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RESEARCH ARTICLE

SCREENING FOR THE EXTENDED SPECTRUM BETA LACTAMASES AMONG SALMONELLA SPP. ISOLATED FROM BROILER CHICKEN IN EGYPT.

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Abstract

The intestinal carriage of Salmonella producing extended spectrum beta – lactamase (ESBL) in broilers may help to contaminate retail meat and thus participate in the presence of these bacteria in humans. The study aimed to investigate Salmonella contamination in 115 breast meat samples from diseased broiler chickens at Dakahlia Governorate, Egypt. A total 46 isolates of *Salmonella* spp. were phenotypically identified. Furthermore, the isolates were screened for ESBL production and revealed 11 (23.9%) isolates were grown on MacConkey agar supplemented by cefotaxime 1 mg⁄ L, and from, only 54.4% isolates were positive with double disc synergy test using different 3rd generation cephalosporins. Also, The ESBL producing isolates were serotyped, where the predominant serotypes were Salmonella Typhimurium and Salmonella Enteritidis (36.4%, each), then, these isolates were the screening for lactamase gene showed that only 5 isolates harbored bla_{TEM}. The current study recommends strict restrictions on using cephalosporins agents in medical and veterinary sectors with a continuous monitoring of ESBL producing bacteria in the broiler farms. Also, further investigations are needed for the detection of various mechanisms for cephalosporins resistance.

Introduction:-

Infection with salmonellae has public health and economic impact (Sanchez et al., 2002). Chickens are considered the main reservoir of Salomnellae, therefore, infection is mainly required through the food chain (Majowicz et al., 2010). The drugs of choice for the treatment of Salmonella infections especially in complicated cases include fluoroquinolones and extended spectrum cephalosporins, which are widely used in the veterinary sector (Le Hello et al., 2011). Emergence of antibiotic resistance in Salmonella spp. was attributed to selective pressure due to the misuse of antibiotics in humans and veterinary fields (Murgia et al., 2015). This was mainly encountered in important classes of antibiotics, such as the β-lactams (Livermore et al., 2007).

β-lactam resistance is attributed to β-lactamase production in Gram-negative bacteria (Livermore, 2003). β-lactamase production is an important factor producing resistance to different types of β-lactam antibiotics, including the expanded-spectrum (3rd generation) cephalosporins (Bradford, 2001). Hence, treatment failure with

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cephalosporins is mainly reported in expanded-spectrum β-lactamase (ESBL) producing bacteria leading to serious consequences for infection control (Paterson and Bonomo, 2005).

The incidence of extended spectrum cephalosporin resistant Salmonella has increased in several countries (Noda et al., 2015). In Egypt, cephalosporin resistant Salmonella isolates from chicken meat have been reported (Ahmed and Shimamoto, 2015). The detection of ESBL-producing bacteria by initial screening test is crucial for therapy and control measures of infections (Wilson and McCabe, 2007). Initial screening of ESBL producers was performed by MacConkey agar containing ceftazidime 1 mg/L or cefotaxime 1 mg/L. This combination enabled the detection of 100% of known ESBL producers. However, confirmatory tests should be applied because any organism with resistance to the antibiotic used in the agar will grow (Wilson and McCabe, 2007). The extended-spectrum β-lactamases are derived from point mutations in the β-lactamase genes, which are inhibited by clavulanic acid (Bush and Jacoby, 2010), while, ampicillin class C β-lactamase (AmpC) enzymes are not (Jacoby, 2009). Thus, it is important in clinical microbiology laboratories to detect and report ESBL-producing organisms.

The study aimed to investigate Salmonella contamination in broiler chicken farms at Dakahlia Governorate, Egypt, with screening for extended spectrum beta lactamase production using both MacConkey agar supplemented by cefotaxime 1 mg/L and the double disc synergy test with different 3rd generation cephalosporins.

Materials and Methods:-

Sample collection

A total of 115 breast meat samples from diseased chickens were collected from four broiler farms around the time of sale at Dakahlia Governorate, Egypt, between December 2017 and May 2018. The clinical symptoms on the birds were observed and recorded (Table 1). The collected samples were aseptically transported in ice box as soon as possible to the laboratory for bacteriological examination.

Isolation and identification of Salmonella spp.

Salmonella spp. isolation were performed according to ISO-6579 (2002) protocol. Twenty five grams of breast meat samples were individually pre-enriched at 37 ºC for 24 h with 225 mL of buffered peptone water broth (Fluka, Sigma Aldrich, France) after homogenization. From each pre-enrichment solution; 100 µl were transferred into 10 mL of Rappaport Vassiliadis broth (VP) (Merck Darmstadt, Germany) and incubated at 42 ºC for 24 h. All enriched samples were streaked on Xylose Lysine Deoxycholate Agar (XLD) (Fluka analytical Steinheim, Switzerland) and incubated at 37 °C for 24 h. Suspected colonies were subjected to further biochemical identification (Murray et al., 2003).

Phenotypic identification and serotyping of ESBL isolates

The phenotypically identified Salmonella isolates were screened for extended-spectrum β-lactamase activity using MacConkey agar containing cefotaxime 1 mg/ L (Wilson and McCabe, 2007). The colonies were confirmed by morphological and culture characters onto MacConky and Salmonella Shigella (S-S) agar media. The identified ESBL producers on MacConkey agar containing cefotaxime 1 mg/ L were subjected to susceptibility testing according to the Kirby Bauer disc diffusion method, using Mueller Hinton agar with the following discs (Oxoid) of third generation cephalosporin antibiotics: Cefpodoxime (CPD, 10 µg), Cefotaxime (CTX, 30 µg), Ceftazidime (CAZ, 30 µg), Cefoxitin (FOX, 30 µg) and Cefepime (FEP, 30 µg). The results were interpreted according to the criteria recommended by the Clinical and Laboratory Standards Institute for antimicrobial susceptibility testing (CLSI, 2011).

Furthermore, these ESBL producers were confirmed in accordance with CLSI (2011) by double disc synergy test. This test was performed using the five cephalosporin antibiotics in combination with the β-lactamase inhibitor (clavulanic acid; CA). The increase in the diameter of the inhibition zone for these drugs by at least 5 mm (compared with results obtained with the cephalosporin alone) indicated a positive result (CLSI, 2011).

All Salmonella isolates phenotypically confirmed as ESBL were serogrouped on the basis of somatic (O) and flagellar (H) antigens by slide agglutination test using commercial antisera (SIFIN, Berlin, Germany) following Kauffman–White scheme (Popoff et al., 2004). Reference strains; Escherichia coli ATCC@25922 and Klebsiella pneumoniae ATCC@700603 as negative and positive ESBL control, respectively, were used.

Genotyping of ESBL isolates

The DNA from phenotypically identified ESBL isolates was extracted using QIAamp DNA mini kit according to the manufacturer's instructions. Screening for the presence of β-lactamases genes (blaTEM, blaSHV, and blaCTX-M) were carried out by conventional PCR (Colom et al., 2003; Mulvey et al., 2003; Dierikx et al., 2010).
Amplification products were detected by electrophoresis, using agarose gels containing SYBR safe (Invitrogen, Leek, the Netherlands), along with a DNA molecular weight marker (BenchTop pGEM®DNA Marker, Promega, Madison, Wisconsin, USA).

Results:-

Isolation and identification of Salmonella spp.

A total of 46 isolates of Salmonella spp. were preliminary identified by their colonial morphology on MacConkey agar, XLD agar and S-S agar, in addition to morphological characters; Gram negative non spore forming short shaped bacilli, and biochemical reactions; negative reaction for oxidase, indole and VP, in addition to yellow and blue coloration on urea and Simmon´s Citrate agar, respectively, in addition, positive reaction was shown on TSI agar. The clinical signs and age of diseased broilers, in addition to incidence of Salmonella spp. were shown in Table 1.

Table 1:-Flock description, isolation rate of Salmonella spp. From broiler chicken farms in the Dakahlia Governorate in delta area of Egypt

Farm N.	Broiler flocks		Samples	
	Age	Clinical signs	N. of breast meat samples	Positive samples N (%)
F 1	39 day	Whitish diarrhea, conjunctivitis and decreased body weight	34	11 (32.3%)
F 2	40 day	Pasty diarrhea, loss of appetite	22	9 (40.9%)
F 3	36 day	Respiratory signs, loss of appetite	28	10 (35.7%)
F 4	42 day	Decreased body weight	31	16 (51.6%)
Total			**115**	**46 (40%)**

Phenotypic identification, Antimicrobial resistance profile and serotyping of ESBL isolates

Out of 46 Salmonella isolates, only 11 (23.9%) isolates were identified on MacConkey agar supplemented by cefotaxime 1 mg⁄ L, of which, only 6 (54.4%) isolates were positive with double disc synergy test (Table 2). High degrees of resistance to different members of cephalosporin were detected in phenotypically identified ESBL isolates by the disc diffusion testing method (Table 3). The serotyping of these isolates revealed that S. Entrritidis and S. Typhimurium were the predominant serotypes.

Table 2:-Phenotypically Identified ESBL Isolates

Identified Serotypes* (N, %)	Phenotypically identified ESBL isolates N (%)		Results of double disc synergy test with different Cephalosporins (mm***)				
	Growth on MacConkey with cefotaxime	Positive double disc synergy test	CTX	FOX	CPD	CAZ	FEP
S. Enteritidis (17, 36.9%)	4	2 (18.1%)	+9	+6	+8	+6	+5
S. Typhimurium (17, 36.9%)	4	2 (18.1%)	+10	+6	+6	+9	+5
S. paratyphi (8, 17.4%)	2	1 (9%)	+8	+5	+5	+5	+5
Un-typed (4, 8.7%)	1	1 (9%)	+5	+6	+5	+5	+5
Total (46, 100%)	**11**	**6 (54.4%) ****					

Serogrouped on the basis of somatic (O) and flagellar (H) antigens by slide agglutination test following Kauffman–White scheme. ** Versus total isolates grown on agar with antibiotic. *** The increase in the diameter of the

inhibition zone for these drugs in combination with clavulanic acid when compared with results obtained with the antibiotics alone. Cefpodoxime (CPD), Cefotaxime (CTX), Ceftazidime (CAZ), Cefoxitin (FOX) and Cefepime (FEP), Clavulanic acid (CA)

Table 3:-Phenotypic Antimicrobial Susceptibility Profiles of ESBL Isolates According To CLSI (2011)

Cephalosporin agent (Conc.)	S. enteritidis (N=4)			S. typhimurium (N=4)			S. paratyphi A (N=2)			Un typed (N=1)		
	R	I	S	R	I	S	R	I	S	R	I	S
Cefotaxime (CTX, 30 µg)	3 (75%)	-	1 (25%)	2 (50%)	1 (25%)	1 (25%)	2 (100%)	-	-	1 (100%)	-	-
Cefoxitin (FOX, 30 µg)	2 (50%)	1 (25%)	1 (25%)	2 (50%)	-	2 (50%)	1 (50%)	-	1 (50%)	1 (100%)	-	-
Cefpodoxime (CPD, 10 µg)	2 (50%)	-	2 (50%)	2 (50%)	-	2 (50%)	2 (100%)	-	-	1 (100%)	-	-
Ceftazidime (CAZ, 30 µg)	2 (50%)	-	2 (50%)	2 (50%)	-	2 (50%)	1 (50%)	-	1 (50%)	1 (100%)	-	-
Cefepime (FEP, 30 µg)	2 (50%)	1 (25%)	1 (25%)	2 (50%)	-	2 (50%)	2 (100%)	-	-	1 (100%)	-	-

* (%) Calculated to total number of examined serotype

Genotyping of ESBL isolates

From 11 phenotypically identified ESBL isolates, five isolates (45.5 %) harbored only blaTEM, (Figure 1).

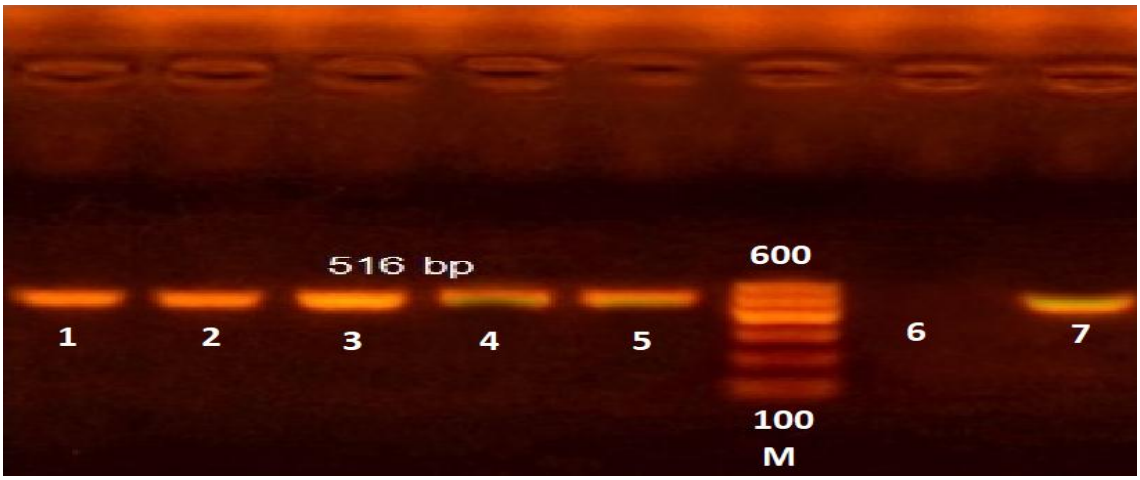

Figure 1:-Agarose gel electrophoresis pattern for amplification product of blaTEM gene of phenotypically identified ESBL Salmonella isolates. 1-5; positive isolates, 6; Negative control, 7; Positive control, M: DNA molecular size marker (100 bp).

Discussion:-

Isolation and identification of Salmonella spp.

Salmonella is one of the major causes of foodborne outbreaks in Egypt (El-Sharkawy et al., 2017). In the present study, Salmonella species were isolated from 40% of breast meat samples obtained at Dakahlia Governorate, Egypt, and the predominant serotypes among ESBL producing isolates were S. Typhimurium and S. Enteritidis (36.9%, each). In accordance, El-Ghany et al. (2012) reported that S. Typhimurium and S. Enteritidis have been identified as the predominant serotypes present in Egyptian poultry farms among ESBL producing isolates. Comparable prevalence rate of Salmonella spp. was reported in Egypt (41%) (El-Sharkawy et al., 2017), South Korea (42.3%) (Hyeon et al., 2011), Algeria (36.6%) (Elgroud et al., 2009) and Japan (45.6%) (Noda et al., 2015). On the other hand, higher prevalence rate was reported in Algeria (60%) (Ayachi et al., 2010).

However, lower prevalence rates were obtained in Egypt (4.7% and 10.9%) (El-Ghany et al., 2012; El-Tawab et al., 2015), France (3.4%), Germany (2.7%) (EFSA, 2013), Spain (1%) (Lamas et al., 2016), Morocco (24%) (Chaiba

and Filali, 2016), UK (10.7%) (Snow et al., 2008), Lithuania (29%), Netherlands (11%) and Italy (20%) (Pieskus et al., 2008). Overall, the variation in Salmonella spp. isolation rates among the previously mentioned studies could be attributed to Salmonella infection control plan and the hygienic state in poultry farms, where Salmonella can persist. In addition to, difference in the study area, sanitation level during handling and processing of chicken, season of sampling and the laboratory methodologies employed for isolation.

Phenotypic identification, Antimicrobial resistance profile and serotyping of ESBL isolates

To our knowledge, this is the first study compare between MacConkey agar supplemented by cefotaxime 1 mg⁄L and double disc synergy test. Also, using five different cephalosporin antibiotics in double disc synergy test for detection ESBL isolates, while, in the previous studies the ceftazidime and cefotaxime only were used (Wilson and McCabe, 2007). The results obtained from different members of 3rd cephalosporin in differentiation of ESBL producers by the double disc synergy test were comparable with the previously reported from cefotaxime or ceftazidime (Wilson and McCabe, 2007). This gave the ability of using different cephalosporin members in double disc synergy test.

Out of 46 Salmonella isolates, only 11 (23.9%) isolates were grown on MacConkey agar supplemented by cefotaxime 1 mg⁄L, of which, only 54.4% isolates were positive with double disc synergy test. In parallel, Djeffal et al. (2017) documented that 26.5% of Salmonella isolates were considered as ESBL producers, while, Noda et al. (2015) reported higher incidence (45.6%), based on resistance to Cefotaxime. The increased incidence of antibiotic resistance in Salmonellae has resulted from the inappropriate use of antibiotics including cephalosporins in chicken farms (Okeke et al., 2005).

Wilson and McCabe (2007) reported that the combination between MacConkey agar containing ceftazidime 1 mg⁄L or cefotaxime 1 mg⁄L enabled 100% detection of known ESBL producers, the author reported that any organism has resistance to cephalosporins can grow on the MacConkey agar supplemented with the antibiotic. Also, Jacoby (2009) reported that ampicillin class C β-lactamase (AmpC) enzymes could not be inhibited by clavulanic acid. On the other hand, Stürenburg et al. (2005) reported a compensating benefit of this agar for detection these isolates that hyper produce ampC enzymes. Thus, MacConkey agar supplemented by cefotaxime 1 mg⁄L unable to detect only ESBL producers due to the ability of other cephalosporins resistant isolates to grow on the agar, and the double disc synergy test showing higher ability for detection of ESBL producer isolates than MacConkey agar supplemented by cefotaxime.

Genotyping of ESBL isolates

The screening of eleven ESBL-producing Salmonella isolates for β-lactamases genes revealed that only five isolates from same farm harboring only blaTEM gene. The detection of these isolates in the same farm in the current study may be an indicator for horizontal transfer of ESBL resistance determinants, which are carried on mobile genetic elements, from other bacterial species (Pfeifer et al., 2010).

Conclusion:-

The dissemination of ESBL producing Salmonella always makes a vital need for continuous monitoring of ESBL producers in the poultry farms to limit its impact on human health by cephalosporin resistance genes transmission. For monitoring strategies, the double disc synergy test using different members of cephalosporin showing more ability for detection of ESBL producer isolates than MacConkey agar supplemented with antibiotics,. Also, strict restrictions on using cephalosporins agents in medical and veterinary fields are needed in Egypt.

Disclosure Statement

No competing financial interests exist.

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