MOLECULAR CHARACTERIZATION OF ANTIBIOTIC RESISTANCE IN GRAM – NEGATIVE BACTERIA ISOLATED FROM DAIRY PRODUCTS

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ABSTRACT

The present work was carried out on 127 dairy products samples (62 buffalo milk, 46 cow milk, 16 kariesh cheeses and 3 yoghurt) were obtained from retail markets in Dakahlia governorate. (214) isolates were assayed for antimicrobial susceptibility, the presence of integrons and antimicrobial resistance genes. 152 out of 214 (71.02%) Gram – negative bacterial isolates showed multidrug resistance phenotypes for two or more of the following antimicrobial agents: ampicillin, streptomycin, gentamicin, tetracycline, trimethoprim/ sulfamethoxazole, nalidixic acid, ciprofloxacin, amoxicillin - clavulanic acid , chloramphenicol and cefotaxime . PCR screening for integrons showed that eight (3.73%) isolates of (Enterobacter aerogens, Klebsiella pneumoniae, Klebsiella oxytoca, Citrobacter diversus, Citrobacter freundii, Proteus vulgaris, Escherichia coli and Serratia marsescens) were positive for class 1 integron and all isolates were negative for class 2 integron .β-lactamase resistance gene bla_{TEM} was identified in 6 (2.80%) isolates of (K. oxytoca , E. coli , S. liquefaciens ,E. cloacae, K. pneumoniae and C. diversus). bla_{CTX-M} was identified in 3 (1.40%) isolates of (E. coli , K. oxytoca, and S. marsescens). All isolates were negative for $bla_{CMY.}$

These results highlighted the role of antimicrobial use in dairy animals and development of transferable gene in bacteria in dairy animals from which such genes can be disseminated by horizontal gene transfer to other bacteria and reach human pathogens.

Key words: Integrons, β-lactamases, bla_{TEM}. bla_{CTX-M}, bla_{CMY}

INTRODUCTION

Over the last 25 years, the global incidence of food borne infections has markedly increased, with nearly a quarter of the population at a high risk of illness ***(Oliver et al., 2005).*** Food borne pathogens are major threat to food safety, especially in developing countries where hygiene and sanitation facilities are often poor ***(Ahmed and Shimamoto, 2014).*** While human illness from milk borne pathogens may be linked to contamination of the product after pasteurization or improper pasteurization, such diseases are usually associated with consumption of raw milk or its by-products **(*Cristine et al., 2014).***

Of particular concern, is the potential transmission of multidrug resistant (MDR) foodborne pathogens to humans through the food supply. Antimicrobial resistance among these foodborne bacteria is not uncommon and is often associated with the use of antimicrobial agents in food animals ***(ThrelFall et al., 2000 Molbak, 2005)***. Since most integrons are carried on plasmids and transposons, a strong antibiotic selective pressure can potentially result in the mobilization and dissemination of antibiotic resistance genes. Therefore, integrons play a major role in the spread of antibiotic resistance genes in Gram-negative

bacteria ***(Lever stein- Van Hall et al., 2002, Lever stein- van Hall et al., 2003)***. Resistance to ampicillin and cephalosporins in Gram-negative bacteria is primarily mediated by β-lactamases, which hydrolyse the β-lactam ring and thus inactivate the antibiotic, many different β-lactamases have been described, but TEM, CTX-M, and CMY type β-lactamase are the most predominant ***(Brad Ford, 2001).*** Therefore, the objective of this study was to characterize the molecular basis of antimicrobial resistance in multidrug-resistant from dairy products in Egypt.

MATERIALS AND METHODS

2.1 Sample collection:

(Harrigan 1998) A total of 127 samples were collected from dairy products as following (62 buffalo milk, 46 cow milk, 16 kariesh cheese and 3 yoghurt samples).These samples were randomly collected from different retail markets in Dakahlia governorate in Egypt as shown in table (1). All samples were aseptically collected in sterile bags, labeled then transferred in ice-boxes to the laboratory under strict hygienic conditions, and frozen samples were left to thaw at refrigerator at $5^{o}C$ for 18 hours.

2.2 Microbiological and molecular analysis:

2.2.1 Isolation and identification of bacteria:

The method described by ***(ISO 7251:2005)*** was followed for isolation of the family *Enterobacteriaceae*. All samples were

centrifuged for 15 min at 3000 rpm and a loopful was taken from the sediment and inoculated on Nutrient broth incubated at 37°C for 24 hrs then subculture on MacConkey's agar. The inoculated plates were then incubated at 37°C for 24 and 48 hrs .Both pink and yellow colonies were selected for further analysis and subsequent biochemical testing ***(Edwards and Ewing, 1986)***. All isolates were tested by TSI, Urease test, MR, VP, Indole , Simmon's citrate ,oxidase and catalase test. All isolates were stored at −80°C in Luria Bertani broth (LB) containing 25% glycerol until used.

2.2.2. Anti-microbial susceptibility testing:

Bacterial isolates were tested for their susceptibility to 10 different antimicrobial discs included, ampicillin (AMP), 10µg; amoxicillin–clavulanic acid (AMC), 30µg; cefotaxime (CTX), 30µg; chloramphenicol (CHL), 30µg; ciprofloxacin (CIP), 5µg; streptomycin (STR) 10µg; nalidixic acid (NA), 30µg; sulfamethazole-trimethoprim (SXT), 23.75/1.25µg; gentamicin (GEN), 10 µg; and tetracycline (TE), 30µg; by the disc diffusion method according to the standards and interpretive criteria described by CLSI ***(Clinical and Laboratory Standards Institute, 2002).*** The incidence of antimicrobial phenotypes shown in table (2).

2.2.3 Bacterial DNA preparation:

(Ahmed et al., 2007) A smooth single colony was inoculated in 5ml nutrient broth and incubated at 37°C for 18 hour, then 200µl from bacterial culture was mixed with 800µl of distilled water then made

vortex for good mixing then heating at 96°C for 5 minutes in heat block. The resulting solution was centrifuged at 10.000 rpm for 5 minutes and the 200µl from supernatant was used as the DNA template.

2.2.4. Bacterial DNA preparation, PCR for the class 1 and 2 integrons:

(Ahmed et al.,2005) Amplification reactions were carried out with 10 µl of boiled bacterial suspensions, 250 mM deoxy nucleoside triphosphate, 2.5 mM MgCl2, 50 pmol of primers and 1 U of Ampli*Taq* Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA). Distilled water was added to bring the final volume to 50 µl. The class 1 integron primers 5'-CS and 3' CS which amplify the region between the 5' – conserved segment (5'-CS) and 3'-CS of class 1 integrons, were used for the detection of class 2 integrons, PCR was performed with the primer pair hep 74 and hep 51,specific to the conserved regions of class 2 integrons as shown in table (3). Both DNA strands of the entire class 1 integrons segments were sequenced using an ABI automatic DNA sequencer **(Model 373; Perkin-Elmer**). Two other primers were designed according to the preliminary DNA sequencing results of class 2 integron segment.

2.2.5. Screening of β-lactamase-encoding genes:

(Ahmed et al.,2007) 25 bacterial isolates were tested for TEM, CTX-M, CMY β-lactamase encoding gene by PCR using universal primers for the bla_{TEM}, $bla_{\text{CTX-M}}$ and bla_{CMY} families as shown in table (3).

RESULTS

A total of 214 Gram-negative bacterial isolates were recovered from 214 samples, *Enterobacter* spp 60 (28.03%), *K. pneumoniae* 42 (19.62%), *K. oxytoca* 28 (13.08%), *Shigella* spp. 12 (5.60%), *Morganella morganii* 11(5.14%), *Yersinia enterocolitica* 10 (4.67%), *S. liquefaciens* 9 (4.20%), *P. vulgaris* 8 (3.73%), *C. freundii* 7 (3.27%), *C. diversus* 6 (2.80%), *E. coli* 6 (2.80%), *P. mirabilis* 5 (2.33%), *S. marsescens* 4 (1.86%), and *C. amalonaticus* 2 (0.93%).

PCR screening results detected class 1 integrons in 8 (3.73%) bacterial isolates as shown in figure (1) and (2) one isolates of *E. aerogens , K. pneumoniae, K. oxytoca , C. freundii, C. diversus , P. vulgaris, E. coli and S. marsescens.* All isolates were negative for class 2 integron as shown in figure (3) and (4).

Molecular characterization of bla_{TEM} was positive in 6 isolates (2.80%) in *K. oxytoca, K. pneumoniae, E. cloacae, E. coli, S. liquefaciens and C. diversus* as shown in figure (5)and (6) . The $bla_{\text{CTX-M}}$ show positive result in 3 isolates: *E. coli, K. oxytoca and S. marsescens* as shown in figure (7) and (8).

All isolates were negative for bla_{CMY} resistance gene as shown in figure (9) and (10).Resistance phenotype and incidence of resistance genes in gram negative bacteria shown in table (5).

DISCUSSION

Food of animal origin has been identified as the main vehicle for the transmission of food borne pathogens to humans ***(EFSA, 2011)***.

Multidrug resistant pathogens which have accumulated resistance genes are the main cause of failure to treat the infectious diseases resulting in increasing of morbidity and higher rates of mortality and greater economic loss on governments individuals and health care ***(lipsitch et al., 2002).*** In this study incidence of gram-negative in the isolates were *Enterobacter* spp. was the predominant (28.03%), followed by *K. pneumoniae* (19.62%), *K. oxytoca* (13.08%) *C. freundii* (3.27%), *C. koseri* (1.86%), *C. diversus* (2.80%), *C. amalonaticus* (0.93%), *Shigella* spp.(5.630%), *E. coli* (2.80%), *P. vulgaris* (3.73%), *P. mirabilis* (2.33%), *S. liquefaciens* (4.20%), *S. marsescens* (1.86%), *M. morganii* (5.14%) *Y. enterocolitica* (4.67%) and *Salmonella spp.* (0%)

compared with *E. coli* (20%), *C. freundii* (10%), *C. diversus* (10%), *E.aerogens* (15%), *E. cloacae* (15%), *K. pneumoniae* (10%) and *K. oxytoca* (20%) by ***El-Jendy (2004)*** in Egypt. But *E. coli* at a higher percentage from kareish cheese (66.3%) followed by yoghurt (50%) and raw milk (41.6%) by ***Abdel-Tawab and Khater (2009)*** in Egypt, while *Shigella* detected in 4(0.5%) raw milk samples, 3(0.4%) buffalo milk and 1(0.13%) cow milk in 7(0.9%) kariesh cheese samples, no *Shigella* were detected in any yoghurt sample,by ***(Ahmed and Shimamoto 2014)*** In Egypt.

In this study sensitivity for the isolated bacteria against 10 antimicrobial agents found the incidence of resistance to streptomycin STR (80.37%) nalidixic acid NA (52.33%) ampicillin AMP (42.52%),

gentamicin GEN (34.57%), tetracycline TE (30.37%), amoxicillin clavulanic acid AMC (24.29%), trimethoprim- sulfamethoxazole SXT (18.69%), ciprofloxacin CIP (13.08%), cefotaxime CTX (12.14%), and chloramphenicol CHL (8.41%). Compared with all *E. coli, K. pneumoniae* and *P. mirabilis* 30% were resistant to cefotaxime by ***Cao et al., (2002)***, *E. coli* show the highest resistance to penicillin (100%) followed by tetracycline (57.44%) by ***Momtaz et al., (2012)***, 73% of *E. coli* show resistance against one or more antimicrobial drugs specially chloramphenicol by ***schlegelova et al., (2002)***, all isolates of *K. pneumoniae* were highly resistant to gentamicin ,cefotaxime (14.9%), gentamicin (83.8%), ciprofloxacin(36.5%) and tetracycline (82.4%) by ***yao et al., (2007)*** among isolated bacteria of *E. cloacae, K. pneumoniae, K. oxytoca ,E. coli* and *C. freundii* they show the highest resistant to ampicillin (97.0%), streptomycin (94.1%), tetracycline (91.2%), trimethoprim/sulfamethazole (88.2%), nalidixic acid (85.3%) and chloramphenicol (76.5%) by ***Ahmed et al., (2009)***, *Shigella* was resistant to tetracycline (73.5%), trimethoprim-sulfamethoxazole (70.4%), amoxicillin - clavulanic acid (50.0%)ciprofloxacin (3.1%) and nalidixic acid (1.0%) by ***MoezArda et al., (2003)***. The isolated *E. coli* were resistant to tetracycline (25%), sulfamethoxazole (9%), streptomycin (7%) and ampicillin (3%).

Integrons play a major role in the spread of antibiotic resistance gene in gram negative bacteria ***(Rowe-Magnus et al., 2001)***. Integrons are capable of capturing individual gene cassettes, which mostly encode antibiotic resistance, by a site-specific recombination system ***(Mazel, 2006).*** In this study, class 1 integrons were detected in 8 (3.73%) of the tested bacterial isolates, the most important capture gene cassettes are

those related of dihydrofolate reductase gene (dfr), aminoglycoside adenyl transferase (aad) and chloramphenicol acetyl transferase (cat) groups which confer resistance to trimethoprim, streptomycin/ spectinomycin and chloramphenicol respectively. Compared with 46% of isolates from the family *Enterobacteriaceae* were positive for class 1 integron by ***(Goldstein et al., 2001)***, 28(25.0%) of gram negative bacterial isolates were positive for class 1 integrons. The gene cassettes within class 1 integrons included those encoding resistance to trimethoprim (dfr A1 , dfr A5 dfr A12 , dfr A15 , dfr A17 and dfr A25), aminoglycoside (aad A1 , aad A2 , aad A5 , aad A7 , aad A12, aad A22 and aad (3) -1d by ***(Ahmed and Shimamoto, 2011)***.

Prevalence of class 1 integron in *E. coli* (56.90%) from bovine mastitis by ***(wang et al., 2008)*** . In this study all isolates were negative for class 2 integron compared with class 2 integron doesn't detected also by ***(wang et al., 2008)***. Penicillin derivatives (β-lactams) was broad spectrum antibacterial agents widely used in human and veterinary medicine. Resistance to ampicillin in Gram-negative bacteria is primarily medicated by β-lactamases. Many different β-lactamases have been described, but TEM, CTX-M and CMY type β-lactamases are the most predominant in gram negative bacteria ***(Bradford, 2001).*** In this study bla_{TEM} was detected in 6 isolates (2.80%) including *K. oxytoca, K. pneumoniae, E. cloacae, E. coli, S. liquefaciens and C. diversus*. Compared with 49 isolates were positive for β-lactamase among 77 examined milk samples by ***(Cui et al., 2007)*** in china, one of mastitic milk samples contains extended spectrum β-lactamase producing strains. 2 isolated (2.2%) contain TEM by ***Geser et al., (2012).***

In this study *bla*$_{\text{CTX-M}}$ (1.80%) was identified by PCR and DNA sequencing screening in 3 isolates: *E. coli, K. oxytoca* and *S. marsescens* compared with 78 isolated (85.7%) produced CTX-M group 1 ESBLs while 6 isolates (6.6%) produced CTX-M group enzymes by ***(Geser et al., 2012)***.

In this study all isolated which are positive to *bla*$_{\text{TEM}}$ are negative to *bla*$_{\text{CTX-M}}$ resistance gene compared with (27) CTX-M carriers were additionally PCR-positive for *bla*$_{\text{TEM}}$ gene by ***(Geser et al., 2012).***

In this study all isolates were negative for *bla*$_{\text{CMY}}$ resistance gene compared with the gene *bla*$_{\text{CMY-2}}$ was identified in four bacterial isolates which isolated from 99 milk samples ***(Ahmed and Shimamoto, 2011).***

The treatment of infection is increasingly complicated by the ability of bacteria to develop multiple mechanism of resistance these multiple resistances are becoming threat to the public health due to complications of treatment and increasing both human morbidity and financial costs. In this study, many tested bacteria showed several mechanisms of antimicrobial resistance which reflected on their resistance phenotypes. These multiple resistances increase the public health hazard of these bacteria.

In conclusion, in this study many multidrug resistant Gram-negative bacteria were isolated from various types of antimicrobial resistance gene were identified from dairy products, strikingly; many of these resistance genes are recorded in clinical bacterial isolated from humans.

Table (1): Sites, and number of samples:

Sites of markets	Number of buffalo milk samples	Number of cow milk sample	Number of kariesh cheese samples	Number of yoghurt samples	Total samples
Sherbin	8	5	1	1	15
Met-Salseel	7	6	2	0	15
Gogar	8	8	1	0	17
El-Senbelawen	9	4	3	0	16
Aga	7	6	2	1	16
Belkas	6	8	1	0	15
Sandoub	7	5	3	0	15
Mansoura	10	4	3	1	18
Total	**62**	**46**	**16**	**3**	**127**

Table (2): Incidence of antimicrobial resistance.

Antimicrobial drug	Number	Percentage
Streptomycin STR	172/214	80.37%
Nalidixic acid NA	112/214	52.33%
Ampicillin AMP	91/214	42.52%
Gentamicin GEN	74/214	34.57%
Tetracycline TE	65/214	30.37%
Amoxicillin-clavulanic acid AMC	52/214	24.29%
Trimethoprim/Sulfamethazole SXT	40/214	18.69%
Cefotaxime CTX	26/214	12.14%
Ciprofloxacin CIP	24/214	11.21%
Chloramphenicol CHL	18/214	8.41%

Table (3): primers used in this study:

Primer	Sequence (5' to 3')	Amplicon size (bp)	Target	Reference or genbank accession no.
Integrons				
5' – CS 3' - CS	GGATCCAAGCAGCAAG AAGCAGACTTGACCTGA	variable	Class 1 integron	Ahmed et al ., 2007b
HEP 74 HEP 51	CGGGATCCCGGACGGCATGCACGATTTGTA GATGCCATCGCAAGTACGAG	variable	Class 2 integron	Ahmed et al ., 2007b
B - lactamases				
TEM – F TEM – R	ATAAAATTCTTGAAGACGAAA GACAGTTACCAATGCTTAATC	1080	bla_{TEM}	Ahmed et al ., 2007b
CTX – M – F CTX – M – R	CGCTTTGCGATGTGCAG ACCGCGATATCGTTGGT	550	bla_{CTX-M}	Ahmed et al ., 2007b
CMY – F CMY – R	GACAGCCTCTTTCTCCACA TGGAACGAAGGCTACGTA	1007	Bla_{CMY}	Ahmed et al ., 2007b

Table (4): PCR Conditions:

Gene / Integrons	Hot start	Denat.	Anneal.	Prim. Ext.	Cy.	Final ext.	target
Class 1 integ	94°c/10min	94°c/1min	55°c/1min	72°c/3min	30	72°c/10min	variable
Class 2 integ	94°c/10min	94°c/1min	55°c/1min	72°c/3min	30	72°c/10min	variable
B- lactamases							
CTX-M	95°c/10min	95°c/30sec	55°c/30sec	72°c/30sec	30	75°c/5min	550bp
CMY	94°c/10min	94°c/1min	55°c/1min	72°c/1min	35	72°c/7min	1007bp
TEM	94°c/10min	94°c/30sec	50°c/30sec	72°c/1min	30	72°c/10min	1080bp

Table (5): incidence of class I integrons and antimicrobial resistance genes in multidrug-resistant gram-negative bacteria isolated from dairy products.

NO	Isolate name	Bacteria	Resistance phenotypes	Integron/resistance gene
1	13a	*Enterobacter cloacae*	TET,SXT,NAL	bla_{TEM}
2	40	*Klebsiella pnpneumoniae*	GEN,TET,STR,NAL	bla_{TEM} ,*aadA2*
3	41x	*Serratia liquefaciens*	GEN,AMP,TET,STR	bla_{TEM}
4	51e	*Citrobacter diversus*	GEN,AMP,TET,STR,NAL	bla_{TEM}
5	57x	*E. coli*	AMC,CTX,AMP,STR,NAL	bla_{TEM}
6	88	*Citrobacter diversus*	AMC,GEN,AMP,TET,STR, SXT, NAL	$bla_{TEM,}$ *dfrA5,aadA1*
7	22a	*Serratia marsescens*	AMC,CTX,GEN,STR,SXT	bla_{CTX-M},*dfrA1*
8	43a	*E. coli*	AMC,GEN,AMP,STR	bla_{CTX-M}
9	108a	*Klebsiella oxytoca*	GEN,STR,SXT,NAL	bla_{CTX-M},*aadA1*
10	113b	*Enterobacter aerogens*	AMP,NAL,STR	*dfrA1,aadA1*
11	127	*Citrobacter freundii*	AMC,AMP,STR,SXT,CIP,NAL	*dfrA5*
12	71ax	*Proteus vulgaris*	GEN,AMP,TET,STR,SXT	*dfrA7,dfrA1,aadA1, aadB,catB3*
13	68cx	*E. coli*	GEN,AMP,STR,TET	*dfrA1*

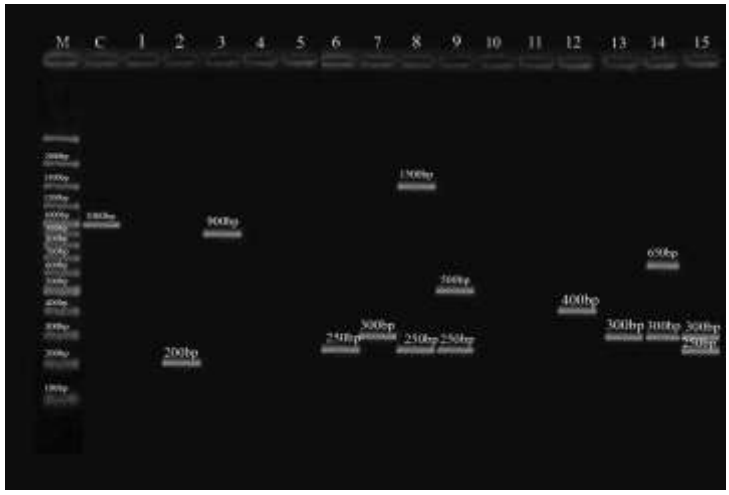

Fig. (1):1%Agarose gel electrophoresis for PCR products of the different types of class 1 integrons. Target variable.

Lanes M is λ DNA digested with HindIII used as size marker. Sample (3):900bp.Sample (8):1500bp.Sample (9):500bp.Sample (14):650bp M is a 100bp ladder used as size marker.

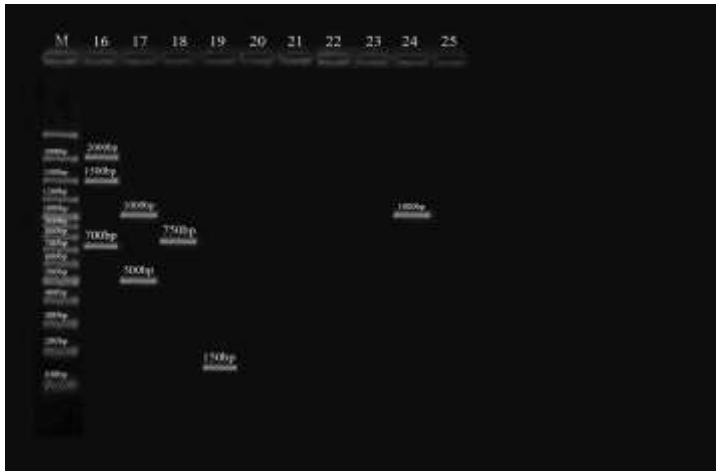

Fig. (2): 1%Agarose gel electrophoresis for PCR products of the different types of class 1 integrons. Target variable.

Lanes M is λ DNA digested with HindIII used as size marker. Sample (16):700bp, 1500bp, 2000bp.Sample (17):500bp, 1000bp.Sample (18):750bp.Sample (24):1000bp M is a 100bp ladder used as size marker.

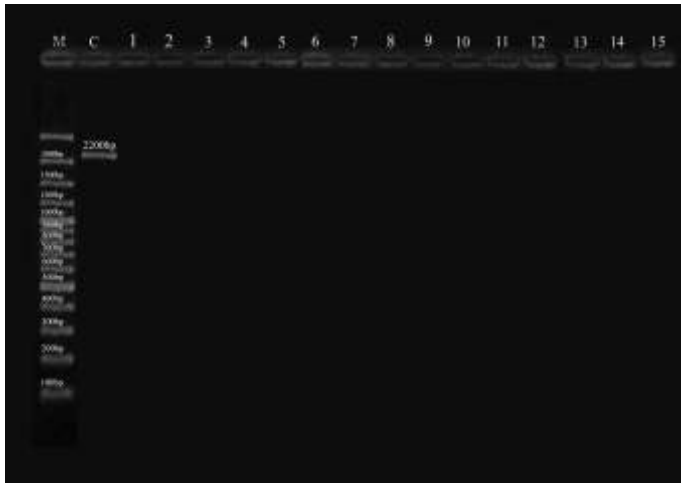

Fig. (3): 1%Agarose gel electrophoresis for PCR products of the different types of class 2 integrons. Target variable. All samples were negative.

M is a 100bp ladder used as size marker.

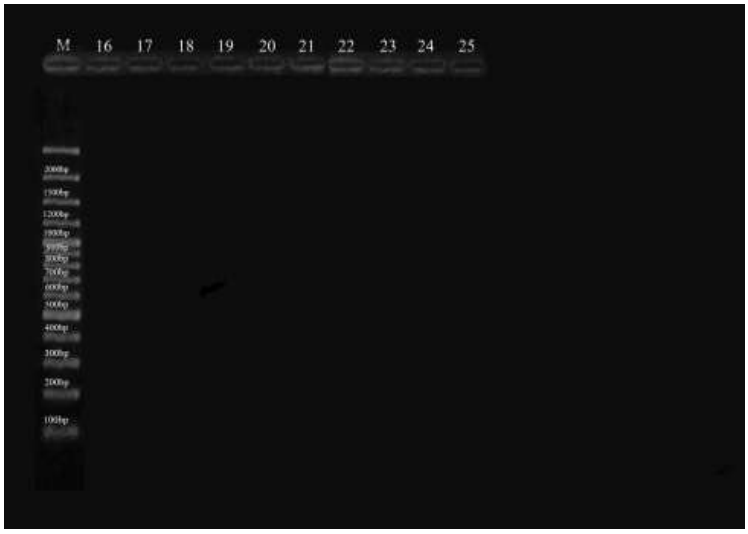

Fig. (4): 1%Agarose gel electrophoresis for PCR products of the different types of class 2 integrons. Target variable. All samples were negative.

M is a 100bp ladder used as size marker.

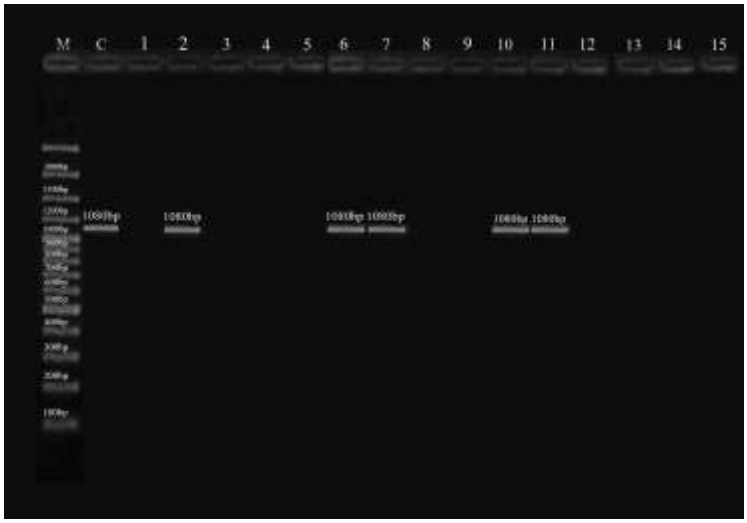

Fig.(5): 1%Agarose gel electrophoresis for PCR results of *bla*$_{TEM}$ genes (1080bp) screening of: Sample:(2),(6),(7),(10),(11) show positive result.

M is a 100bp ladder used as size marker.

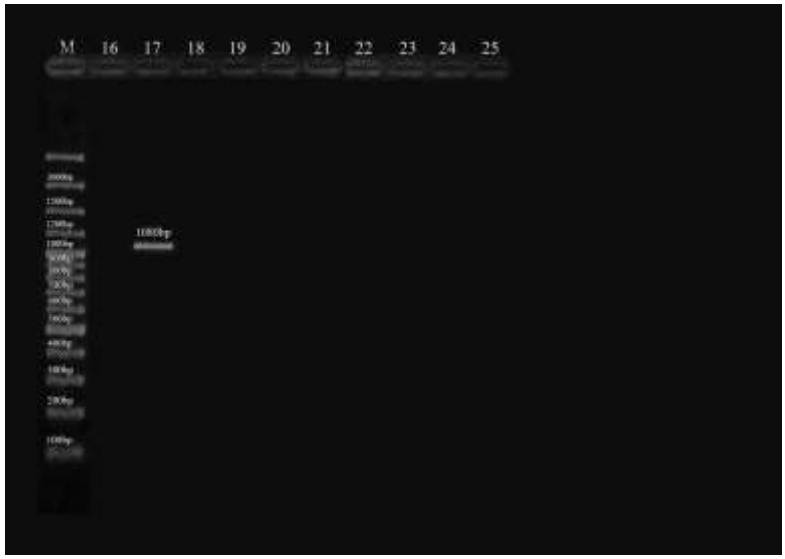

Fig.(6): 1%Agarose gel electrophoresis for PCR results of *bla*$_{\text{TEM}}$ genes (1080bp) screening of: Sample: (17) show positive result.

M is a 100bp ladder used as size marker.

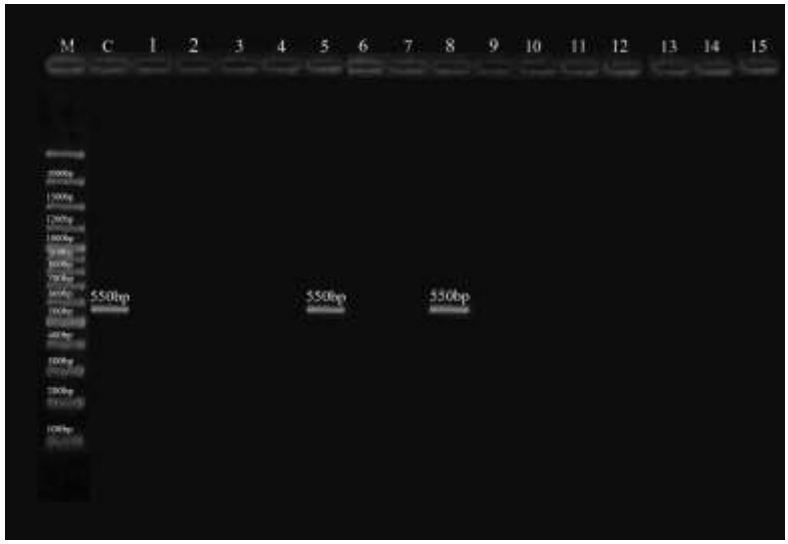

Fig. (7): 1%Agarose gel electrophoresis for PCR results of *bla*$_{\text{CTX-M}}$ genes (550bp) screening of: Sample: (5) and (8) show positive result.

M is a 100bp ladder used as size marker.

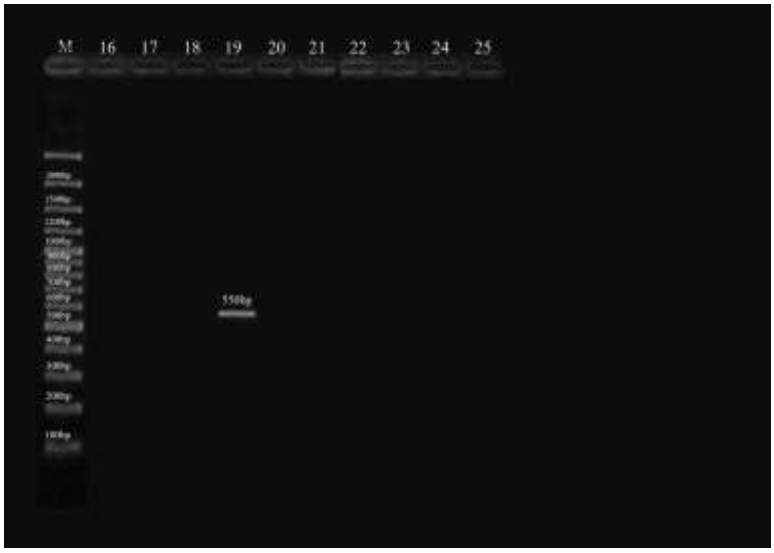

Fig. (8): 1%Agarose gel electrophoresis for PCR results of *bla*$_{\text{CTX-M}}$ genes (550bp) screening of: Sample: (19) show positive result.

M is a 100bp ladder used as size marker.

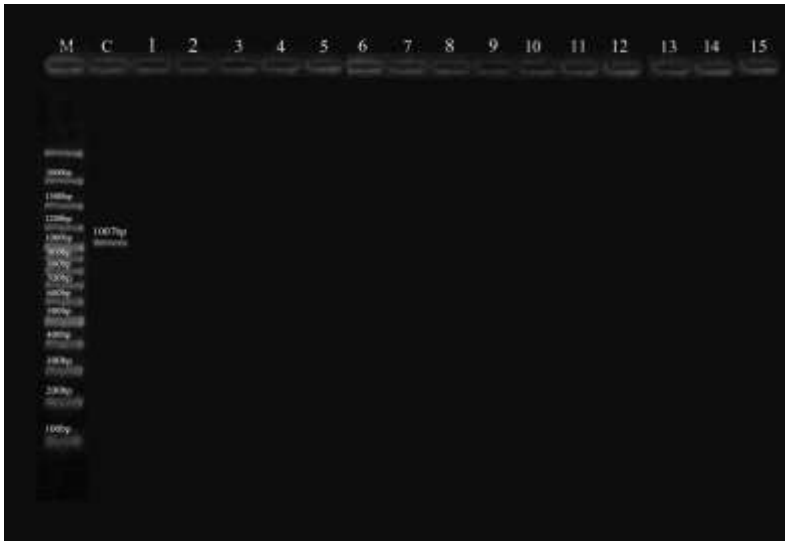

Fig. (9): 1%Agarose gel electrophoresis for PCR results of *bla*$_{\text{CMY}}$ genes (1007bp).All results were negative for bla$_{\text{CMY}}$ genes.

M is a 100bp ladder used as size marker.

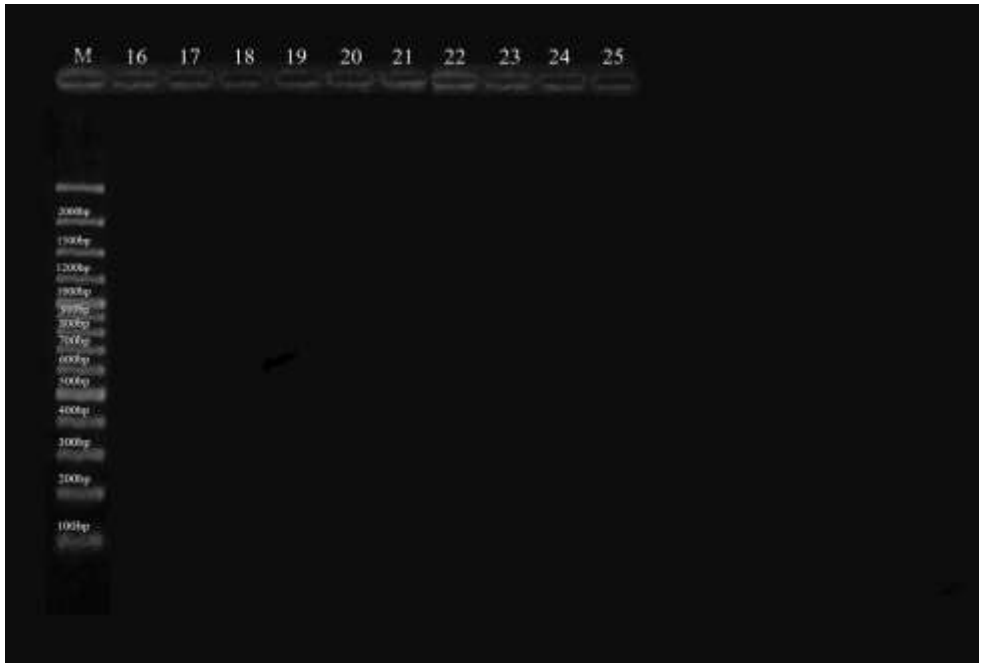

Fig. (10): 1%Agarose gel electrophoresis for PCR results of *bla*$_{CMY}$ genes (1007bp).All results were negative for bla$_{CMY}$ genes.

M is a 100bp ladder used as size marker.

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