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**CHARACTERIZATION AND GENOTYPING OF RESISTANT ENTERIC PATHOGEN  
STRAINS ISOLATED FROM FEED MILLS IN EGYPT**

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**ABSTRACT**

Samples were collected from feed ingredients and finished products and tested for the presence of Salmonella, E.coli and clostridium spp. Positive samples for all spp. were subjected to serotyping, antimicrobial susceptibility and genotyping of isolated resistant enteric pathogens strains. *S. typhi* was resistant to tested antibiotics (inhibition degrees, ID 6–16). *S. entititidis* was only resistant to streptomycin (ID, 12). *S. typhimurium* was resistant to all antibiotics (ID,6-16) except colistin sulphate. *E.coli* O26, H9 was resistant to all antibiotics, while un-typed strains were resistant to all antibiotics except for colistin sulphate (ID, 10). Detected *aadA2* gene in all E-coli isolates indicating high virulence of these isolates. The *TetA(A)* and *blaTEM* genes for the *salmonella* isolates as well the virulence genes (*invA*, *avrA*, *sopB* and *stn*) were detected. The 100% positive results of the *avrA*, *sopB* and *stn* genes have confirmed the virulence profile of the tested salmonella isolates. The *tetA(A)* gene in Salmonella and *aadA2* gene in *E. coli* isolates were matching the resistance state obtained by the tetracycline and streptomycin sensitivity tests

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respectively. The *bla*TEM gene positive PCR was compatible with that of the antibiogram confirming the resistance of the isolated *Salmonella* to ampicillin, penicillin and amoxicillin. Alpha toxin gene of *Clostridium perfringens* was detected by PCR confirming the isolation of type A *Clostridium perfringens*. Characterization and genotyping of resistant enteric pathogens and recognition of virulent and toxin genes are more and rapid diagnostic methods for proposed control and preventive protocol against zoonotic diseases of poultry feed origin.

**Keywords: feed ingredients, enteric pathogen, toxin, genotyping, antibiotic resistant.**  
**Inhibition degree**

## INTRODUCTION

Animal feed ingredients are frequently contaminated with *Salmonella* species either from the source or from the processing plant. Steaming during the pelleting process is known to decrease the number of microorganisms in chicken feed (1). A potential and more deadly hazard has been associated with the consumption of microbial toxins of bacterial and fungal origin in feed (2). The antimicrobial agents are of great value for devising curative measures against bacterial infections. The indiscriminate use of antibiotics has led to the emergence of antimicrobial resistance in various isolates of bacteria (3). Multi-resistance was considered the biggest threat for public health and disease therapy (4). *C. perfringens* strains are classified into five toxinotypes (A, B, C, D and E), based on the production of four major toxins (a, b, o and i) (5, 6). *C. perfringens* type A strains produce the chromosomal-encoded alpha-toxin, while *C. perfringens*

type C strains produce alpha toxin together with beta toxin (6). Some strains of *C. perfringens* type A produce an enterotoxin at the moment of sporulation, causing disease in humans (7,8). Alpha toxin is a phospholipase C sphingomyelinase that hydrolyzes phospholipids and promotes membrane disorganization (9,10). *C. perfringens* type A, producing the alpha toxin, and to a lesser extent type C, producing both alpha toxin and beta toxin. Some strains of *C. perfringens* type A produce an enterotoxin at the moment of sporulation and are responsible for foodborne disease in humans (11). Feed may be contaminated during processing, storage or transport. Contaminated feed frequently causes zoonoses, it is necessary to establish surveillance programs for microbiological feed hazards. Microbiological quality control programs are increasingly applied throughout food chain production in order to minimize

the risk of infection for the consumer. Even with improved methods for detecting pathogens in foods, microbiologists so mandated often face a “needle-in-a-haystack” challenge (12,13, 14). Lynne et al. (15) characterized the genetic basis of Multi-Antibiotic Resistance (MAR) in *Salmonella* serovar Newport isolates collected from food animals in the United States during 1997-2003. They found an increase of salmonella from 4.6-10.3% and multidrug resistance to Ampicillin, Cephalothin, Cefoxitin, Ceftiofur, Amoxicillin-clavulanic acid, Ceftriaxone, Streptomycin, Sulfisoxazole, Trimethoprim-Sulfamethoxazole, Chloramphenicol and Tetracycline. Nthenge et al. (16) reported that *E. coli* was resistant to ampicillin, nalidixic acid and Kanamycin. *Salmonella* strains also were resistant to ampicillin, kanamycin and nalidixic acid. Most of the antibiotic resistance has emerged as a result of mutation or through transfer of genetic material between microorganisms. (17). The genetic means by which *Salmonella* spp has evolved to resist antimicrobials which explained either by mutations in chromosomally encoded genetic elements or by acquisition of exogenous mobile resistance genes by plasmids, integrons and transposons. The greatest resistance in isolates from poultry was to tetracycline >

streptomycin> sulfamethoxazole> gentamicin > kanamycin> ampicillin> amoxicillin-clavulanate> ceftiofur . The *E. coli* strain types can be divided into several pathogroups, resistant to ampicillin, amoxicillin/clavulanic acid, piperacillin/sulbactam, piperacillin/tazobactam, cefuroxime . The strain carries plasmid-borne blaCTX-M-15 and a blaTEM-1 genes (18). The antibiotic susceptibility indicated alarming multi-resistance frequencies for *Salmonella* and *E. coli* isolates. Antibiotic use preferentially eliminates nonresistant bacteria and increases the proportion of resistant bacteria that remains (19).

The present work was designed to identify and characterize and genotyping the resistant enteric pathogen of commercially available poultry and fish feed ingredients and finished feeds sold in Egypt which potentiate human health risk .

## MATERIALS AND METHODS

Traditional and standardized analysis of animal feed ingredient for the presence of bacteria relies on culture enrichment step, selective and differential plating, followed by subsequent identification by morphological, biochemical and/or immunological tests. These methods have low limits of detection and can be used in complex food sample

matrices, but they are labour-intensive and typically require days from initiation to readout (13).

**1-Sample collection:** Samples of feed ingredients and finished feed were obtained from different feed mills (Table 1) according to the method described by (20). The samples were transported in a cooler to the laboratory within 1 hr.

## **2. Bacteriological isolation:**

The samples were analyzed within 2-6 hours from collection. The different media such as nutrient agar (NA), nutrient broth (NB), SS agar (Salmonella-Shigella Agar), BGA (Brilliant Green Agar), EMB (eosin methylene blue) and McConkey were prepared separately.

Serotyping was performed according to the procedure described in Identification and Serotyping of Salmonella (21) in FDA's microbiological laboratories.

## **3--Antimicrobial susceptibility tests according to Osman et al., (22):**

Each Salmonella and E.coli isolate have been isolated from all samples was further characterized by serotyping and antimicrobial susceptibility testing. The antimicrobial susceptibility of *Salmonella* and *E. coli* isolates from different feed ingredients (**unpublished data**) was determined by standard agar disc diffusion

technique in accordance with the Clinical and Laboratory Standards Institute (23). The panel of antibiotic disks (Becton, Dickinson and Company) used in the panel screens belonged to certain drug classes ranked by - **FAO, -OIE and -WHO (24)**. The following antimicrobials were chosen given their common use in treating and preventing salmonella and *E. coli* infection in poultry and human. The antimicrobial agents (**13 agents**) tested and their corresponding concentrations were recorded in **table (2)**. Antibiotic discs were placed onto Mueller-Hinton Agar (Difco), the diameters of the inhibition zones were measured. Resistant isolates are defined according to the interpretation criteria to *E. coli* standard (ATCC No. 25922) established by CLSI. Antibacterial agents used in antibiotic sensitivity test (25) as illustrated in **table 2**.

## **4- PCR virulotyping & antimicrobial resistance genes:**

### **DNA extraction:**

DNA extracted from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. 200 µl of the sample was added to 20 µl of proteinase K and 200 µl of lysis buffer and incubated at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was

added to the lysate. The sample was then washed and centrifuged according to the manufacturer's instructions. DNA was eluted with 100 µl of elution buffer supplied in the kit.

#### Oligonucleotide Primers:

Primers were supplied from Metabion (Germany) and are listed in **Tables (3) and (4)**.

#### 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 20 pmol conc. of each primer, 4.5 µl of water, and 6 µl of template. The reactions were performed in an applied biosystem 2720 thermal cycler.

For multiplex PCR of different *Clostridium perfringens* toxins, Primers were utilized in a 50- µl reaction containing 25 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of 20 pmol conc. of each primer, 9 µl of water, and 8 µl of template DNA.

#### Analysis of the PCR Products:

The PCR products were electrophoresed in 1.5% agarose gel (Lonza) in 1x TBE buffer using 5V/cm gradients. 15 µl of the PCR products were loaded in each gel lane. A 100 bp DNA Ladder (Fermentas) was used to determine the fragment sizes. A gel documentation system (Alpha Innotech, Biometra, Germany) was used to photograph

and analyze the bands through computer software.

## RESULTS AND DISCUSSION

### Antimicrobial susceptibility tests:

**Data in table (2)** the antimicrobial susceptibility testing reveal that, *Salmonella Typhi* was resistant to all tested antibiotics with different inhibition degrees ranged from 6 - 16, while *Salmonella Enteritidis* was only resistant to streptomycin (inhibition degree was 12). *Salmonella Typhimurium* and the untypeable strain were resistant to all antibiotics with inhibition degrees ranged from 6 - 16, except colistin sulphate (**table 2**). It was found that 58.1% of the salmonella spp. was resistant to one or more antibiotics in Greece ( **26**) or 9.2 to 11.1% in Denmark by **Seyfarth et al., (27)** and 100% in Spain by **-Carraminana et al. (28)**. *S. Enteritidis* showed low resistance characteristics. Low percentages of multiple resistance in *S. Enteritidis* strains have been pointed out by other authors (**29, 30,31,32,33**). *Salmonella Typhimurium* showed resistant to ampicillin, tetracycline and gentamycin (**34**), which was in contrast to the findings of **Tjaniadi et al,(4)**, who recorded that salmonella species were resistant to ciprofloxacin and norfloxain, while *Salmonella typhi* strains were resistant to streptomycin and tetracycline. The resistance to

oxytetracycline was observed in 63.3% of the isolates in USA, 46% in Senegal (35) and 36% in Portugal (36). Resistance to streptomycin was also clear and is in conformity with other findings (37). The isolate *E.coli* O26, H9 was resistant to all antibiotics, while the isolates number 3 and 4 were resistant also to all antibiotics except for colistin sulphate, where the inhibition zone was 10. However, isolate number 2 was susceptible to amoxicillin, ampicillin, colistin and quinolones (table 2). It was reported by many authors that isolate originating from different animal species, feed or environment showed different resistance patterns (38,39). The antibiotic susceptibility results of *E. coli* isolates were in agreement with those recorded by Salehi and Bonab (40) who reported that *E. coli* isolates showed high percentage of resistance to the antibiotics. Yadav et al. (41) studied the antibiogram of isolates of *Escherichia coli* and mentioned that 26.67% of the isolates were resistant to norfloxacin. It was also reported that all *E. coli* isolates were susceptible for colistin sulphate and this result was agreed with Habrun et al. (42) who found a high rate of susceptibility (94%) of the *E. coli* for colistin. One study on chickens fattening evaluated the incidence and distribution of antibiotic resistance in 197 commensal

*Escherichia coli* strains. The effects of supplementation with antimicrobial agents approved bambarmycin, penicillin, salinomycin and bacitracin or a combination of salinomycin more bacitracin. All isolates showed some degree of resistance to multiple antibiotics and resistance to tetracycline (68.5%), amoxicillin (61.4%), ceftiofur (51.3%), spectinomycin(47.2%), and sulfonamides (42%). These data demonstrate that the multidrug resistance of *E.coli* can be found in broilers, regardless of antimicrobial growth promoters used. (18).

#### **PCR virulotyping and antimicrobial resistance genes:**

Positive amplification of the 720 bp of the *phoA* alkaline phosphatase housekeeping gene which is usually used to detect *E. coli* was reported confirming the bacteriological results of isolating *E. coli* from the feed samples (unpublished data). The *E. coli* isolates virulence was checked by testing them for *iss*, *iutA* and *eaeA* genes that were detected in each of the tested isolates (table 3, photo 1). There are many virulence-associated genes that act individually or in combination in many virulence mechanisms as adhesion, ferric transport system and toxin-production of the pathogenic *E. coli* have been reported (43, 44, 45). *eaeA* gene encodes intimin which is an outer membrane

protein that mediates the attachment to the intestinal epithelium (46).

The *eaeA* gene was detected in all isolates indicating the high virulence of these *E. coli* strains as this gene aids the attachment of the bacteria to the cytoplasm membrane of the epithelial cells and leads to the disappearance of the brush border microvilli.

Iss (increased serum survival) gene that was detected also in all isolates is considered as a promising virulence gene of the extra intestinal pathogenic *Escherichia coli* (ExPEC) such as uropathogenic *E. coli* (UPEC), neonatal meningitis-associated *E. coli* (NMEC), and avian pathogenic *E. coli* (APEC) (47). An outer-membrane protein (IutA) was also identified in all isolates. The IutA protein is involved in the binding and internalisation of the bacteriocin cloacin DF13 and the bacteriophage 74 (48).  $\beta$ -lactamases of Enterobacteriaceae were caused by plasmid-mediated  $\beta$ -lactamases, such as TEM-1, TEM-2 and SHV-1. These  $\beta$ -lactamases are resistant to inactivation by  $\beta$ -lactamase inhibitors (clavulanic acid, sulbactam, tazobactam) (49). *Salmonella Typhimurium* resistance to colistin was described by Sun et al., (50), who assessed spontaneous mutations in PmrA and PmrB genes in *S. Typhimurium* LT2 that showed

reduced susceptibility to colistin. Resistance rate of *Salmonella* to ciprofloxacin was 35% in USA (51), 10.2 to 16.8% in Germany (52) and 9.6% in Austria (53). High level of ampicillin resistance (90 to 100%) was observed in almost all salmonella isolates, which is in agreement with the findings of Suresh et al., (54). They also observed a higher proportion of ampicillin-resistant *Salmonella* from eggs.

The salmonella *inv A* gene was present in all samples as it is highly conserved among all *Salmonella* serotypes (55). The four isolated *Salmonella* strains showed positive amplification the 284 bp of the *inv A* gene which is considered as an important factor for the *Salmonella* invasion of the epithelial cells (56).

The 100% positive results of the *avrA*, *sopB* and *stm* genes (table 3, photo 2) have confirmed the virulence profile of the tested isolates. As *avrA* gene is an effector protein of the TTSS complex which usually translocated into host cells by a type 3 secretion system and share in the virulence of *Salmonella* spp. by rearrangement of the host cellular cytoskeleton through cell apoptosis of macrophages, and by the inhibition of IL-8 and TNF- $\alpha$ . (57, 58, 59). The positive results of the *sopB* gene is a strong indicator of the *Salmonella* virulence because it activates the

host cell's small GTPase proteins which are involved in the regulation of the formation of F-actin filaments, membrane ruffling and bacterial internalization by macropinocytosis (60,61). Also, the positive PCR result of *Salmonella Stn* gene proved

the high risk expected from this strains (62) as this gene encodes for the heat labile exotoxin which is one of the causative agents of diarrhea (63). The result of the *bla*TEM gene PCR was compatible with that of the antibiogram confirming the resistance of the isolated Salmonella to ampicillin, penicillin and amoxicillin.

Although the negative *qepA* gene PCR results for all Salmonella and *E. coli* isolates and also the negative results of the *aac*(6')-Ib-cr gene of the Salmonella isolates, this don't conflict with the resistance profile reported in the different quinolones sensitivity. As, Quinolone resistance in the Enterobacteriaceae is mostly mediated by point mutations in the quinolone resistance-determining regions (QRDR) of the target genes (*gyrA* and *gyrB*, which encode DNA gyrase, and *parC* and *parE*, which encode topoisomerase IV) (64, 65).

The current positive PCR results for the *tetA*(A) gene in Salmonella isolates and *aadA2* gene in *E. coli* isolates were completely matching the resistance state

obtained by the tetracycline and streptomycin sensitivity tests respectively. However, one *S. Enteritidis* isolate was sensitive to most of the antibiotics, different antibiotic resistance genes was detected for it by PCR especially the *bla*TEM which may be due to poor expression of a functional TEM-1 enzyme created by a mutation in the promoter region of the gene, or due to the production of an inactive mutant TEM enzyme.

The PCR result for *Clostridium perfringens* toxins showed amplification of the 402 bp of the alpha toxin only (table 4, Fig. 3), which indicates that the isolated *Clostridium* was related to type A *Clostridium perfringens* which has severe toxigenic power and can cause myonecrosis, hemolysis, an increase in vascular permeability, and platelet aggregation (66, 67). Alpha toxin is a phospholipase C sphingomyelinase that hydrolyzes phospholipids and promotes membrane disorganization (9,10). Hydrolysis of lecithin results in the formation of diacylglycerol, resulting in activation of protein kinase C, and subsequent stimulation of the arachidonic acid cascade. This induces the synthesis of inflammatory mediators. These mediators cause blood vessel contraction, platelet aggregation and myocardial dysfunction, leading to acute death. (11)

Table (1): No. of examined samples of feed ingredients and finished ones

Samples	No. of sample
<b>Feed ingredients</b>	
Soya	10
Yellow corn 44%	10
DDGs	5
Fish meal 65%	10
Corn gluten meal 60%	5
Bran mash	5
Rice polish	10
Poultry offal	10
Bone meal	5
Water	4
<b>Finished feed mills</b>	
Breeder production I (mash)	5
Breeder production I (pellet)	5
Broiler starter (mash)	10
Broiler finisher (pellet)	10
Tilapia floating feed 25%	5
Tilapia sinking feed 25%	5

Table (2) Antibacterial agents used in antibiotic sensitivity test (25)

Antibiotic	Concentration
Amoxicillin (AX)	25 mg
Nalidixic acid (NA)	30 µG
Flumoquine (UB)	30 µG
Tetracycline (T)	30 µG
Gentamycin (CN)	10 µG
Colistin sulphate (CT)	10 µG
Streptomycin (S)	10 µG
Penicillin	10 µG
Enrofloxacin(ENR)	5 µG
Norofloxacin (NX)	10 µG
Danofloxacin (DO)	30 mg
Ciprofloxacin (CF)	5 µG
Ampicillin (Amp)	10 µG

Table (3): Sequences and cycling conditions of the different PCR primers used for amplification of different *E. coli* and *Salmonella* genes.

Gene	Primers sequences	Amplified segment	Secondary denaturation	Annealing	Extension	References
<i>eaeA</i>	GACCCGGCACAAGCATAAGC	384bp	94°C	54°C	72°C	(68)
	CCACCTGCAGCAACAAGAGG		45 sec.	45 sec	45 sec	
<i>qepA</i>	CGTGTGCTGGAGTTCTTC	403bp	94°C	50°C	72°C	(69)
	CTGCAGGTAAGTGCATG		45 sec.	45 sec	45 sec	
<i>aac(6)-Ib-cr</i>	CCCGCTTCTCGTAGCA	113bp	94°C	52°C	72°C	(70)
	TTAGGCATCACTGCGTCTC		30 sec.	30 sec	30 sec	
<i>blaTEM</i>	ATCAGCAATAAACCAGC	516bp	94°C	54°C	72°C	(71)
	CCCCGAAGAACGTTTC		45 sec	45 sec	45 sec	
<i>Iss</i>	ATGTTATTTCTGCCGCTCTG	266bp	94°C	54°C	72°C	(46)
	CTATTGTGAGCAATATACCC		30 sec.	30 sec.	30 sec.	
<i>iut A</i>	GGCTGGACATGGGAACTGG	300bp	94°C	63°C	72°C	
	CGTCGGGAACGGGTAGAATCG		30 sec.	30 sec.	30 sec.	
<i>tetA(A)</i>	GGTCACTCGAACGACGTCA	576bp	94°C	50°C	72°C	(72)
	CTGTCCGACAAGTTGCATGA		45 sec.	45 sec.	45 sec.	
<i>aadA2</i>	TGTTGGTACTGTGGCCGTA	622bp	94°C	50°C	72°C	(73)
	GATCTCGCCTTTCACAAAGC		45 sec.	45 sec.	45 sec.	
<i>avrA</i>	CCT GTA TTG TTG AGC GTC TGG	422bp	94°C	58°C	72°C	(74)
	AGA AGA GCT TCG TTG AAT GTC C		30 sec.	30 sec.	30 sec.	
<i>sopB</i>	TCA GAA GRC GTC TAA CCA CTC	517bp	94°C	58°C	72°C	
	TAC CGT CCT CAT GCA CAC TC		30 sec.	30 sec.	30 sec.	
<i>Stn</i>	TTGTGTCGCTACTGGCAACC	600 bp	94°C	59°C	72°C	(75)
	ATTCGTAACCCGCTCTCGTCC		1 min.	1 min.	1 min.	
<i>phoA</i>	CGATTCTGGAAATGGCAAAAG	720 bp	94°C	58°C	72°C	(76)
	CGTGATCAGCGGTGACTATGAC		45 sec.	40 sec.	45 sec.	
<i>invA</i>	GTGAAATATCGCCACGTTCTGGGCAA	284 bp	94°C	55°C	72°C	(77)
	TCATCGCACCGTCAAAGGAACC		30 sec.	30 sec.	30 sec.	

Table (4): Sequences and cycling conditions of the different PCR primers used for detection of different *Clostridium perfringens* toxins

Gene	Primers sequences	Amplified segment	Secondary denaturation	Annealing	Extension	References
Alpha Toxin	GTTGATAGCGCAGGACATGTTAAG	402bp	94°C 1 min.	55°C 1 min.	72°C 1 min.	(78)
	CATGTAGTCATCTGTCCAGCATC					
Beta Toxin	ACTATACAGACAGATCATTCAACC	236 bp				
	TTAGGAGCAGTTAGAACTACAGAC					
Epsilon Toxin	ACTGCAACTACTACTCATACTGTG	541 bp				
	CTGGTGCCTTAATAGAAAGACTCC					
Iota toxin	GCGATGAAAAGCCTACACCACTAC	317 bp				
	GGTATATCCTCCACGCATATAGTC					

5	4	3	L	2	1
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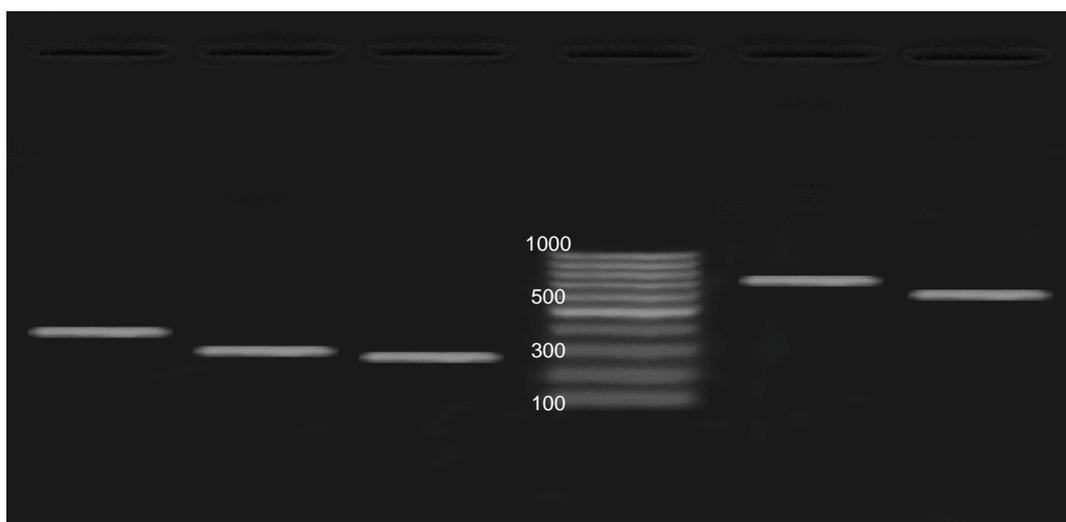


Photo. 1 PCR results for *E. coli* virulence genes and quinolone resistant gene. L, 100 bp ladder (Fermentas) (100-1000 bp). 1-5 lanes are shown respectively: 1) *aadA2* (622 bp), 2) *phoA* (720 bp), 3) *iss* (266 bp), 4) *iutA* (300 bp) and 5) *eaeA* (384 bp).

6	5	4	L	3	2	1
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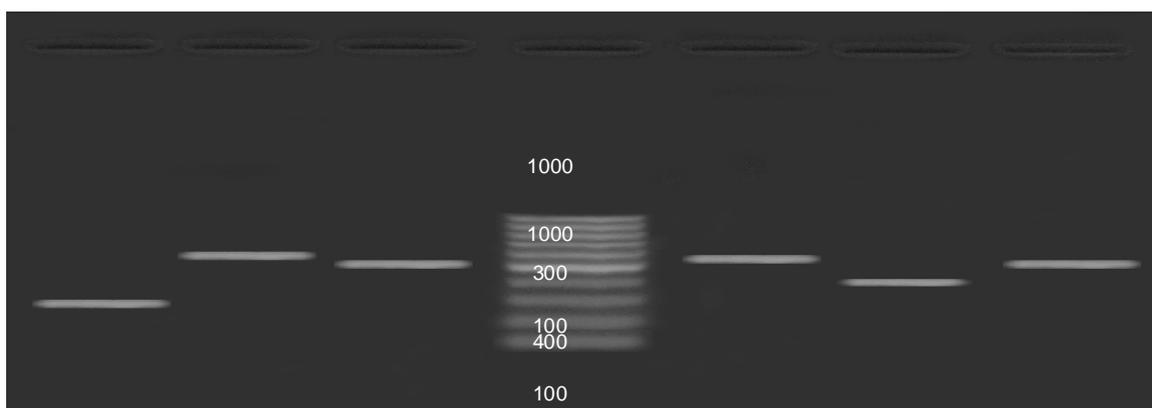


Photo 2. PCR results for *Salmonella* virulence genes and quinolone resistant genes. L, 100 bp ladder (Qiagen, Germany, GmbH) (100-600 bp). 1-6 lanes are shown respectively: 1) *sopB* (517 bp), 2) *avrA* (422 bp), 3) *tetA(A)* (576 bp), 4) *blaTEM* (516 bp), 5) *stn* (600 bp) and 6) *invA* (284 bp).

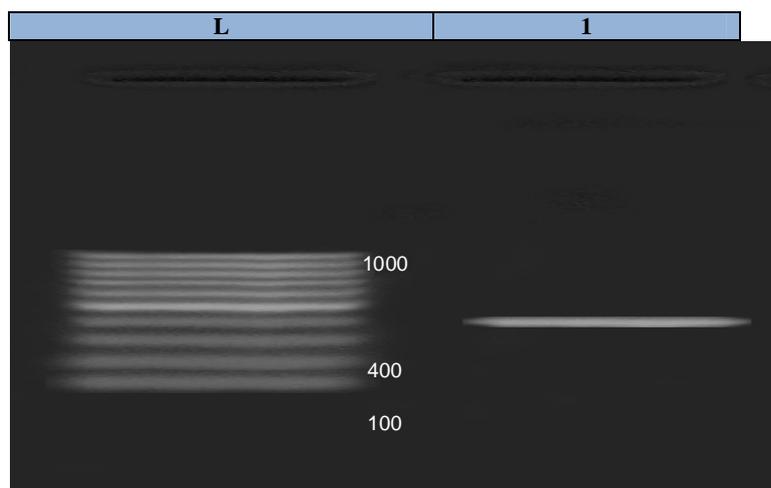


Photo 3. PCR results for *Clostridium perfringens* toxin. L, 100 bp ladder (Qiagen, Germany, GmbH) (100-600 bp). One lane show positive amplification of 402 bp of alpha toxin gene only

**Conclusion:** Presence of *Salmonella* strains on raw materials is a very influential factor in order to find *Salmonella* on meal finished feeds. Clostridial toxin is an indicator of the prospected lethal effect that may be shown in the ingesting animals

## CONCLUSION

*Salmonella typhi*, *Entitritidis*, *typhimurium*, *E.coli* O26, H9 and *C.perferengens* showed different levels of drug resistance. Their isolated virulent genes were compatible to their antibiogram. Characterization and genotyping of resistant strains of isolated enteric pathogens and recognition of virulent and toxin genes are more and rapid diagnostic methods for proposed control and preventive protocol against zoonotic diseases of poultry feed ingredients and product origin.

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