacin (KA)	30 µg	0	0	5	100
Gentamicin (CN)	10 µg	0	0	5	100
β-lactams (Cephalosporine)					
Cefotaxime (TX)	30µg	0	0	5	100
Cephalothin (KF)	30µg	2	40	3	60
Ceftazidime (caz)	30µg	1	20	4	80
β-lactams (Penicillin)					
Ampicillin (AM)	10 µg	1	20	4	80
Amoxicillin	10 µg	0	0	5	100
Amoxicillin + clavulenic acid	30 µg	2	40	3	60
Sulfonamides					
Sulfa methaxozole trimethoprim (SXT)	25 µg	0	0	5	100
Macrolide					
Erythromycin (E)	10 µg	0	0	5	100
Tetracyclin					
Tetracycline (TE)	30 µg	1	20	4	80
phenicols					
Chloramphenicol (C)	30 µg	1	20	4	80

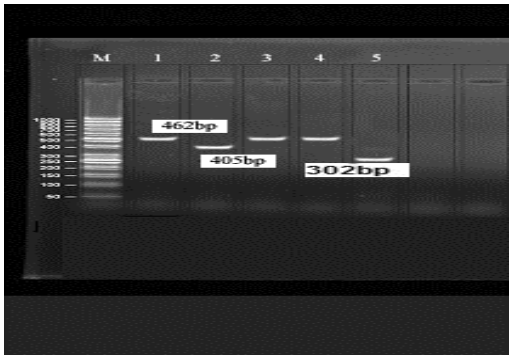

Fig. (10): Multiplex PCR for the detection of β-lactams multidrug resistance O157:H7 isolates Lane M: ladder 50bp; Lane 1:control negative, Lanes 2, 4,5 isolates showed presence of LAT-1 to LAT-4, CMY-2 to CMY-7; lanes 3, isolates showed presence of DHA-1, DHA-2 and lane 6: isolate showed presence of MIR-1T ACT-1 gene.

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