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Detection of Beta lactamase -producing *E. coli* in broiler chickens and *in vitro* evaluation of antibacterial activity of chitosan nanoparticles

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Abstract

The main challenge to the poultry industry is antimicrobial resistance and the emergence of multi-drug resistant bacteria that threaten the safety of the food chain. Beta-lactam antibiotics is one of the most popular classes of antibiotics used in treatment of bacterial infection in poultry. The present study aimed to isolate beta-lactamase producing *E.coli* from (16) apparently healthy and (48) diarrhoeic broiler chickens. Pathogenic *E. coli* could be recovered with percentages of 31.3 and 43.8% from apparently healthy and diarrhoeic birds respectively. The antibiogram profiles of 10 selected *E. coli* isolates revealed that all isolates were resistant to ampicillin and cephalexin; and most of them resistant to more than three types of beta-lactam antibiotics tested. Detection of beta-lactamase genes (*blaCXT*$_M$, *blaSHV, blaTEM*) were carried out by using PCR and the results showed that 3 isolates positive for all tested genes and the other isolates have at least one of tested genes. The antibacterial activity of Chitosan nanoparticles (CNPs) against one *E. coli* isolate positive for beta-lactamase genes was evaluated by using well diffusion method. The obtained results revealed that CNPs have inhibitory effect on the growth of beta- latamase producing *E. coli.*

Keywords: *E. coli, resistance, beta-lactamase, broilers, chitosan nanoparticles, antibiogram, antibacterial activity.*

Introduction

Escherichia coli (*E. coli*) is considered as a member of the normal microflora of all warm-blooded animals including poultry **(Kaper *et al*., 2004).** However, in the debilitated or in immune suppressed hosts, or when gastro-intestinal barriers are violated, even normal "non-pathogenic" strain of *E. coli* can cause infection to poultry, humans and animals. Moreover, there are certain *E. coli* strains designated as avian pathogenic *E. coli*, spread into various internal organs and cause colibacillosis characterized by systemic fatal disease **(Nakazato *et al*., 2009).** Diseases associated with *E. coli* in poultry are manifested by yolk sac infection, omphalitis, respiratory tract infection, septicaemia, polyserositis, enteritis, cellulitis and salpingitis **(Lutful, 2010).**

Increasing consumption of chicken and their products contributed to an increasing use of antibiotics in a poultry farm. Antibiotics that are used to improve chicken growth performance and to protect chicken from pathogenic microorganisms are known as antibiotic growth promoters (AGPs) **(Gaggia *et al*., 2010)**. However, application of AGPs in poultry can cause development of bacterial resistance to antibiotics and it can affect human health, due to the residues in chicken products **(Jannaha *et al*., 2014).**

Bacteria in food-producing animals are spread through the food chain, which is important in terms of food shelf life and for transmission of pathogenic bacteria to the consumer. Food-borne pathogens and bacteria with zoonotic potential are in focus worldwide because of immense health loss and costs that arise from foodborne infection associated with bacteria **(Felix *et al*., 2013).**

β-lactam antibiotics are an important group of broad-spectrum antibiotics used in both human

and animal for the treatment of bacterial infections **(Li *et al.*, 2007).** The intensive use of these antibiotics has contributed to the emergence of resistant bacteria, including bacteria of animal origin **(Dias *et al*., 2018)**

In Gram-negative bacteria, one of the most important mechanisms of antibiotic resistance is the production of β-lactamases **(Hawkey & Jones, 2009).** These enzymes are usually acquired by horizontal gene transfer and confer resistance to β-lactams, the most commonly used class of antibiotics for the treatment of human and animal infections **(Henriques *et al.*, 2006)**

The presence of enterobacteria, especially *E. coli* that produces extended-spectrum β-lactamases (ESBLs), has increased during past decades in terms of the worldwide distribution of such resistance traits and of the evolution of different genes **(Paterson and Bonomo, 2005).** The main ESBL types are *TEM, SHV*, and *CTX*-M. Rates of *CTX*-M infections have increased during the last decade compared with rates of *TEM* and *SHV* infections. These enzymes confer resistance to β-lactam antibacterial drugs, particularly cephalosporins, and may be accompanied by co-resistance to drugs of other classes **(Paterson and Bonomo, 2005; Cantón and Coque, 2006).** Resistance genes of the ESBL type are mostly plasmid associated and therefore can spread among bacteria. In addition to human sources of transmission in hospitals and communities, animals pose a reservoir for different pathogenic bacteria with zoonotic potential, especially with food-producing animals **(Felix *et al*., 2013).**

ESBL-producing enterobacteria were shown in different sources of food-producing animals at the farm and from products **(Dierikx *et al*., 2010).**

The resistance of the bacteria and fungi to the innumerous antimicrobial agents is a major challenge in the treatment of the infections demands to the necessity for searching and finding new sources of substances with antimicrobial properties **(Raphaël and Meimandipou, 2017).** Chitosan (CS) is a natural biodegradable and biocompatible non-toxic polymer extracted from crustacean shells having immune-stimulating, antimicrobial, wound-healing properties **(Kaur *et al*., 2015).**

CS, as a cationic natural polymer, has been used widely as an antimicrobial agent for preventing and treating infectious disease **(Bangun *et al*., 2018)**. It is due to its intrinsic antimicrobial properties and its ability to deliver the antimicrobial compound to the infected area **(Kong *et al*., 2010; Dai *et al*., 2011)**. CS inhibited and suppressed microbial activities through their electrostatic charge interaction between positive charges on polycationic CS molecules (amino groups) with negative charges on the microbial surface **(Aziz *et al*., 2012).** This interaction caused disruption of the microbial cells, which then changed their metabolism and led to cell death **(Leceta *et al*., 2013).** CS can form nanoparticles using various methods, one of them is the biological method.

The present work was aimed to detect beta-lactamase producing *E. coli* in broiler chicken and evaluate the antibacterial activity of CNPs on the resistant *E. coli* and to achieve these follow the following:

Isolatation and biochemical identification of *E. coli* from caecal contents and intestinal wall of apparently healthy and diarrhoeic chickens.

Detection of pathogenicity of the isolates.

Antimicrobial susceptibility test for some *E. coli* isolates (10 isolates).

Confirmation of the isolates by detection of alkaline phosphatase gene (*pho*A*)* using polymerase chain reaction (PCR).

Detection of some beta -lactamase genes (*blaCXT*$_{M}$*, blaSHV, blaTEM*).

Evaluation of antibacterial activity of CNPs using well diffusion method.

Materials and Methods

Collection of samples

Sixty four chicken broilers were collected from some farms in Alexandria governorate, 16 were apparently healthy and 48 were diarrhoeic, transported to the lab and bacteriological examination was carried out for isolation of *E. coli* from their intestinal wall and caecal contents.

Isolation of *E. coli* from examined samples

Caecal contents and scraping of intestinal wall from each bird were inoculated into *E. coli* broth media and incubated aerobically at 37ºC for 24 hours. A loopful was taken from the liquid media and subcultured on MacConkey and

Eosin methyline blue agar media (EMB) obtained from Himedia then incubated at 37°C for 24-48 hrs.

The isolated colonies were picked up, purified and streaked on slope agar as stock culture for identification (morphologically by microscopical examination and cultural characteristics; and biochemically by Oxidase, Catalase, Indole, Citrate, Methyl red, Voges Proskauer (**CruickShank *et al*., 1975)** and in to semisolid agar for preservation and motility.

Detection of pathogenicity of isolates:

Pathogenic *E. coli* were identified by alpha haemolysis on blood agar **(Ryan *et al.,* 2004)**, growing of red colonies on Congo red agar media **(Berkhoff and Vinal., 1986).**

Antibiogram pattern of *E. coli* isolates:

Antimicrobial susceptibility test was performed for 10 selected isolates by agar disk diffusion method according to **Bauer *et al.* (1966)** using the following antimicrobials: Ampicillin (AM) (10µg), Amoxicillin-clavulanic acid (AMC) (30µg), Cefepime (FEP) (30µg), Cefotaxime (CTX) (30µg), Ceftazidime (CAZ) (30µg), Cephalexin (CL) (10µg), Ciprofloxacin (CIP) (5µg), Doxacycline (DO) (30µg), Gentamycin (CN) (10µg), Streptomycin (S) (10µg) and Trimethoprimsulphamethoxazole (COT) (25µg). The discs were distributed over the surface of Muller-Hinton agar (MHA) plates swabbed with the inoculum of isolate and incubated at 37ºC for 18-24 hours then the diameters of inhibition zones were measured and interpreted according to **Clinical and laboratory standards institute (CLSI, 2006)**.

DNA extraction:

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer:

Primers used were supplied from **Metabion (Germany)** are listed in table (1).

PCR amplification:

Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

Analysis of the PCR Products.

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. Gelpilot 100 bp ladder (Qiagen, Gmbh, Germany) and Gene ruler 100 bp ladder (Fermentas, Germany) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table (1). Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences 5´ - 3´	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
***pho*A (Alkaline phosphatase)**	CGATTCTGGAAATGG-CAAAAG CGTGATCAGCGGTGACTAT GAC	720	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Hu *et al.*, (2011)**
BlaTEM	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Colom *et al*., (2003)**
blaSHV	AGGATTGACTGCCTTTTTG ATTTGCTGATTTCGCTCG	392	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	
***blaCTX*$_{M}$**	ATG TGC AGY ACC AGT AAR GTK ATG GC TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Archambault *et al*., (2006)**

Evaluation of anibacterial activity of CNPs:

CNPs were synthesized by biological method using Ulva fasciata was obtained from Genetic Engineering and Biotechnology Research Institute (GEBRI), Egypt in the form of suspension with average nanoparticle size<50nm measured by using Transmission Electron Microscope.

The procedure of evaluation was carried out according to **Bangun *et al.*, (2018)** with some modifications).

The evaluation was performed using one *E. coli* isolate proved to have beta-lactamase genes on MHA. Where as much as 0.1 ml of inoculum was placed in a Petri dish, then added to 60 ml of sterile MHA media, homogenized, and waited until the media were solidified. Then, with the help of the sterile cork borer, four wells of 6 mm diameter were created in the MHA plate. The wells were labelled and loaded each with 25, 50,100 µl of CNPs suspension and the fourth well loaded with sterile distilled water. The plate was incubated at 37°C for 24 hours and observed for the diameter of Zone of Inhibition (ZOI) around the wells and were measured. This test was repeated three times.

Results

Table (2). *E. coli* isolated from examined chicken samples

Apparently healthy chicken			Diarrhoeic chicken			Total		
No.	+ve	%	No.	+ve	%	No.	+ve	%
16	5	31.3	48	21	43.8	64	26	40.6

Table (2) showed that Clinically sick chickens and those with diarrhoea are more likely to be *E. coli* positive as compared to apparently healthy and non-diarrheic chicken.

Table (3). *E. coli* isolated from intestinal wall and caecal contents

Intestinal wall			Caecal contents		
No.	+ve	%	No.	+ve	%
64	18	28.1	64	8	12.5

Table (3) indicated that *E. coli* could be recovered from intestinal wall more than caecal contents.

Table (4). Antibiogram profile of 10 selected *E. coli* isolates

Antimicrobial disk	Interpretation of inhibitory zone diameter					
	Susceptible		Intermediate		Resistant	
	+ve	%	+ve	%	+ve	%
Ampicillin (AM) (10µg)	-	0	-	0	10	100
Amoxicillin-clavulanic acid (AMC) (30µg)	1	10	3	30	6	60
Cefepime (FEP) (30µg)	-	0	4	40	6	60
Cefotaxime (CTX) (30µg)	6	60	1	10	3	30
Ceftazidime (CAZ) (30µg)	1	10	2	20	7	70
Ciprofloxacin (CIP) (5µg)	5	50	1	10	4	40
Cephalexin (CL) (10µg)	-	0	-	0	10	100
Doxacycline (DO) (30µg)	5	50	2	20	3	30
Gentamycin (CN) (10µg)	4	40	2	20	4	40
Streptomycin (S) (10µg)	2	20	1	10	7	70
Trimethoprimsulfamethoxazole (COT) (25µg)	2	20	-	0	8	80

Table (4) showed that all tested isolates resistant to ampicillin and cephalexin; and most of isolates resistant to more than three types of beta- lactam antibiotics (ampicillin, Amoxicillin-clavulanic acid ,cephalexin, cefepime, cefotaxime, ceftazidime).

Table (5) demonstrated that the most prevalent gene was *bla TEM.*, combination among *bla TEM,bla SHV* and *bla* CTX_M found in 3 isolates, combination between *bla TEM* and *bla* CTX_M found in another 2 isolates while 4 isolates carry only *bla TEM.*

Table (5). Alkaline phosphatase and beta-lactamase genes detected in examined *E. coli* samples

***E. coli* sample**	***pho*A**	***blaTEM***	***blaSHV***	***blaCTX*$_M$**
1	+	+	-	+
2	+	+	-	-
3	+	+	-	+
4	+	+	+	+
5	+	+	-	+
6	+	+	-	-
7	+	+	-	-
8	+	+	-	-
9	+	+	+	+
10	+	+	+	+

Table (6). Percentage of beta-lactamase genes detected in the examined samples

Gene	+ve	%
bla TEM	10	100
bla SHV	3	30
***bla CTX*$_M$**	6	60

No. of examined samples=10

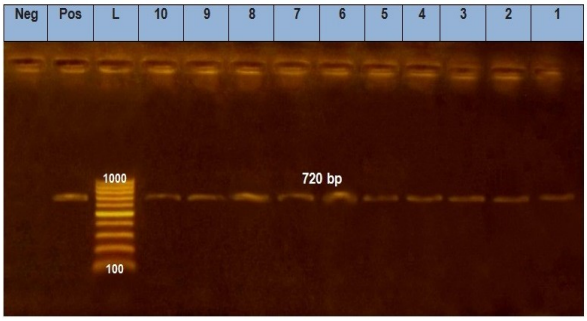

Photo. (1): Amplification of *E. coli phoA* gene by PCR. lane L: DNA size marker (100bp ladder); lane 1-10: positive

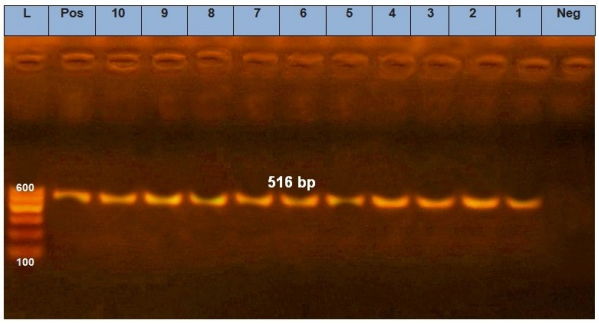

Photo. (2): Amplification of *blaTEM* gene by PCR. lane L: DNA size marker (100bp ladder); lane 1-10: positive

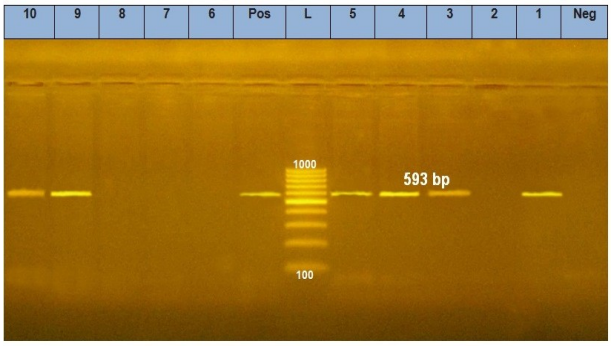

Photo (3): Amplification of *blaSHV* gene by PCR. lane L: DNA size marker (100bp ladder); lane 1, 3, 4, 5, 9, 10 positive, lane 2, 6, 7, 8 negative.

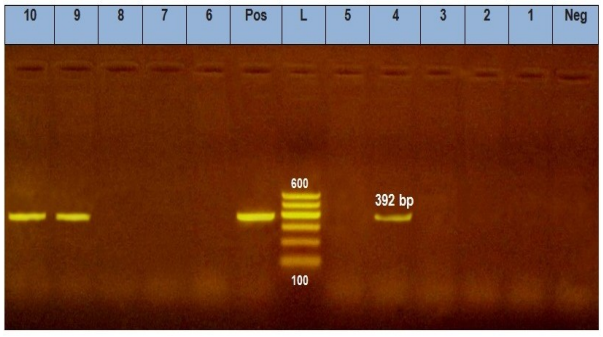

Photo. (4): Amplification of *blaSHV* gene by PCR. lane L: DNA size marker (100bp ladder); lane 4, 9, 10 positive, lane 1, 2, 3, 5, 6, 7, 8 negative.

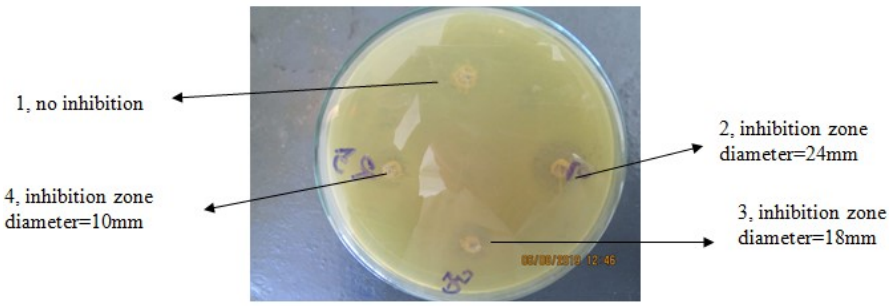

Photo. (5): Antibacterial test results for *E.coli* after 24hrs. of incubation: 1, distilled water; 2, 100µl CNPs; 3, 50µlCNPs; 4, 25µlCNPs

Discussion

E. coli is a member of the family Enterobacteriaceae, which may constitute a great hazard to poultry industry causing high mortality, loss of weight and reduction of egg production **(Bandyopadhay and Dhawedkar, 1984).** *E. coli* infection is one of the serious problems that cause a great threat to the profitability of birds' enterprises all over the world. Although *E. coli* is a normal inhabitant of the intestinal tract of birds can cause disease under the influence of predisposing factors, like inadequate and faulty ventilation, overcrowding, hunger, thirst, extremes of temperatures **(Kaul *et al*., 1992).**

Table (2) illustrated that *E. coli* could be isolated from intestinal wall and caecal contents of apparently healthy, diarrhoeic broilers and total examined samples with percentages of 31.3, 43.8% and 40.6% respectively. The obtained results were nearly similar to **Hossain *et al*. (2008)**; higher than that reported by **Gokben and Adile (2006)** while lower than that recovered by **AbdElatif (2004); Abd El Tawab *et al*. (2015); Amer *et al*. (2015)** and **Ibrahim *et al*. (2019).**

E. coli can be isolated from intestinal wall more than caecal contents and that clear from the results in table (3) as it could be isolated from intestinal wall of 64 examined birds with in a percentage of 28.1% while from caecal contents of the same examined birds with in a percentage of 12.5%.

The development of antimicrobial resistance in *E. coli* is one of major concern worldwide **(Sabra *et al*., 2019).**

Antibiogram profiles of the ten selected *E. coli* proved to be pathogenic represented in table (4) showing that resistance to ampicillin and cephalexin was 100%. Also, most of the isolates were resistant to more than three types of beta-lactam antibiotics tested and resistant to other types of antibiotic classes meaning that multidrug resistant *E. coli* were recovered from the examined birds.

The obtained results were nearly similar to that obtained by **Subedi *et al*. (2018)** and **Gundran *et al.* (2019).**

E. coli is one of the most important bacteria that causes resistance problem. These bacteria produce an enzyme called extended-spectrum β-lactamase (ESBL) that allows it to become resistant to a wide variety of penicillins and cephalosporins **(Gundran *et al*., 2019).**

Table (5), photo (1, 2, 3, 4) illustrated that Alkaline phosphatase gene (*pho*A*)* which is confirmatory for *E. coli* could be detected in all examined isolates. Also, The ESBL-producing *E. coli* isolates carried the beta- lactamase genes of either *bla*CTX_M, *bla*SHV, and *bla*TEM or a combination. Beta-lactamase genes: *bla TEM* could be detected in all examined isolates, *bla SHV* could be detected in 3 isolates and *bla* CTX_M could be detected in 6 isolates.

The percentages of beta-lactamase genes detected in the examined *E. coli* samples are represented in table (6) as 100, 30 and 60% of *bla TEM, bla SHV* and *bla* CTX_M respectively. The predominant gene in the present study was *bla TEM* followed by *bla* CTX_M, then *bla SHV* while in the study carried out by **Falgenhauer *et al*. (2018)** and **Gundran *et al*. (2019)** the predominant gene was *bla* CTX_M followed by

bla SHV **.** On the other hand **Kameyama *et al.* (2013); Rao *et al*. (2014)** recorded low prevalence of *bla* CTX_M*,* in contrast to **Alonso et al. (2017)** who recorded high prevalence of *bla* CTX_M in poultry. Also, **Chishimba *et al.* (2016); Chah *et al.* (2018)** could detect *bla TEM, bla SHV* and *bla* CTX_M or a combination in chickens.

The development of antimicrobial resistance necessitate the searching for new substance of antimicrobial properties. CS, as a cationic natural polymer, has been used widely as an antimicrobial agent for preventing and treating infectious disease **(Bangun *et al*., 2018),** it has a wide spectrum of antimicrobial activity against gram-positive and gram-negative bacteria **(Kong *et al*., 2010)**. CS can form nanoparticles using various methods, one of them is the biological method. Biosynthesis of CNPs using green sources like Ulva fasciata is a better alternative to chemical synthesis, since this green synthesis is pollutant free and eco-friendly.

Photo (5) showing well diffusion method used to evaluate the antibacterial activity of CNPs, Inhibitory zone diameters around wells: 2,3,4 loaded with 100, 50, 25 µl of CNPs measured as 24,18 and 10 mm respectively; it means that increasing of CNPs amount, increase its inhibitory effect. The antibacterial activity of CNPs against *E. coli* was previously proved by **Hassan *et al*. (2016), Rasaee *et al*. (2016)** and **Divya *et al*. (2017).**

Conclusion

The present study concluded that broiler chickens could act as a potential reservoir of ESBL producing *E. coli* and multidrug resistant *E. coli* which could be transmitted to humans through direct contact or ingestion of contaminated meat. Also, CNPs can over come the infection with beta-lactamase producing *E. coli.*

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