

ORIGINAL ARTICLE

# Down Regulation of Biofilm and Quorum Sensing Genes in *E. coli* Isolated from Broiler Chickens Pericarditis Lesions by the Action of Some Essential Oils

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## Abstract

*E. coli* is one of the most important pathogenic bacteria in poultry industry. It causes high economic losses, high morbidity and mortality rates due to colibacillosis. Moreover, it is multidrug resistant bacteria. In the last few years, scientists directed their efforts to the use of essential oils which have antimicrobial actions by inhibition of some virulence properties such as biofilm formation and quorum sensing. In this study, out of 150 examined samples, 139 were found to be bacteriologically positive for G-ve bacilli (92.7%) and 84 *E. coli* isolates (60.4%) were recovered from broilers suffering from pericarditis. Twenty random recovered isolates were selected for antimicrobial susceptibility test. All isolates were completely resistant to amoxicillin, gentamicin, lincomycin and florfenicol and 90% resistant to trimethoprim-sulfamethoxazole, kanamycin, ciprofloxacin and doxycycline while sensitivity to amoxicillin+clavulanic acid and cefotaxime was 40% and 20% respectively. We screened 15 randomly selected MDR isolates by CR assay for detection of biofilm formation at which CR positive isolates represent 80%. Cinnamon and clove oils showed antimicrobial effect and cause down regulation in both biofilm gene (*sfa*) and quorum sensing gene (*luxS*).

**Keywords** Antimicrobial resistance; Biofilm; *E. coli*; Essential oils; Quorum sensing; Virulence

## 1. Introduction

*Escherichia coli* is a normal flora in both animals and human's intestine that can induce enteric and extra intestinal infections. Avian pathogenic *E. coli* (APEC) is the main etiology of colibacillosis in poultry farms. It causes air sacculitis, perihepatitis, pericarditis, and sometimes fatal septicemia (Sola Giens et al. 2012) causing severe economic losses to poultry industry (Schouler et al. 2012). Several virulence genes are involved in avian colibacillosis such as adhesins, toxins, antihost defense factors, iron

acquisition systems and autotransporters (Nakazato et al. 2009). According to (Johnson et al. 2003) a strain could be considered ExPEC if reveals two or more of the following virulence genes; *pap* (P fimbriae), *sfa/foc* (S/F1C fimbriae), *afa / dra* (Dr Binding adhesins), *iutA* (aerobactin receptor), and *kpsM II* (group 2 capsule synthesis). Successful treatment of avian colibacillosis depends on antimicrobial agents. However, the lack of veterinary supervision on farms in Egypt and failure of treatment by fluoroquinolones and aminoglycosides, force some

owners of small farms to use last resort antibiotics intended for human therapy (Solà-Ginés et al. 2015). These include cefotaxime injection solutions, which is a third-generation cephalosporin not allowed for use in poultry and have been classified as critically important antimicrobials in human medicine by the World Health Organization (WHO 2012). Such resistance can be transmitted to humans via the food supply (Johnson et al. 2007; Mora et al. 2010). *E. coli* producing extended-spectrum beta-lactamases (ESBLs) and plasmid mediated AmpC beta-lactamases which are found on mobile genetic elements (e.g., plasmids or integrons), can be transferred to other bacteria and other bacterial species by horizontal gene transfer (Pfeifer et al. 2010). Mixing of species in the intestines allows *E. coli* to accept and transfer plasmids from one to other bacteria in a process called horizontal gene transfer (Salysers et al. 2004). The pili (Pap) and s fimbrial adhesion (*sfa*) which are encoded within the “operon” region of *sfa* gene are responsible for resistance against the host’s immune system and a wide range of antibiotics (Emody et al. 2003). The products of *sfa*, *afa* and *foc* genes are particularly involved in biofilm formation (Lee et al. 2016) In accordance with (Sawhney and Berry 2009; Romling 2012) biofilm formation around the bacteria results in increased morbidity and persistent infections and this might led to difficulty in treatment of bacterial diseases with antimicrobial agents.

Some types of *E. coli* infection are associated with biofilm formation which leads to an inability to eradicate the infection due to its intrinsic nature to resist high doses of antibiotics (Chen et al. 2010) and is considered as an important virulence property (Zalewska-Piatek et al. 2009). Biofilm formation in *E. coli* needs a group of gene expression facilitating its initiation, attachment, and subsequent maturation. A variety of virulence factors are concerned in biofilm formation in *E. coli*, which permit attachment and more forming biofilm. So, the virulence factors related to adherence and concerned in biofilm production by APEC have been became major concerns (de Pace et al. 2011; Han et al. 2013; Zhuge et al. 2013)

Quorum sensing (QS) is an intercellular signal mechanism that bacteria use to control their gene expression for adapting to changes in their surroundings. This process involves the production, secretion, and recognition of signal molecules and regulation of gene expression (Xavier and Bassler

2005). Gene *luxS* exists in a broad range of species that participates in interspecies cell-to-cell communication (Xavier and Bassler 2003). A new approach of antimicrobial chemotherapy as anti-QS compounds (Rasmussen and Givskov 2006; Roy et al. 2011) which inhibits a wide varieties of prokaryotic phenotypes including biofilm formation, virulence factor expression, and motility (Antunes et al. 2010; Li and Tian 2012), so the present study aimed to control biofilm formation and quorum sensing ability of *E. coli* isolated from cases of broiler chicken pericarditis by using some essential oils.

The essential oils of aromatic plants are commonly used in food preservation and flavoring as they have phenolic groups against foodborne microorganisms (Rezaei et al. 2010). Inhibition of cell growth is a traditional method for antimicrobial agents although, however there is bacterial drug resistance, so essential oils have been studied as new approach for antimicrobial agents as they inhibit biofilm formation, toxin production, bacterial quorum sensing, and adhesive factors (Kim et al. 2016).

## 2. Materials and methods

### 2.1. Sample collection

Samples were collected from 150 broiler chickens of different ages (3-5weeks) and from different Egyptian farms. These chickens were suffering from high mortalities and respiratory manifestations, so the chickens were subjected to postmortem examination for detection of the affected tissues lesions. All samples were given a serial numbers and detailed information (Quinn et al. 2011).

### 2.2. Isolation and identification of *E. coli*

The specimens were inoculated into MacConkey's broth and incubated aerobically at 37°C for 24 hrs. Then, a loopful of this culture was streaked out onto MacConkey's agar and EMB agar and incubated at 37°C for 18-24 hours. Colonies showed characteristic green metallic sheen on EMB agar were picked up and considered presumptively *E. coli*. All oxidase negative Gram-negative bacilli were identified biochemically according to schemes described by Quinn et al. (2011).

### 2.3. Antimicrobial susceptibility and biofilm formation assay

Twenty randomly biochemically identified *E. coli* isolates were subcultured into Muller–Hinton broth and incubated at 37°C for 18 hours. The standardized

culture was swabbed thoroughly on completely dried Muller Hinton agar plates then dried for 5-10 minutes at 37°C then the antibiotic discs were placed aseptically; incubated over night at 37°C then examined by measuring the visible and clear zone of inhibition of growth produced by diffusion of antibacterial agent from the discs into surrounding medium. The recovered isolates were examined for the antimicrobial sensitivity using disc diffusion according to CLSI (2018). The Multi drug resistant isolates were selected for detection of biofilm formation using YESCA-CR medium according to Zhou et al. (2013)

#### 2.4. Antibacterial activity of some essential oils

The antibacterial activity of some essential oils as; cinnamon, clove, peppermint, black pepper, ginger, oregano and capsicum oil” against 15 MDR random selected isolates was tested. Briefly, the tested bacteria were grown on tryptone soya agar at 37°C for 24hr, then cells were suspended in physiological saline (0.9% NaCl), and the suspension was adjusted

to  $1.5 \times 10^8$  cfu/ml. Tryptone soya agar was prepared and autoclaved at 121°C for 15 min. and kept at 55°C. The tested oils were mixed with polyethylene glycol+ tween 80 which they act as a surfactant with ratio 1:1, then mixed with TSA according to the concentration. The oil-agar medium was poured into sterile petri dishes and was solidified. Bacterial suspensions were swabbed and speared onto the agar plates. The plates were then incubated at 37°C for 24-48hr (Jeff-Agboola et al. 2012).

#### 2.5. Real-time PCR screening of biofilm and quorum sensing genes expression before and after exposure to some essential oils

Bacterial RNA extraction was performed following the “Enzymatic Lysis” procedure of QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH). PCR reaction was applied in a Stratagene MX3005P real-time PCR machine where the specific primers were utilized according to Yuan et al. (2006). Primers sequences for reference housekeeping and target genes for each microbial agent are listed in Table (1, 2).

**Table 1. Oligonucleotide's sequence used for screening of virulence genes in *E. coli*.**

| Target bacteria | Gene & Function                   | Primer sequence (5'-3')                                | Reference             |
|-----------------|-----------------------------------|--|-----------------------|
| <i>E. coli</i>  | <i>16s rRNA</i><br>(Housekeeping) | GCTGACGAGTGGCGGACGGG<br>TAGGAGTCTGGACCGTGTCT           | Tivendale et al. 2004 |
|                 | <i>Sfa</i><br>(Biofilm)           | CTCCGGAGAACTGGGTGCATCTTAC<br>CGGAGGAGTAATTACAAACCTGGCA | Yazdi et al. 2018     |
|                 | <i>luxS</i><br>(Quorum sensing)   | ATGCCGTTGTTAGATAGCTTCA<br>GATGTGCAGTTCCTGCAACTTC       | Wang et al. 2016      |

**Table 2. Cycling conditions for SYBR green real time PCR.**

| Target bacteria | Gene        | Reverse transcription | 1st denaturation | Amplification (40 cycles) |                  |                  | Dissociation curve (1 cycle) |                 |                    |
|-----------------|-------------|-----------------------|------------------|---------------------------|------------------|------------------|------------------------------|-----------------|--------------------|
|                 |             |                       |                  | 2nd denaturation          | Annealing        | Extension        | 2nd denaturation             | Annealing       | Final denaturation |
| <i>E. coli</i>  | 16S rRNA    | 50°C/<br>30 min.      | 94°C/<br>15 min. | 94°C/<br>15 sec.          | 55°C/<br>30 sec. | 72°C/<br>40 sec. | 94°C/<br>1 min.              | 55°C/<br>1 min. | 94°C/<br>1 min.    |
|                 | <i>Sfa</i>  |                       |                  |                           | 53°C/<br>40 sec. |                  |                              | 53°C/<br>1 min. |                    |
|                 | <i>luxS</i> |                       |                  |                           | 55°C/<br>40 sec. |                  |                              | 55°C/<br>1 min. |                    |
|                 |             |                       |                  |                           |                  |                  |                              |                 |                    |

### 3. Results and discussion

Sampled chickens from diseased flocks show postmortem lesions including general congestion, with characteristic fibrinous lesions (air sacculitis, perihepatitis, and pericarditis) and fatal septicemia (Saif et al. 2003; Sharada et al. 2010). In this study out of 150 examined samples, 139 were found to be bacteriologically positive for G-ve bacilli (92.7%). A total of 84 (60.4%) *E. coli* isolates were recovered, in

contrast with (Amer et al. 2018) 56 samples (35%) were positive.

Antibiogram results demonstrated in Table (3) and Fig. (1) recorded that we selected randomly 20 *E. coli* isolates. All *E. coli* isolates were resistant to amoxicillin, gentamicin, lincomycin and florfenicol showed 90% resistance to trimethoprim- sulfamethoxazole, kanamycin, ciprofloxacin and doxycycline

while sensitivity to amoxicillin+ clavulanic acid and cefotaxime was **40%** and **20%** respectively. On the other hand, **El-Shazly et al. (2017)** revealed that the resistance was highest against cefazolin (**100%**), streptomycin (**98%**), and ampicillin (**96%**), followed by doxycycline (**90%**), ciprofloxacin (**82%**), cotrimoxazole (**66%**), and gentamicin (**45%**), while all strains were susceptible to ertapenem.

*E. coli* is a highly adaptive microorganism that can create biofilms under certain conditions and become critical for diseases (**Chen et al. 2010; Silva et al. 2014**). In our study randomly 15 MDR isolates were screened by CR assay for detection of biofilm formation at which CR positive isolates represent **80%**. Out of 256 clinical samples of *E. coli* the results of biofilm formation were divided to strong, moderate, weak and no form a biofilm with a percentage **25.39%**, **31.25%**, **28.90%** and **18.36%** respectively (**Wang et al. 2016**).

Plant extracts or essential oils are the new approach to overcome the bacterial resistance mechanisms either singly or in combination. It also protects the birds from adverse effects of the chemical antibiotics and moreover, protects the consumers from the harmful effect of the residues of the antibiotics that remain in birds' meat and organs (**Diaz-Sanchez et al. (2015)**,

so in the present investigation the 15 MDR isolates were selected to detect the effect of some essential oils as; cinnamon, clove, peppermint, black pepper, ginger, oregano and capsicum oil, the results in **Table (4)** showed that all examined isolates grown when exposed to black pepper, peppermint, capsaicin, and ginger oils in both concentrations 0.1% and 0.01% and 0.01% concentration of oregano, cinnamon and clove oils, while 0.1% concentration of oregano, cinnamon and clove oils inhibited the growth of all isolates. The current results are in agreement with **Franz et al. (2010); Hippenstiel et al. (2011); Bassole and Juliani (2012)** who mentioned that thymol, eugenol and carvacrol have high antimicrobial activity against pathogenic bacteria such as *Escherichia coli* and *Salmonella typhimurium*. In the current study the isolated *E. coli* were proved to be pathogenic as RT-PCR was able to detect specific virulence genes related to different virulence determinants; biofilm (*sfa*) and quorum sensing (*luxS*). **Table (5)** and figure (2,3,4) discussed the down regulation in fold change for *sfa* gene after exposure to 0.01% concentration of cinnamon oil was 0.1267 and 0.3392 after exposure to 0.01% concentration of clove oil on other hand down regulation in fold change for *luxS* was 0.0390 and 0.1869 after exposure to 0.01% concentration of cinnamon and clove oil respectively.

**Table 3. Antibiogram of *E. coli* recovered from examined samples.**

| Chemotherapeutic discs         | Conc. (µg) | <i>E. coli</i> (n=20) |     |           |    |
|--------------------------------|------------|-----------------------|-----|-----------|----|
|                                |            | Resistant             |     | Sensitive |    |
|                                |            | No.                   | %   | No.       | %. |
| Amoxicillin                    | 10         | 20                    | 100 | 0         | 0  |
| Amoxicillin+Clavulanic acid    | 30         | 12                    | 60  | 8         | 40 |
| Doxycycline                    | 30         | 18                    | 90  | 2         | 10 |
| Kanamycin                      | 30         | 18                    | 90  | 2         | 10 |
| Gentamicin                     | 10         | 20                    | 100 | 0         | 0  |
| Cefotaxime                     | 30         | 16                    | 80  | 4         | 20 |
| Ciprofloxacin                  | 5          | 18                    | 90  | 2         | 10 |
| Trimethoprim- sulfamethoxazole | 25         | 18                    | 90  | 2         | 10 |
| Florfenicol                    | 30         | 20                    | 100 | 0         | 0  |
| Lincomycin                     | 2          | 20                    | 100 | 0         | 0  |

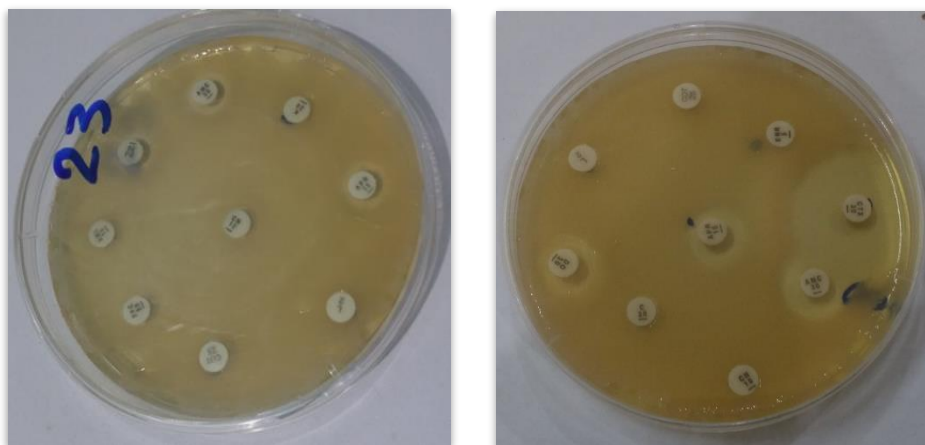


Fig. 1. Antimicrobial sensitivity tests of selected bacterial isolates

Table 4. Results of antibacterial effect of some organic oils on some MDR isolates.

| Oils         | Concentration          |     | 0.1%                      |     | 0.01%                  |     | 0.01%                     |   |
|--------------|------------------------|-----|---------------------------|-----|------------------------|-----|---------------------------|---|
|              | No. of growth isolates | %   | No. of inhibited isolates | %   | No. of growth isolates | %   | No. of inhibited isolates | % |
| Black pepper | 15                     | 100 | 0                         | 0   | 15                     | 100 | 0                         | 0 |
| Peppermint   | 15                     | 100 | 0                         | 0   | 15                     | 100 | 0                         | 0 |
| Capsaicin    | 15                     | 100 | 0                         | 0   | 15                     | 100 | 0                         | 0 |
| Ginger       | 15                     | 100 | 0                         | 0   | 15                     | 100 | 0                         | 0 |
| Oregano      | 0                      | 0   | 15                        | 100 | 15                     | 100 | 0                         | 0 |
| Cinnamon     | 0                      | 0   | 15                        | 100 | 15                     | 100 | 0                         | 0 |
| Clove        | 0                      | 0   | 15                        | 100 | 15                     | 100 | 0                         | 0 |

Table 5. Results of some virulence genes before and after exposure to 0.01% concentration of cinnamon and clove oils.

| <i>E. coli</i> Sample No. | 16S rRNA |  | luxS  |             | sfa   |             |
|---------------------------|----------|--|-------|-------------|-------|-------------|
|                           | CT       |  | CT    | Fold change | CT    | Fold change |
| E                         | 20.52    |  | 21.19 | -           | 20.87 | -           |
| E1                        | 19.87    |  | 23.52 | 0.1267      | 24.90 | 0.0390      |
| E2                        | 20.41    |  | 22.64 | 0.3392      | 23.18 | 0.1869      |

E. *E. coli* (control), E1. After exposure to 0.01% concentration of cinnamon oil, E2. After exposure to 0.01% concentration of clove oil.

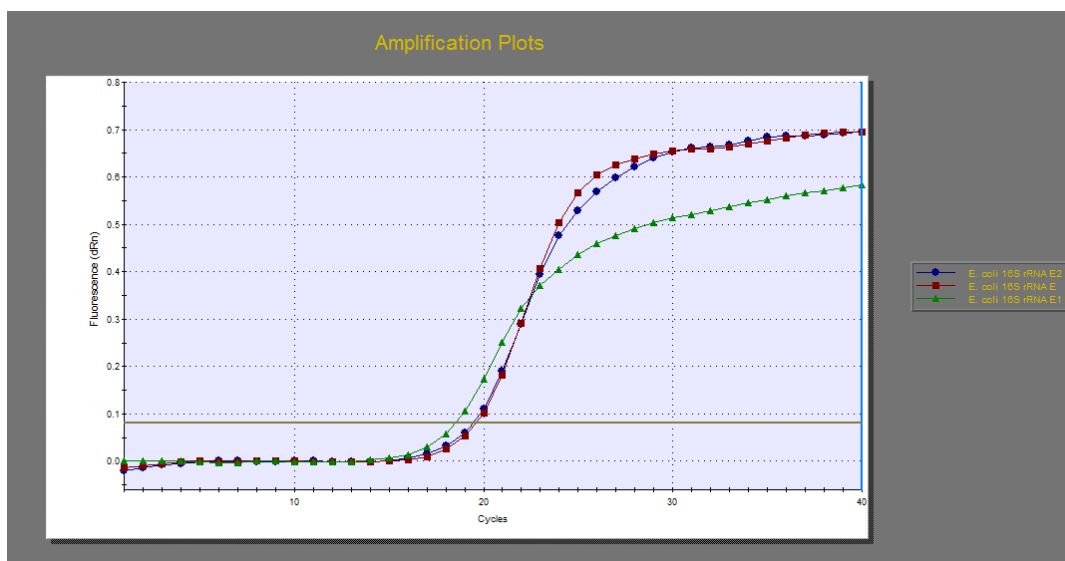


Fig. 2. Down regulation in 16s rRNA before and after exposure to 0.01% concentration of cinnamon and clove oils.

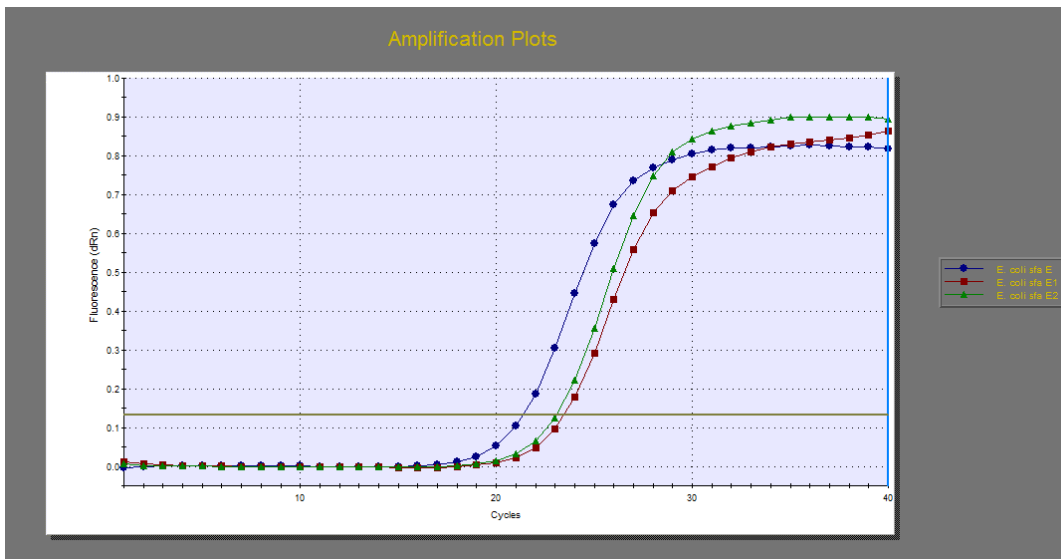


Fig. 3. Down regulation in *sfa* before and after exposure to 0.01% concentration of cinnamon and clove oils.

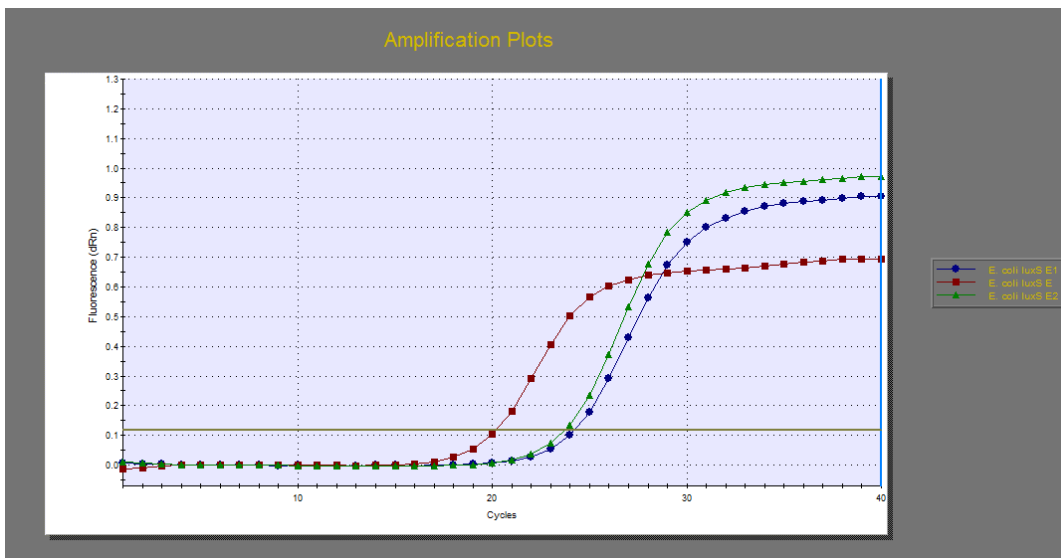


Fig. 4. Down regulation in *luxS* before and after exposure to 0.01% concentration of cinnamon and clove oils.

#### 4. Conclusion and Recommendations

The reported results have shed some light on the possible use of cinnamon oil and other essential oils for the control of antibiotic-resistant bacteria isolated from pericarditis lesions in Egyptian broilers chickens' instead of antibiotics that usually develop bacterial resistance or cause harmful effects for birds' vital organs in addition to the possible residues that remain in the poultry meat causing public health hazard.

#### 5. Authors Contributions

All authors contributed equally to study design methodology, interpretation of result and writing of the manuscript.

#### 6. Conflict of interest

The authors declare no conflict of interest.

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