Research article

***Escherichia coli* strains as Major secondary bacterial pathogen isolated from an outbreak of swollen head syndrome in layers, in Al-Diwaniyah, Iraq**

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(Received 23/8/2017, Accepted 23/12/2017)

Abstract

A cross-sectional study was conducted to identify the involvement of *E. coli* as secondary pathogen in cases of Swollen Head infection and to detect drug susceptibility pattern of these isolates. This study was carried out on 20 chickens that were purchased from Al-Safaa Company from an outbreak of a sudden increase in mortality in layers farm and experienced clinical signs included difficult breath, coughing, rales, swollen of infraorbital and supraorbital sinuses, and conjunctivitis, as well as severe depression. The main gross lesions demonstrated among the infected chicken including gaseous exudate in trachea, nasal passages and sinuses. There was yellowish gaseous exudate on the air sacs, ovaries and the peritoneum. The samples were inoculated on different bacteriological culture media, the isolates were identified by morphological, and biochemical tests, in which the result revealed that the major pathogens associated with swollen head syndrome in layers was *E.coli*. The majority of isolates were resistant to ciprofloxacin (94.4%), Ampicillin (100%), Erythromycin (100%), azithromycin (100%), trimethoprim (88.9%), and levofloxacin (94.4%). Nonetheless, however majority of APEC isolates were susceptible to nitrofurantoin (72.3%). Moreover, all *E.coli* strains recovered in the current study showed multidrug resistant to three or more different antibacterial classes. In conclusion, the present findings showed that MDR *E. coli* is prevalent SHS. The MDR *E. coli* is alarming signal because these bacteria can transfer their MDR trait to potential human and animal pathogens. Therefore, the introduction of surveillance programs to monitor antimicrobial resistance strains is strongly recommended to protect human and animal health.

Keywords: *Escherichia coli*, Biofilm, Swollen head syndrome, layers.

Introduction

Swollen-head syndrome (SHS) is a recently described, acute respiratory disease that is observed in a variety of domestic poultry, which characterized by congestion and edema of the periorbital and infraorbital sinuses; also, nervous signs may be seen like torticollis, opisthotonos, and incoordination. SHS or SHS like disease has been reported in many countries such as the Netherland, United Kingdom, France, Spain, Israel,

Germany and Canada. Typically, the disease episodes last for 2 to 3 weeks, during which time there is a many cases, diseased bird become reluctant to move and eventually die as a result of their inability to feed. Although the etiology of SHS is uncertain and it is clinical, appearance is quite variable. The disease is thought to result from a mixed infection of paramyxovirus, coronavirus, or avian pneumovirus and *E.coli* in which the

primary viral infection causes acute rhinitis that predisposes *E.coli* to invade the subcutaneous tissues in the head (1). *Escherichia coli* (*E. coli*) are gram-negative, flagellated microbes that belong to Enterobacteriaceae family. *E. coli* are causing widely distributed infections in poultry (2). Avian pathogenic *E. coli* (APEC) infections are implicated in millions of dollars losses in the poultry production industry annually, because of increased mortality, decreased egg production and carcass condemnation (3). It is well known that APEC strains causing extraintestinal infections are characterized by the possession of several determinants that enable to develop extraintestinal life (4). Many of these virulence factors have been identified (5). However, the mechanisms of APEC virulence remain largely unknown (6, 7). Biofilm is an aggregation of microorganisms that grow on living or inert surfaces and surround themselves with secreted polymeric substances. Moreover, this community displays an altered physiology of growth and gene transcription in comparison to planktonic cells of the same organisms (8). Biofilm formation represents a crucial factor in the pathogenic mechanism of extraintestinal APEC. Inappropriate antimicrobial practice in both human and veterinary medicine is regarded as a major contributing factor that creates an antibiotic selective pressure that select for development and spread of antibiotic-resistant strains. These resistant strains could transfer resistant traits to other pathogenic bacteria or even to microbiota of exposed individuals (human and animals) or populations (9). The present study was aimed to investigate the role of *Escherichia coli* as secondary bacterial pathogen in an outbreak of swollen head syndrome among laying hens farm in Al-Safaa Company, Al-Diwaniyah Province, Iraq, and identification of isolates using conventional methods, as well as study the antimicrobial susceptibility profile and biofilm production.

Material and Methods

Ethical approval

The Animal Ethical Committee of Veterinary Medicine College, University of Al-Qadisiyah, Iraq, has approved the present study under permission No: 414

Sampling collection and Bacterial isolation:

An outbreak of suspected swollen head syndrome infection was occurred in AL SAFA Company for poultry production in Al-Diwaniyah Province, during the period between 2-16 January 2017 in layers. The age of infected hens about 21 weeks. There was signs of depression, decreased feed consumption, and egg production with morbidity rate about 70% and mortality rate was approximately between 150- 200 hens per day. Twenty chickens were collected from layer's farm. Twenty serum samples were collected aseptically, and transported in cold box for serological examination. All hens were brought to and sacrificed at the Microbiology Laboratory, College of Veterinary Medicine, University of Kufa, clinical signs and necropsy findings were recorded and swabs from lesions were collected under aseptic technique. Each sample was initially inoculated onto brain-heart infusion broth (Oxoid, UK), which incubated at 37°C aerobically for 18-24h, and then a loopful broth was streaked on sheep blood agar and selective media including MacConkey's agar, Eosin-Methylene Blue agar (Oxoid, UK), Chromagar *E. coli* (Chromagar, France), and Mannito Salt (OXOID). Based on colonial morphology Gram's stain, each suspected colony was selected and sub-cultured on Chromagar *E. coli* in order isolate pure culture for further identification (10, 11).

Biochemical identification:

The following specific biochemical test were done to identify the isolates including, catalase test, oxidase, Triple Iron Sugar agar, Simon citrate, Indole and Methyl Red tests and results were recorded according to (10,11,12)

Rapid serological test:

Rapid test of IB (Korea) was used for screening for infectious bronchitis for all specimens the test was run depending on manufacturer's instructions.

Enzyme-Linked Immunosorbent Assay (ELISA):

ProFLOK® IBV Ab test kit (SINBIOTIC, USA) was used to confirm the rapid test results. The test was performed according to manufacturer's instructions.

Biofilm formation:

Biofilm formation assay was performed following a method described by (13) with few modifications. Eight strains of APEC were incubated in tubes with 5 ml Luria Bertania (LB) broth medium at 37°C for 24hrs. Aliquots of 1:100 from incubated cultures were inoculated in 5m LB broth tubes mixed well and then 200 µl of each broth media were transported into wells of a 96-well polystyrene microtiter plates (Germany), the plates were incubated without shaking at 37°C for 18 hrs. The optical density (OD) of each well was measured at $\lambda = 620$ nm. After that, broth was discarded and the non-adherent cells were removed from wells by washing with 150 µl sterile saline. Then all wells were dried for 20 min and 150 µl of a 1% crystal violet (CV) solution (Panreac, Barcelona, Spain) was added for each well and left for five minutes. The non-adherent dye was discarded and the wells were washed three times with 200 µl of saline to remove excess dye. The microtiter plates were left in inverted position in hood for 1 h to be air-dried. The bound stain to the attached

Results

The Clinical pictures were difficult breath, coughing, rales, swollen of infraorbital and supraorbital sinuses, and conjunctivitis (figure-1). Also there were changes in the egg shell and drop in egg production and feed consumption as well (figure-2). The morbidity rate was 70% while mortality rate was approximately 150-200 hens per day.

bacteria was dissolved in 130 µl of absolute ethanol and the OD of the stained adherent cells and stained control wells were measured at $\lambda = 492$ nm. The rate of biofilm formation was calculated by using the following formula: $BF = AB - CW$, as BF represents the biofilm formation, AB is the OD₄₉₂ nm of stained attached bacteria and CW is the OD₄₉₂nm of dye impregnated control wells containing plain broth only (14). The test was done in duplicate for test and control.

Antibacterial susceptibility testing:

Susceptibility of isolates to antibiotics was screened by the standard disk diffusion method in Mueller-Hinton agar (OXOID) according to Bauer *et al.* (15), and the results were interpreted in accordance to the criteria of the CLSI (16). The isolates were screened for resistance to the following antimicrobial agent CIPROFLOXACIN, ERYTHROMYCIN, AZITHROMYCIN, AMPICILLIN, LEVOFLOXACIN, TRIMETHOPRIM, and NITROFURANTOIN, all antibacterial discs were purchased from MAST DIAGNOSTICS (UK).

Interpretation of susceptibility test results: APEC isolates that are resistant to a minimum of three classes of antibiotics are regarded as MDR strains (17). Prevalence of antibiotic resistance among the APEC isolates was calculated as the percentage of isolates that showed resistance to one or more of the test antibiotics, according to the formula (Percentage of Resistance = Number of resistant isolates/ Number of isolates tested with the antibiotic x 100).

Figure (1): A layer with depression-swollen conjunctivitis

Figure (2): discoloration and changes in eggshell of layer eggs

Figure (5): Elisa test result showed high titer of IBV antibodies in hens' samples

Figure (3): Necropsy findings: Gaseous exudate in sinuses and trachea (B) Affected ovaries of the Hen (yellow gaseous exudate)

Figure (6): Colonial morphology of E.coli on A MacConkey agar; B CHROM agar E. coli; C Eosin Methylene blue agar

Figure (4): Rapid kit for detection of IBV infection

Figure (7): Some biochemical test used for identification of E.coli Urea agar B TSI C Simon citrate test

The main gross lesions demonstrated among necropsied chicken were shown in figure- 3 A and B, briefly, the infected chicken had gaseous exudate in trachea, nasal passages and sinuses. There was yellowish gaseous exudate on the air sacs, ovaries and

the peritoneum. All sera (20) samples examined were positive for infectious bronchitis by Rapid test and ELISA as shown in the figure 4 and 5. The isolates were characterized on selective media and by Gram staining and biochemical

identification. Twenty-two isolates were recovered from 20 hens samples, 18 (81.8%) isolates were confirmed to *E. coli* by sub culturing on selective media, and

biochemical tests as shown in figure- 6 and 7. The other four (18.2%) isolates were *Staphylococcus aureus*, *Klebsiella spp*, *Citrobacter spp*, *Proteus spp*.

Isolates	The OD values		BF
	AB (mean)	CW (mean)	
1	0.549	0.337	0.212
2	0.4875	0.35	0.1375
3	0.4525	0.436	0.0165
4	0.512	0.38	0.132
5	0.4005	0.385	0.0155
6	0.53	0.394	0.136
7	0.4915	0.46	0.0315
8	0.7015	0.4	0.3015

The levels of biofilm growth were calculated and the results recorded as follows: BF, biofilm formation; AB, stained attached bacteria; CW, stained control wells. The results showed that 62.5% (5/8) of tested isolates able to produce biofilm, particularly, one isolate with strong ability to produce biofilm (≥ 0.300), one isolate with moderate ability (0.200-0.299), 3 isolates showed weak ability (0.100-0.199), while 3 isolates did not produce biofilm (< 0.100). Table 1 showed the results of antibacterial susceptibility testing of 18 *E. coli* isolates to a panel of 7

most commonly used antimicrobials in the veterinary practice. The majority of isolates were resistant to Ampicillin (100%), ciprofloxacin (94.4%), Erythromycin (100%), azithromycin (100%), trimethoprim (88.9%), and levofloxacin (94.4%). Nonetheless, however majority of APEC isolates were susceptible to nitrofurantoin (72.3%). Moreover, all *E. coli* strains recovered in the current study showed multidrug resistant to three or more different antibacterial classes.

Table (1): Antibacterial susceptibility pattern of *E. coli* strains isolated from layers with swollen head syndrome.

<i>E. coli</i> (n=18) isolates			
Antimicrobial agent, symbol and potency	Resistant <i>n</i> (%)	Intermediate <i>n</i> (%)	Susceptible <i>n</i> (%)
Ampicillin (AM) 25 µg	18 (100)	0 (0)	0 (0)
Erythromycin (E) 15µg	18 (100)	0 (0)	0 (0)
Ciprofloxacin (CIP) 10 µg	17 (94.4)	1 (5.6)	0(0)
Trimethoprim (TM)5µg	16 (88.9)	0 (0)	2 (11.1)
Nitrofurantoin (NIT) 300µg	5 (27.7)	4 (22.3)	9(50)
Azithromycin (ATH) 15µg	22 (100)	0 (0)	0 (0)
Levofloxacin (LEV)5 µg	17 (94.4)	1(5.6)	0 (0)

Discussion

Swollen head syndrome (SHS) is an important problem in broilers, broiler breeders and layers, where the primary causative agent is viral (avian pneumovirus) which complicated by secondary bacterial infection of *E. coli*. The affected flocks usually

show respiratory and nervous signs (18). All the examined hens suffered from respiratory problems summarized by hard to breath and wheezing, there are inflammatory secretions from nostrils and beak, and edema of face. Similar findings were reported by (19) in

which flocks showing respiratory symptoms, swollen heads, severe depression and unwillingness to move. The drop in egg production could be the result of pathological involvement of the ovaries (like sero-fibrinous oophoritis) and the oviduct that lead to changes in the color and texture of the eggshell. The present study also found that most of the lesions characterized by post-mortem examination are in coexistence with clinical picture of SHS that reported in the literatures (19). The primary cause of the outbreak was detected by serological tests to be infectious bronchitis virus. Swollen head syndrome may be caused by avian metapneumovirus, IBV, or coronavirus, which may permit for secondary bacterial infection especially *Escherichia coli* to be established (1). Present study showed that the major bacterial pathogen associated with swollen head syndrome was *E. coli* isolates which in coexistence with data reported by (20). It is worth noting that the majority of tested strains of *E. coli* for biofilm production are able to form such biofilm. Currently, the efforts for control infections with biofilm producing bacteria are problematic because of increased risk of antibiotic resistance in comparison to treatment of infection caused by planktonic mode of growth of bacterial infection (21). Several researchers have been recorded an elevated resistance among APEC to commonly used antibiotics such as beta-lactam antibiotics, quinolones, and Erythromycins (22, 23, 24, 25). High levels of resistance to these antimicrobial agents in present study could be reflect the widespread, excessive and blind use for treatment and/or prophylactic purposes or as growth promoters which select for resistance strains in the poultry field (26). The percentage of Fluoroquinolones Resistance was increased many years after approval of this class for

use in poultry (27). In current study, the detection of 94.4% of APEC isolates resistant to Ciprofloxacin and Levofloxacin is of public health importance as this drug is used for the treatment of many urinary tract infection and other serious clinical cases like typhoid infections and should be monitored in veterinary field. All *E. coli* isolates were MDR to three or more different antimicrobial classes. Similar findings have been reported by others (28, 29). The misuse of antibiotics may result in development of cross-resistance (the resistance to several structurally related antimicrobials) and co-resistance (the resistance to several structurally unrelated antimicrobials), via specific resistances mechanisms and select for drug resistant zoonotic bacteria. Poultry and livestock infections due to MDR bacteria lead to higher morbidities and prolonged treatment of sick animals, higher treatment failure rates and mortalities, and costly drug changes that add to the production costs for meat, dairy and eggs, and lower farmers' profits. Similarly, MDR bacteria have grave implications for public health in terms of increased morbidities, prolonged hospitalizations, the need for use of newer and expensive drugs, and the greater risk of mortalities in the elderly and infants (17, 30). In conclusion, swollen head syndrome is a significant clinical infection among layers that may be caused by mixed bacterial and viral infections and improper hygienic measures which predisposing for its occurrence. The present findings showed that all APEC were multidrug resistant, which make treatment decision difficult and increase public health hazard. Therefore, the introduction of surveillance programs to monitor antimicrobial resistance strains is strongly recommended to protect human and animal health.

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