CLEANACTIV® Combatting Crisis of Multidrug Resistant Avian Pathogenic E. coli in Broiler Chickens

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ABSTRACT

The dramatic increase in antimicrobial resistance for avian pathogenic E. coli constitutes a key threat to Poultry industry. With the misuse and overuse of antibiotics to treat diseases, resistance to the drugs has begun to appear and has become more serious. In this paper, we studied the antibacterial effects of CLEANACTIV® essential oil (CEO) both alone, using a twofold dilution method, and combined with antibiotics, using a microtitre assay, against multidrug resistant avian pathogenic Escherichia coli. The result indicated that multiple drug-resistant APEC was very sensitive to CEO. The antibacterial effects of CEO in combination with fluoroquinolones, doxycycline, lincomycin, and florfenicol displayed synergism against E. coli, so this combination used to treat infections caused by MDR-APEC which may lower, to a great extent, the effective dose of these antibiotics and thus minimize the side effects of antibiotics.

Key words: CLEANACTIV®; Antibiotic resistance; Avian Pathogenic, E. coli

INTRODUCTION

Avian Pathogenic Escherichia coli is a pathogen of the chickens and other avian species especially broilers, it is considered as a member of the extra-intestinal pathogenic E. coli (Anatao et al., 2008). In the broiler chickens, Avian Pathogenic Escherichia coli (APEC) infections lead to respiratory tract infection, air sacculitis, pericarditis, peri-hepatitis, and swollen head syndrome. APEC is amongst the greatest health threats to the developed poultry industries with evidence of zoonotic risk (Rodriguez et al., 2005). Recently, APEC has become accepted as a primary pathogen rather than a consequence of immunosuppressive infections, so E. coli found in the intestinal tract are likely to form a reservoir of potential infection, as it has a great genomic diversity and plasticity (Moulin-Schouleur et al., 2006). Although virulence genes associated with pathogenicity are common in APEC including iss, iroN, hly, tsh and lue, certain E. coli serotypes such as O78 and O1 are more frequently associated with infections (Johnson et al., 2006). It is difficult to control APEC bacteria that are normally a part of the chickens microbiota and this represent as a crisis in broilers industries as it is considered to be one of the principal causes of morbidity and mortality in poultry worldwide (Lagragione et al., 2013). During the last years, APEC becomes one of the microorganisms that are commonly resistant to antimicrobials (Zhao et al., 2012), as in the poultry sector antimicrobial agents not only used for therapy but also for growth promotion (Van den Bogaard and Stobberingh, 2000), which further imposes selective pressure on the microorganisms leading to development of drug resistance and this has serious public health implications (Szmolka et al., 2012). ESβLs is one of microbial drug resistance and infections caused by ESBL-producing bacteria isolated from poultry have become a clinical and therapeutic problem (Velasco et al., 2007). A current approach to limiting the drug resistant APEC is to use essential oils as alternative agents in disease control. It was reported that oregano oil has antibacterial activity, However There is a little knowledge about its mechanism against ESBL-producing E. coli isolated from poultry especially in combination with antibiotics, it may be in its principal components, i.e. thymol and carvacrol, which have antimicrobial properties (Baydar et al., 2004). Garlic oil has generated a lot of interest for decades as it has antimicrobial activities against many pathogens, which related to related to a non-protein amino acid “alliin” and

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an enzyme "alliinase" (Mnayer et al., 2014). Hesperidin and naringin is a flavonoids found mainly in citrus fruits and it has a great variety of biopharmaceutical activities, e.g. antioxidant and antimicrobial Consequently, it is widely used in the treatment of many diseases, and serve as a raw ingredient for different drugs in pharmaceutical industry (Sosa et al., 2002; Devi et al., 2015). *Urtica dioica* L., commonly known as nettle, globally known as a medicinal plant as it has antibacterial properties (Kiaei et al., 2010). Saponins is the active principle of quillaya Extract which is are glycoside compounds with many biological and antibacterial activities differ according to type of the bacteria (Hassan et al., 2010), also one of the most proved criteria of cinnamon extracts as cassia oil is the antibacterial activity against Gram-positive and Gram-negative bacteria (Keskin et al., 2011),so the current study aimed to analyze the ability of CLEANACTIV® essential oil (CEO), which is a mixture of volatile oils such as; nettle extract 3.3%, cassia oil 1.2%, oregano oil 0.5%, carvacrol 0.3%, garlic oil 0.3%, quillaya Extract 0.15%, hesperidin 0.1% and naringin 0.05% in resolving the problem of ESβLs–MDR–APEC and in broiler chickens.

**MATERIALS AND METHODS**

**Bacterial strains**

*Escherichia coli* isolates were recovered from broilers chickens suffered from chronic respiratory disease. Strains were isolated, purifed identified using an API 20E system (bioMérieux, France) and serotyped according to (Quin et al., 2011), while the antibiogram disk diffusion technique was adapted according to (CLSI, 2018).

**Screening for ESBLs by Combined Disc Diffusion Test (CDD)**

Using disk of Cefotaxime and Ceftazidime, alone and in combination with Clavulanic acid. 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, 2018).

**Identification of Virulence and ESβLs genes**

Different sets of primers used for detection of ESβLs *E. coli* and some of its virulence genes showed in (Table 1) according to (Hasman et al., 2005 and Johnson et al., 2008) respectively.

**CLEANACTIV® essential oil (CEO)**

It is a mixture of essential volatile oils as; nettle extract 3.3%, cassia oil 1.2%, oregano oil 0.5%, carvacrol 0.3%, garlic oil 0.3%, quillaya Extract 0.15%, hesperidin 0.1% and naringin 0.05% manufactured by phytosynthese company, France.

**CEO minimal inhibitory concentration (MIC)**

*E. coli* adjusted in sterile saline with 0.5% Tween-80 to enhance oil solubility to match the 0.5 McFarland. MIC of CEO alone or both with antibiotics as enrofloxacin 5µg, lincomycin 30µg, florfenicol 30µg and doxycycline 20µg were examined by broth dilution method in 96-well, microtiter plates. The MICs of the agents against the isolated strain were determined and interpreted in according to (CLSI, 2018).

**CEO Fractional inhibitory concentration (FIC)**

Determined by dividing the MIC of the combination of antibacterial and essential oil by the MIC of antibacterial or essential oil alone. The FIC index, obtained by adding both FICs, was interpreted as synergistic when it was ≤0.5, as additive (indifferent) when it was >5 and ≤2.0 and as antagonistic when it was >2 (White et al., 2000).

**Experimental design**

Three hundred commercial Ross broiler chicks were used and vaccinated with common vaccines as IB, ND and IBD, also they divided in three groups each one consists of 100 chicks, Gp1: CLEANACTIV® treated, Gp2, enrofloxacin treated, Gp3: treated with CLEANACTIV® plus enrofloxacin, All groups challenged with MDR–APEC in concentration 1.5x10⁸ cfu/ml by eye dropping and treating begin after 48hr from challenge, livability, clinical signs, postmortem changes were detected (Barnes et al., 2003), while the re-isolated challenged strains is screened by using RT-qPCR for determination of down-regulation and fold change of *E.coli* virulence and ESβLs genes (in vivo) compared with its (in vitro) record when the challenged isolates exposed to 0.001% conc. of CLEANACTIV® (Sun et al., 2011). Intestinal samples from each group were admitted to Electron Microscope for Histomorphological study according to (Bozzola and Russell, 1991).

**RESULTS**

**E. coli identification, serotyping, ESβL and virulence genes screening**

*E. coli* isolates were recovered from broilers chickens CRD disease with an incidence of 22% which confirmed biochemically by API 20E and serotyped by using specific antisera to O78 (35%), O8 (30%), O158 (25%), O111 (10%), these isolates tested against 10 different chemotherapeutic: amoxicillin, floraphenicol, colistinsulphate, enrofloxacin, doxycycline, gentamycin, lincomycin, ciprofloxacin, Cefotaxime, Sulfamethxazole / Trimethoprim. The multidrug resistance isolates were in percentage of 92% (Fig. 1), with phenotypically ESβL positive in an incidence of 85%, while molecular screening in Table 2 for some ESβL genes as (*blaTEM*, *blaCTX*, and *blaSHV*) showed that these genes detected in percentage of 66% regarding the characterization of some virulence genes (*iss, hlyF* and *iutA*) these genes detected in a percentage of 37%.

**CEO; minimal and fractional inhibitory concentration**

CLEANACTIV® essential oils has MIC and FIC against selected MDR–APEC illustrated in Table 3 at which CEO MIC is 0.72 µ/l/ml and has synergetic effect with enrofloxacin, florafenicol, doxycycline, also has additive effect with lincomycin.
Barros, enrofloxacin treated livability were 90% with Table 4 (Johnson et al., 2005).

Table 4: RT-qPCR for determination of down-regulation and fold change of virulence and ESBLs genes in MDR-APEC used in challenged study

<table>
<thead>
<tr>
<th>Experimental challenge</th>
<th>Virulence genes</th>
<th>ESBL</th>
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<tbody>
<tr>
<td>CT</td>
<td>FC</td>
<td>CT</td>
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<tr>
<td>hlyF</td>
<td>iss</td>
<td>iutA</td>
</tr>
<tr>
<td>MDR, ESβL</td>
<td>In vitro</td>
<td>12.3</td>
</tr>
<tr>
<td>Virulent, APEC</td>
<td>In vivo</td>
<td>13.6</td>
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CT: Cycle Threshold, FC: Fold Change.

Experimental challenge with MDR-APEC and RT-qPCR for determination of down-regulation and fold change of E. coli virulence and ESBLs genes

Livability in Gp3 (treated with CLEANACTIV® plus enrofloxacin) were 96% with mild respiratory and digestive signs and mild enteritis, while Gp1 (CLEANACTIV® treated) livability were 90% with moderate respiratory and digestive signs and moderate enteritis, in the other hand Gp2 (enrofloxacin treated), livability was 92%, with rales, sneezing and diarrhea signs, postmortem changes were detected moderate enteritis. Down-regulation and fold change of E. coli virulence were recorded in Table 4 as hlyF, iss, iutA cycle threshold were 12.3,11.6,10.7 and their fold change were 0.34,0.56,0.43 respectively, while ESBLs genes CT were blasrv, blaCTX, blasrv were 16.3,14.8,17.3 and their fold change were 0.92,0.45,0.16 respectively compared with value of in vitro study.

Histomorphology

In Gp3 (treated with CLEANACTIV® plus enrofloxacin) intestinal histomorphology in (Fig. 2) showed intestinal villi healthiness with immune cells infiltration mainly plasma cells and macrophages and, while increase proliferation of lymphoid cells, endocrine cells, goblet cell and in Gp1 (Fig. 3), unfortunately in Gp 2 edematous and destructive microvilli with massive heterophiles was detected (Fig. 4).

DISCUSSION

Avian colibacillosis causes significant economic losses, either as a primary disease or as a secondary infection, to broilers chicken (Lutful Kabir., 2010), as the presence of resistance plasmids in avian E. coli strains could facilitate horizontal transfer of virulence gene between pathogenic and non-pathogenic strains (Barros., 2012). Several factors have been shown to contribute to the virulence of avian E. coli ,and many of the genes encoding these factors have been found on large conjugative plasmids, so in the current study characterization of some virulence genes (iss, hlyF and iutA) these genes detected in a percentage of 37% (Table 2). Because of the occurrence of antimicrobial resistance genes on these plasmids, it is possible that the use of antimicrobial agents may select for persistence of E.coli containing such plasmids (Johnson et al., 2004). In the present study phenotypically investigate incidence of APEC in broilers chickens which is 22% with high isolation rate for O78, O8, O158 and O111 (10%) serotypes...
De Kievit and Iglewski, Nabavi

lincomycin, and Camellia sinensis

pathogens is the most which.

bla ()., CS; Colistin sulphate ENR;

rofloxacin, CTX; Cefotaxime, SXT;

edematous and destructive microvilli with heterophiles infiltration.

Fig. 1: showing resistance patterns for MDR-APEC. AMX: Amoxicillin, FFC; Flor phenicol, CS; Colistin sulphate ENR; Enrofloxacin, DO; Doxycycline GM; Gentamycin, L; Lincomycin, CIP; Ciprofloxacin, CTX; Cefotaxime, SXT; Sulfamethxazole/Trimethoprim.

Fig. 2: Micrograph of the Gp3 treated intestinal tissue showing healthy villi with plasma and macrophages cells infiltration.

Fig. 3: Micrograph of the Gp1 treated intestinal tissue showing healthy microvilli with proliferation of lymphoid cells, endocrine cells, and goblet cell.

Fig. 4: Micrograph of the Gp2 treated intestinal tissue showing edematous and destructive microvilli with heterophiles infiltration.

respectively, also the antibiogram profile for the recovered isolates showed high level of MDR serotypes in rate of 92%. Production of beta-lactamases represents the most important contributing factor to resistance against beta-lactam antibiotics in Gram-negative bacteria (Liebana, 2013). ESβLs can hydrolyze the newer beta-lactam antibiotics, including 3rd and 4th generation of cephalosporins (WHO, 2017). The current study explore the incidence of ESβLs incidence between the recovered MDR-APEC which revealed that phenotypically ESβL positive present in an incidence of 85%, while molecular screening in Table 2 for some ESβL genes as (blaTEM, blaCTX and bladSHV) showed that these genes detected in percentage of 66%. Multiple antimicrobial resistances may be acquired through mobile genetic elements which contributing in the creation of multi drug-resistant phenotype, and the widespread use of extended-spectrum cephalosporins creates a reservoir of resistant bacteria, also continuous emergence of resistance to antimicrobial agents among the prevalent pathogens is the most dangerous threat for the treatment of infectious diseases (Roca et al., 2015). There are many edible and medicinal plants with high antimicrobial effects, such as thyme (Thymus vulgaris L.), tea (Camellia sinensis L.), garlic (Allium sativum L.), turmeric (Cinnamomum longa L.), berries belonging to Rosaceae family, and cinnamon (species belonging to Cinnamomum genus) (Nabavi et al., 2013). The present work MIC and FIC For CLEANACTIV® essential oils against VIRULENT-MDR – APEC illustrated in Table 3 at which CEO MIC is 0.72 µl/ml and has synergetic effect with enrofloxacin, florafenicol, doxycycline, also has additive effect with lincomycin, and in the experimental challenge with VIRULENT-MDR – APEC in broilers chickens Livability was high in Gp3 (treated with CLEANACTIV® plus enrofloxacin) were 97% with mild signs which mainly refer to Down-regulation and fold change of E.coli virulence and resistance genes as showed in Table 4, hlyF, iss, iutA CT were 12.3, 11.6, 10.7 and their fold change were 0.34, 0.56, 0.43 respectively, while ESβLs genes CT were blatEM, bladCTX, bladSHV were 16.3, 14.8, 17.3 and their fold change were 0.92, 0.45, 0.16 respectively, this may related to that various essential oils can disrupt the Quorum sensing of pathogenic bacteria (Zhou et al., 2013), which referred to a regulatory system in bacteria that regulates gene expression (De Kievit and Iglewski, 2000).

Conclusions
Combination between antibiotic and CLEANACTIV® essential oils act as lifeline in control of multidrug resistant avian pathogenic E. coli and avoidance emergence of ESβL E. coli in broilers chickens.

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