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CHARACTERIZATION OF EXTENDED SPECTRUM B-LACTAMASES (ESβLS) GENES OF COLISEPTICEMIC *E.COLI* IN BROILER CHICKENS

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ABSTRACT

A total of 120 broiler chickens (76 diseased living and 44 freshly dead), 2 - 8 weeks old were collected from different localities in Egypt (Giza, Kaliobyia, Monofyia, Dakahlyia, Sharkyia, Kafr El-sheikh and Behyira).The internal organs " heart, liver, bone marrow, spleen and kidney" were collected from the examined broilers. The percentage recovery rate of *E. coli* from broiler chicks was (18.33%). The antimicrobial sensitivity for the recovered isolates against 10 antibiotic agents revealed that resistance to Doxycycline was (100%), Amoxicillin (90.91%), Ciprofloxacin (86.37%), Gentamycin (81.82), Sulfamethoxazole/Trimethoprim (77.27%), Chloramphenicol (72.73%), Danofloxacin (68.18%), Nitrofurantoin (68.18%), cefotaxime (40.91%) and Colistin sulphate (27.27%). The molecular characterization of some ESβL genes *(bla_{TEM}, bla_{CTX}, bla_{CMY1}, bla_{SHV} and bla_{OXA1})* in the plasmid of the isolated pathogenic *E. coli* revealed that bla_{TEM}, bla_{CTX} and bla_{SHV} genes detected in 18/22 isolates at a percentage of 81.82%, while bla_{CMY1} and bla_{OXA1} could not be detected. Meanwhile none of these genes could be recovered from the chromosomal DNA of the examined isolates. This study suggests that emergence of ESβL Colisepticemic *E.coli* magnify the disease condition and failure of control.

Key words:

E.coli, Colisepticemia, ESβL, Broilers

INTRODUCTION

Avian Pathogenic *E.coli* (APEC) is a substantial burden to the global poultry industry. It causes a syndromic poultry infection known as colibacillosis ,which has been previously associated with broiler chickens over two weeks old' **Kemmett *et al.* (2014)**. Avian colibacillosis causes significant economic losses, either as a primary disease or as a secondary infection, to broiler chickens, youth, of laying hens and of breeding hens. It is produced by

strains of *E. coli* falling within APEC pathotypes (with the penetration site, the body's respiratory mucosa), being considered systemic extra intestinal infection **Iancu *et al.* (2015)** the detection of the first ß-lactamase was reported before the use of penicillin in the medical field. Extended-spectrum-ß-lactams have been introduced in the medical practice in the 1980s for the treatment of serious gram negative bacteria but the resistance to this class of antibiotic has emerged rapidly due to production of a new class of ß-lactamase later termed Extended Spectrum β-Lactamases (ESβLs). **Al-Jasser (2006)**.(APEC) strains harbor chromosomal and plasmid pathogenicity-related genes. The presence of resistance plasmids in avian *E. coli* strains could facilitate horizontal transfer of virulence genes between pathogenic and non-pathogenic strains **(Barros, 2012)** .In Gram-negative bacteria, the production of beta-lactamases represents the most important contributing factor to resistance against beta-lactam antibiotics. During the last few years increasing numbers of antibiotic-resistant bacteria have become a problem in the field of infection control **(Liebana, 2013).** (APEC) have the ability to hydrolyze various types of the newer beta-lactam antibiotics, including extended-spectrum cephalosporins of the 3rd and 4th generations (e.g .cefotaxime ,ceftriaxone and ceftazidime) and monobactams (e.g .aztreona) ,which were assessed as ‘‘critically important antimicrobials’ **(WHO, 2011).** Nowadays the predominant ESβL-gene families encountered are *bla*$_{CTX}$-M ,*bla*$_{TEM}$ and *bla*$_{SHV}$ **(Poirel *et al.*, 2012).** Multi-drug resistant **(**MDR) *E. coli* among poultry may colonize in the human intestinal tract via food and may also contribute resistance genes to human endogenous flora **(Savita, 2007).** So the present study aimed at characterization of the commonly isolated *E coli* from septicemic broiler chickens with the determination of their antimicrobial susceptibility, resistance, and resistance genes.

MATERIAL AND METHODS

Samples:

A total of 600 heart, liver, bone marrow, spleen and kidney collected from 120 broiler chicks to investigate the prevalence of *E. coli.* All samples were brought to the bacteriology laboratory, Poultry Department, Animal Health Research Institute, Dokki, Giza in sterile wide-mouth screw capped bottles undercooling and then analyzed for the presence of *E.coli.*

Isolation of Colisepticemic *E.coli* according to Swayne *et al*., (1998) and *Quinn et al*., (2002).

Under complete sterile condition broilers internal organs (Heart, Liver, Bone marrow, Spleen and Kidney) were examined for isolation and identification of *E.coli*. Samples were

first inoculated into peptone buffer water and incubated at 37 °C for 18 - 24 hours. A loopful from each culture was subcultured on MacConkey's agar medium at 37 °C for a further 24 hr. the suspected colonies (lactose fermenter pink colonies) were subcultured on E.M.B agar medium for further identification of *E. coli* organisms. The biochemical identification of the isolated *E. coli* was confirmed according to the API 20 E system.

Antimicrobial susceptibility testing

The disc diffusion technique was applied to detect the antimicrobial sensitivity of the isolated *E.coli* against 10 antimicrobials (Doxycycline, Amoxicillin, Ciprofloxacin, Gentamycin, Sulfamethoxazole/Trimethoprim, Chloramphenicol, Danofloxacin, Nitrofurantoin, cefotaxime and Colistin sulphate) .The test was carried out according to **Cruickshank *et al.* (1975).** Cartridges of antimicrobial-containing discs were obtained from Oxoid (Hampshire, UK). Interpretation was done according to **NCCLS, (1990).**

β -lactamase detection using Combined Disc Diffusion Test (CDD).

The identification of the ESβL genes was carried out by using both Cefotaxime and Ceftazidime, alone and in combination with Clavulanic acid (cefotaxime 30 µg, cefotaxime/clavulanic acid 30/10 µg) and (Ceftazidime 30 µg, Ceftazidime/Clavulanic acid 30/10 µg). The plats were incubated at 37 °C overnight. The organisms were considered to be producing ESβL when A ≥ 5 mm increase in a zone diameter for either antimicrobial agent tested in combination with Clavulanic acid versus the zone diameter of the agent when tested alone equals ESβL. **(CLSI, 2016).**

Molecular Detection of ESβL Colisepticemic *E.coli* genes by PCR.

The molecular detection of ESβL genes was carried out by using primers for detection of chromosomal DNA and plasmid. Theses primers in addition to the cyclic conditions are demonstrated in (Tables1and 2).

RESULTS

Incidence of *E. coli* recovered from Septicemic broiler chickens.

AS demonstrated in (Table 3) out of 120 examined broiler chickens (76 diseased living and 44 freshly dead), 22 *E. coli* isolates were recovered with an incidence of 18.33 % (22 isolates /120 bird). Regarding the diseased living birds, the incidence was 22.37% (17/76), while from dead chichens was 11.36% (5/44).

Results of Antibiogram for the recovered *E. coli.*

22 *E. coli* isolates tested against 10 chemotherapeutic agents are tabulated in (Table 4). Doxycycline recorded the highest resistance percentage (100%), while Amoxicillin, Ciprofoxacin , Gentamycin Sulfamethxazole/Trimethoprim, Chloramphenicol, Danofloxacin, Nitrofurantoin and Cefotaxime recorded 90.91%, 86.37%, 81.82%, 77.27%, 72.73%, 68.18%m 68.18% and 40.91% resistance respectively. Colistin sulphate recorded the lowest percentage of resistance (27.27%) against *E.coli* isolates.

Results of β -lactamase detection using Combined Disc Diffusion Test (CDD).

Results of phenotypic detection of of β –lactamase producing *E. coli* revealed that out of 22 examined *E. coli* 19 (86.36%) found ESβLs producers (Table 5).

Results of molecular detection of ESβL genes in the isolated *E. coli*.

The molecular characterization of some ESβL genes (*bla*$_{TEM}$, *bla*$_{CTX}$, *bla*$_{CMY1}$, *bla*$_{SHV}$ and *bla*$_{OXA1}$) in the chromosomal DNA revealed that these genes could not be detected. While in the plasmid *bla*$_{TEM}$, *bla*$_{CTX}$ and *bla*$_{SHV}$ genes detected in 15/22(68.18 %), 6/22 (27.27%) and 18/22 (81.82%) respectively, while *bla*$_{CMY1}$ and *bla*$_{OXA1}$ could not be detected in the examined isolates, these results are demonstrated in (Table 6) and illustrated in Figs. (1 and 2). Results of the relationship between phenotypic and genotypic characters of ESβLs in chromosomal and plasmid DNA revealed that 18/22 (81.82%) found harboring ESβLs plasmid genes, sample number 5 found positive phenotypically but negative genotypically, all the phenotypically negative and positive ESβLs are found negative for ESβl genes in the chromosomal DNA (Table 7)

DISCUSSION

Avian colibacillosis is caused by a group of pathogens designated avian pathogenic *E. coli* (APEC). Despite being known for over a century, avian colibacillosis remains one of the major endemic diseases afflicting the poultry industry worldwide. **(Francis *et al.*, 2008).** In this study, the incidence of *E. coli* in broiler chicks was 18.33%, as shown in (Table 3), this result may be due to many factors such as, environmental stresses, keeping large number of birds in a poorly ventilated houses or due to infection with Mycoplasma gallicepticum or viral infection **(Seidavi *et al.*, 2010; and Bopp *et al.*, 2005)** or due to vertical contamination **(Gross, 1994).** These results mostly agreed with that results obtained by **Ammar *et al.* (2015)** who recovered pathogenic *E. coli* from 20% of the examined poultry collected from different sources in Egypt. Also **Mona *et al.* (2015)** isolated pathogenic *E. coli* from broilers infected

with swollen head syndrome in Egypt at a percentage of 28%. Higher results obtained by **Ashraf *et al.*(2014)** who isolated the organism from 38% from the examined cases in four different centers in Kaliobia governorate**. Heba *et al.* (2012)** examined 800 chickens suffering from colisepticaemia, collected from different governorates in Egypt for detection of pathogenic *E. coli*; they isolated the organism from 43% from the examined birds**. Hamza *et al.*(2016)** investigated 100 diseased broiler chickens in Sharkia province, Egypt, the incidence of *E. coli* isolation was 60%. **Sarah *et al.* (2013)** recovered pathogenic *E. coli* from 510 samples collected from different sources of poultry broiler farms with overall prevalence of 75.2%. As shown in (Table 4), the antibiogram was carried out against all *E. coli* isolates by using 10 different antibiotic discs. The results revealed that all isolates expressed multiple antimicrobial phenotypes (greater than or equal to 3 antimicrobials). This pattern of *E. coli* resistance was reported by **(Savita *et al.*, 2007; Wang *et al.*, 2010; Foder *et al.*, 2011 and Meena, 2015)**. The results reported in this study are due to the misuse of antimicrobial agents in poultry farms for treatment of infections caused by pathogenic bacteria, this widespread usage of large quantities of antimicrobials in poultry farms in Egypt without veterinary consultation together with using antibiotics as growth promoters create a great problem in controlling diseases. These finding agreed with that recorded by **(Aarestrup, 2005).** In this work genotypic characterization of ESβLs was carried out through amplification of 5 genes (*bla*$_{TEM}$, *bla*$_{CTX}$, *bla*$_{CMY}$, *bla*$_{SHV}$ and *bla*$_{OXA}$) by PCR in both chromosomal DNA and plasmid of the isolated pathogenic *E.coli* as demonstrated in (Table 6). None of these genes could be detected in the chromosomal DNA, this agreed with **George *et al.* (2014)** how found that extended spectrum β-lactamases are typically plasmid mediated and seen mainly in *E. coli* and *Klebsiella pneumonia*, although other authors like **Chishimba *et al.* (2016)** recovered *bla*$_{TEM}$, *bla*$_{CTX}$ and *bla*$_{SHV}$ genes from the chromosomal DNA of *E. coli* isolates . Meanwhile three genes (*bla*$_{TEM}$, *bla*$_{CTX}$ and *bla*$_{SHV}$) could be recovered from the plasmid, bla$_{TEM}$ was detected in 15/22 *E. coli* isolate (68.18%), *bla*$_{CTX}$, in 6/22 (27.27%) and *bla*$_{SHV}$ in18/22 (81.82%). Also there were combinations between 2 or 3 genes in the same *E. coli* isolate. A combination between *bla*$_{TEM}$ and *bla*$_{SHV}$ was noticed in 9 isolates (40.91%), meanwhile a combination between the 3 genes was detected in 6 isolates (27.27%).Here are some reports recorded in Egypt about detection of ESβLs, In Egypt **Ahmed *et al.* (2013)** could recover beta-lactamase encoding genes, *bla*$_{TEM}$, *bla*$_{OXA}$, *bla*$_{CTX}$ and *bla*$_{SHV}$ from 94.5% coli cepticemic broiler chicks. **Abdelhakim *et al.* (2011)** tested Seventy six non-typhoid

Salmonella isolated from both human and poultry in Egypt and Algeria, by Polymerase chain reaction (PCR), he could detect *bla*$_{TEM}$ and *bla*$_{SHV}$ genes. **Sascha *et al.* (2016)** in their study in Nile Delta, Egypt reported 113 of 114 phenotypically 3rd generation cephalosporin-resistant isolates harbored at least one of the ESβL resistance genes *(bla*$_{CTX}$-M15, *bla*$_{CTX}$-M9, *bla*$_{TEM}$ and *bla*$_{SHV}$). **Mohammed *et al.* (2015)** detected *bla*$_{TEM-1}$ gene in 57.6% and *bla*$_{SHV-1}$ in 6.8% of the isolates from chickens, whilst they could not detect *bla*$_{OXA}$ gene.

CONCLUSION

It was concluded that emergence of ESβL Colisepticemic *E.coli* magnify the disease condition and failure of control.

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Table (1): Primers Used for Molecular Detection of Esβl's Colisepticemic *E.coli* Genes by PCR **(Henrik *et al.*, 2005)**

Primer		Sequence	Amplified product	Reference
blaTEM	F	GCGGAACCCCTATTTG	964	Henrik *et al.*, (2005)
	R	TCTAAAGTATATATGAGTAAACTTGGTC		
blaCTX	F	ATGTGCAGYACCAGTAARGTKATGGC	593	Henrik *et al.*, (2005)
	R	TGGGTRAARTARGTSACCAGAAYCAGCGG		
blaCMY-1	F	GTGGTGGATGCCAGCATCC	915	Henrik *et al.*, (2005)
	R	GGTCGAGCCGGTCTTGTTGAA		
blaSHV	F	TTCGCCTGTGTATTATCTCCCTG	854	Henrik *et al.*, (2005)
	R	TTAGCGTTGCCAGTGYTCG		
BlaOXA1	F	ATGAAAAACACAATACATATCAACTTCG	820	Henrik *et al.*,(2005)
	R	GTGTGTTTAGAATGGTGATCGCATT		

Table (2): Thermal cycle conditions Used For Molecular Detection of Esβl's Colisepticemic *E.coli* Genes *by PCR* **(Henrik *et al.*, 2005)**

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
balTEM	94 ˚C\3 min.	94 ˚C\60 sec.	50-52 ˚C\60 sec.	72 ˚C\60 sec.	25	72 ˚C\10 min.
balCTX	94 ˚C\3 min.	94 ˚C\60 sec.	50-52 ˚C\60	72 ˚C\60	25	72 ˚C\10 min.
balCMY-1	94 ˚C\3 min.	94 ˚C\60 sec.	50-52 ˚C\60	72 ˚C\60	25	72 ˚C\10 min.
balSHV	94 ˚C\3 min.	94 ˚C\60 sec.	50-52 ˚C\60	72 ˚C\60	25	72 ˚C\10 min.
balOXA-1	94 ˚C\3 min.	94 ˚C\60 sec.	50-52 ˚C\60	72 ˚C\60	25	72 ˚C\10 min.

Table (3): Incidence of *E. coli* in broiler chickens.

Governorate	No. of diseased living chicken	No. of isolates	No. of dead chicken	No. of isolates	Total No. of isolates	Total No. of examined chicken	% of isolation
Giza	13	3	7	1	4	20	20
Kalyobyia	10	2	6	1	3	16	18.75
Monofyia	10	3	5	0	3	15	20
Dakahlyia	9	1	9	1	2	18	11.11
Sharkyia	13	4	6	0	4	19	21.05
KafrEl-Sheikh	10	1	5	1	2	15	13.33
Behyra	11	3	6	1	4	17	23.53
Total	76	17	44	5	22	120	18.33

Table (4): Results of antimicrobial sensitivity tests.

Isolate No.	Chemotherapeutic agent										% of resistance
	AML	C	CT	DFX	DO	GM	F	SXT	CIP	CTX	
1	R	R	I	R	R	R	R	R	R	S	8/10 (80%)
2	R	S	S	R	R	R	R	R	R	R	8/10 (80%)
3	R	R	S	R	R	R	I	R	R	S	7/10 (705)
4	R	R	S	R	R	R	R	R	R	S	8/10 (80%)
5	R	R	S	I	R	R	R	R	R	R	8/10 (80%)
6	R	R	S	R	R	R	R	R	R	I	8/10 (80%)
7	R	R	S	R	R	R	R	R	R	R	9/10 (90%)
8	R	R	S	R	R	R	R	R	R	S	8/10 (80%)
9	R	R	S	I	R	R	S	R	R	S	6/10 (60%)
10	R	R	S	R	R	R	S	S	R	S	6/10 (60%)
11	R	R	S	R	R	S	S	S	R	S	5/10 (50%)
12	R	R	S	I	R	S	R	S	R	S	5/10 (50%)
13	S	S	R	S	R	R	R	R	S	R	6/10 (60%)
14	R	R	R	R	R	R	R	R	R	R	10/10(100%)
15	R	R	R	R	R	R	S	R	I	R	8/10 (80%)
16	R	R	S	R	R	R	R	R	R	R	9/10 (90%)
17	R	R	R	R	R	S	R	R	R	S	8/10 (80%)
18	R	R	R	R	R	R	S	R	R	R	9/10 (90%)
19	R	R	I	R	R	R	R	R	R	S	8/10 (80%)
20	R	S	R	S	R	R	R	R	S	R	7/10 (70%)
21	R	R	S	S	R	R	S	S	R	S	5/10 (50%)
22	I	S	S	S	R	S	R	S	R	S	3/10 (30%)
No. of resistant strains	20	16	6	15	22	18	15	17	19	9	
% of resistant strains	90.91	72.73	27.27	68.18	100	81.82	68.18	77.27	86.37	40.91	

Table (5): Phenotypic identification of ESβLs.

Isolate No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	Total
ESβL phenotype	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	19/22 86.36%

T able (6): Results of ESβL genes by PCR.

Isolate No.	**ESBL *gene***									
	DNA					***Plasmid***				
	TEM	**CTX**	**CMY1**	**SHV**	**OXA1**	**TEM**	**CTX**	**CMY1**	**SHV**	**OXA1**
1	-	-	-	-	-	+	+	-	+	-
2	-	-	-	-	-	+	+	-	+	-
3	-	-	-	-	-	+	-	-	+	-
4	-	-	-	-	-	+	+	-	+	-
5	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	+	+	-	+	-
7	-	-	-	-	-	+	-	-	+	-
8	-	-	-	-	-	+	-	-	+	-
9	-	-	-	-	-	+	-	-	+	-
10	-	-	-	-	-	-	-	-	+	-
11	-	-	-	-	-	+	-	-	+	-
12	-	-	-	-	-	+	-	-	+	-
13	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	+	-	-	+	-
16	-	-	-	-	-	+	-	-	+	-
17	-	-	-	-	-	+	-	-	+	-
18	-	-	-	-	-	-	-	-	+	-
19	-	-	-	-	-	+	+	-	+	-
20	-	-	-	-	-	+	+	-	+	-
21	-	-	-	-	-	-	-	-	+	-
22	-	-	-	-	-	-	-	-	-	-
Total	**0**	**0**	**0**	**0**	**0**	**15**	**6**	**0**	**18**	**0**
%	**0**	**0**	**0**	**0**	**0**	**68.18**	**27.27**	**0**	**81.82**	**0**

Table (7): Relationship between phenotypic and genotypic characters in Chromosomal DNA and plasmid.

Sample No.	ESBL (Phenotypic)	Chromosome (Genotypic)	Plasmid (Genotypic)
1	Pos.	-	+
2	Pos.	-	+
3	Pos.	-	+
4	Pos.	-	+
5	Pos.	-	-
6	Pos.	-	+
7	Pos.	-	+
8	Pos.	-	+
9	Pos.	-	+
10	Pos.	-	+
11	Pos.	-	+
12	Pos.	-	+
13	Neg.	-	-
14	Neg.	-	-
15	Pos.	-	+
16	Pos.	-	+
17	Pos.	-	+
18	Pos.	-	+
19	Pos.	-	+
20	Pos.	-	+
21	Pos.	-	+
22	Neg.	-	-

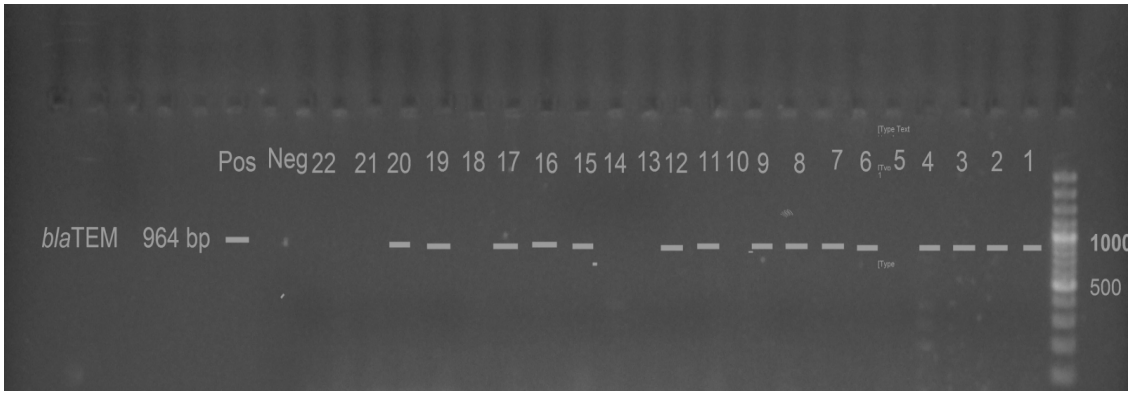

Fig. (1): Detection of ***bla*$_{TEM}$** gene in plasmid; positive samples produced band (964 bp); DNA Ladder,1Kb; lanes 1,2,3,4,6,7,8,9,11,12,15,16,17,19 and 20 were the positive samples.

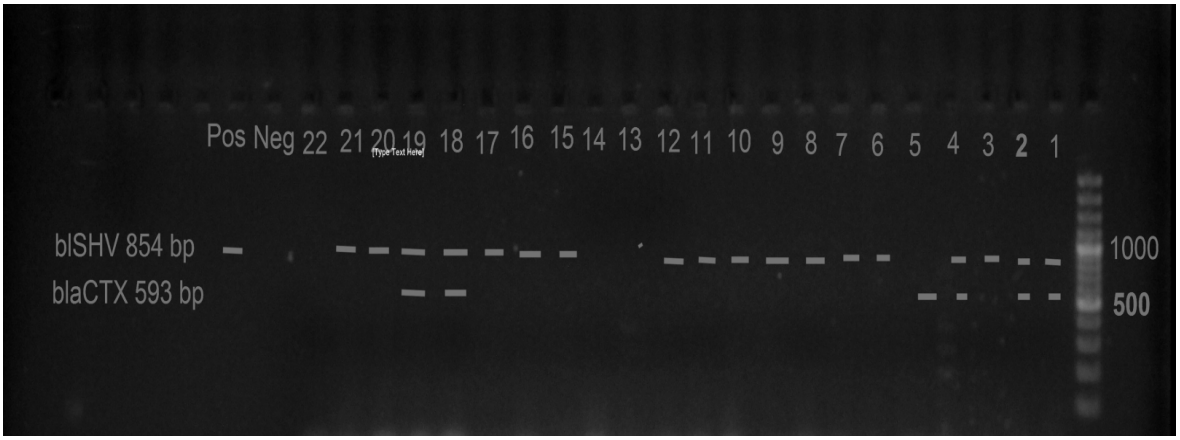

Fig. (2): Detection of ***bla*$_{SHV}$ and *bla*$_{CTX}$** genes in plasmid; samples No1,2,3,4,6,7,8,9,10,11,12,15,16,17,18,19,20 and 21 were the positive samples produced band (964 bp) for *bla*$_{SHV}$ gene ,DNA Ladder,1Kb.While samples 1,2,4,5,19 and 20 were the positive samples of *bla*$_{CTX}$ gene produced band (593bp);DNA ladder,1kb.