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Surveillance of Multi Drug Resistant Bacteria Isolated from Virally Infected Broilers

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ABSTRACT

One health call to ensure human, animal and environment safety put on a major responsibility on the animal health care sector starting with identifying the circulating pathogens and its multi drug resistant (MDR) patterns. This study was conducted on total 283 diseased broilers collected from small broilers flocks in Giza and El-Kalubia province, Egypt. The clinical signs and autopsy findings were highly suggestive for: Infectious Bronchitis (IB), Avian Influenza (AI), Newcastle Disease (ND), and Chicken Infectious Anemia (CIA). Trachea, lungs, and kidney were collected during the autopsy and examined using molecular tests (PCR &RT-PCR) for rapid diagnosis of the viral pathogen revealing a high incidence of IB and CIA (71.4 and 61.3% respectively). The 165 liver and intestine samples of the virally infected broilers were subjected to bacteriological examination and all were positive for Escherichia coli (*E. coli*) or salmonella or both representing an extra challenge facing the infected flocks. *E. coli* isolates were serotyped into O_{125}, O_{158} and O_{111} while Salmonella were serogrouped into: *S. entraitidis, S. gaille* and S. Altona. The MDR pattern was identified by disk diffusion method using 12 different antimicrobial discs: (nalidixic acid, neomycin, trimethoprim, streptomycin, norfloxacine, sulfamethazine, chloramphenicol, tetracycline, doxycycline, oxytetracycline, gentamycin, and fosfomycine). The results showed complete resistance to sulfamethazine, nalidixic acid and oxytetracycline. High resistance to chloramphenicol, trimethoprim, tetracycline, and streptomycin, low resistance to gentamycin, and all isolates were sensitive to fosfomycine. This study revealed MDR bacterial pathogens are highly prevalent among the small poultry flocks and greatly interacts with the viral avian diseases here in Egypt.

Key words: Broiler, Viral, Bacterial, Multi-drug resistant.

INTRODUCTION

Small poultry flocks provide a real mirror for the current situation in the animal sector here in Egypt. Where a significant increase of the poultry production in the last five years reaching self-sufficiency level (1.4 Billion bird annually), small poultry flocks act as a greater supplier for poultry industry (small bird numbers few hundreds up to few thousands, inadequate biosecurity practices with restricted access of veterinary services and increased risk of contact with wild birds "reservoir for many infectious disease").

Poultry production in Egypt is challenged by serious avian diseases either viral or bacterial. On the top of viral diseases: Infectious Bronchitis (IB), Avian Influenza (AI), Newcastle Disease (ND) and Chicken Anemia (CIA) which are routinely detected in broilers flocks with or without bacterial infections complicating the situation (Radwan et al. 2013; Hassan et al. 2016; Ahmed and Naguib 2018). IB is an acute, rapid, spread viral disease of chickens causing respiratory signs, drop in egg production, and poor egg quality or nephritis/nephrosis (Jackwood 2012), it is reported in all over the worldwide, Middle East and Egypt (Valastro et al. 2016).

AI is the cornerstone in "respiratory diseases complex syndrome" since its first discovery in poultry farms in Egypt 2006, circulating in the Egyptian poultry farms and backyard fields with both highly (Aly et al. 2008) and low pathogenic (El-Zoghby et al. 2012) strains.

Despite the vaccination programs, ND still causes a very serious problem in the poultry production all over the world and Egypt (Waheed et al. 2013). Natural reservoirs (wild birds) usually transmit virus to domestic birds causing subclinical infections and upper respiratory disease with high mortalities.

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A great economic impact on poultry industry in all major chicken-producing countries of the world is caused by CIA (Mahzounieh et al. 2005) with severe destruction of the bone marrow cells, resulting in aplastic anemia in the very young ages and immunosuppression (Yuasa et al. 1979) giving a greater chance for another viral or bacterial infection.

Viral infections enhance secondary bacterial infection especially *E. coli*: normal inhabitant of chickens intestine but it is upgraded into a pathological condition under stress " Avian colibacillosis" which is considered the most widespread bacterial infection causes losses and a decrease in the production (Mehmood et al. 2020). Or acquired infection through ingestion of contaminated food as in "salmonellosis" which causes heavy economic losses through reduced meat, egg production and mortality. These secondary bacterial infections not only exaggerate the pathogenesis but also increase the bacterial density in the surrounding environment (Tan et al. 2012).

The worst case scenario happens when the secondary bacterial infection is a multi-drug resistance (MDR) bacteria introducing a greater challenge as it causes prolonged infections with high morbidity & mortality and massive costs associated with prevention, treatment and control measures of infection (Boerlin and White 2013). Consequently, this work was designed to provide a clear idea of the circulating viral and bacterial pathogens with proper identification of it MDR pattern in the Egyptian small poultry flocks.

MATERIALS AND METHODS

Sample Collection

283 freshly dead broilers (within one hour after death) were collected from different flocks in Giza and El-Kalubia province, Egypt. Birds were handled in accordance to the regulations of collecting samples from dead birds, and this study was approved by the animal care committee of the Animal Health Research Institute. The freshly dead broilers had a history of depression, respiratory stress, anorexia, paleness and reduced growth performance with high morbidity and mortality. During the autopsy different organs (trachea, lung, kidney, liver and intestine) were collected aseptically and according to findings the organs were examined as clarified in Table 1.

Detection of Viral Pathogens by Polymerase Chain Reaction (PCR)

Sample Preparation

Lung, trachea and kidney samples were grounded with sterile sand and PBS using a mortar and pestle forming a homogenate, after centrifugation at 3000rpm for 5min. The supernatant is used for viral RNA and DNA extraction.

Viral Nucleic Acid Extraction

Homogenates of tissue samples of kidney, trachea and lung were extracted using commercially available extraction kit Thermo Scientific Gene JET Viral DNA and RNA purification (Thermo Fisher Scientific Inc.,) according to the manufacturer's instructions.

Real-Time PCR for AI Detection

Multiplex PCR amplifications for AI H5 (Löndt et al. 2008) and H9 (Ben Shabat et al. 2010) were performed in 25µL final volume of: 5µL of RNA template, 12.5µL of 2x QuantiTect Probe RT-PCR Master Mix, 5.75µL PCR grade water, 0.25µL of each primer (50pmol conc.) and 0.125µL of each probe (30pmol conc.) and 0.25µL of QuantiTect RT Mix. Reverse transcription was done at 50˚C for 30min trailed by primary denaturation at 94˚C for 15min, followed by 40 cycles of denaturation at 94ºC for 15s, annealing at 54˚C for 30s and last extension step at 72˚C for 10s, using a stratagene MX3005P real time PCR machine.

Real-Time PCR for IB and ND Detection

Using Verso 1-Step qRT-PCR Kit plus ROX Vial (Thermo Scientific, US) RT-PCR with specific oligonucleotide primers and probes for IBV (Meir et al. 2010) and vNDV (Wise et al. 2004) was conducted. 25µL final reaction volume of: 5µL RNA template, 12.5µL 2X 1-step PCR ready mix, 1.25µL RT-enhancer, 0.25µL Verso enzyme mix, 1µL of primers, 0.25µL probe and 3.75µL nuclease free water. RT-PCR conditions started with 50˚C for 15min trailed by 95˚C for 15min, then 40 cycles at 95°C for 15s and 30s at 60˚C (for IBV) or at 54˚C (for NDV) with reading of fluorescence in this step.

Detection of CIA by PCR

25µL final reaction of: 12.5µL of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1µL of each primer of 20pmol conc., 5.5µL of water, and 5µL of DNA template. Using applied biosystem 2720 thermal cycler. Started with primary denaturation step at 95˚C for 5min, trailed by 35 cycles of 94˚C for 30s, 50˚C for 40s and 72˚C for 45s. Ending with final extension step at 72˚C for 10min. Electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1xTBE buffer at room temperature by gradients of 5V/cm was used to view the results of the PCR: 15µL of the products was loaded in each gel slot for gel analysis. Using a gene ruler 100bp DNA ladder (Fermentas, Thermo) to determine the fragment sizes. A gel documentation system (Alpha Innotech, Biometra) photographed the gel and computer software analyzed the data.

Isolation and Identification of Bacterial Pathogens

165 liver and intestine samples from positive samples for the presence of viral pathogen were bacteriologically tested for the presence of *E. coli* and salmonella. Isolation and identification of salmonella were done according to standard methods ISO 6579-1(en) 2017. Samples were cultured on modified semi solid Rappaport Vassillidis base media (OXOID,CM1112) and incubated at 37˚C for 24h followed by sub cultured on XLD Agar (Neogen, LAB032) and Brillent Green agar (Neogen, LAB034) incubated at 37˚C for 18-24h, a morphological and bio-chemical identification were carried out on the suspected colonies, the *E. coli* isolation was conducted according to Lee et al. (2008) using peptone water (Neogen, LAB 104) incubated at 37˚C for 18h followed by sub culturing on MacConkey (Neogen, NCM 0174A) & EMB (Neogen, LAB 061) incubated at 37˚C for 24h. The assumed *E. coli* colonies were morphologically and biochemically identified.

Antigenic Characterization

One randomly selected isolate from each positive flock (representative for the circulating bacterial pathogen among the infected flock) was serologically identified. Ten

E. coli were serotyped according to Lee et al. (2008) using standard polyvalent and monovalent *E. coli* antisera that are prepared by DENKA SEIKEN CO. LTD, while five Salmonella isolates were serologically identified conferring to Kauffmann-White scheme using slide agglutination with O and H antisera provided by SIFIN.

Antimicrobial Sensitivity Test

All identified bacterial isolates were examined for determination of their MDR pattern using disk diffusion method according to Koneman et al. (1997) by representatives of different groups of antibiotics that are usually used in the field. Antimicrobial discs were provided by OXOID: Nalidixic acid (NA30mg), neomycin (N5mg), trimethoprim (T5mg), streptomycin (ST10mg), norfloxacine (NX5mg), sulfamethazine (SMZ250mg), chloramphenicol (C30mg), tetracycline (TE30mg), doxycycline (Do30mg), oxytetracycline (OT30mg), gentamycine (CN10mg), fosfomycine (FOS200mg), were distributed throughout the surface of Mueller Hinton agar (HIMEDIA, M173-500G) plates covered with a bacterial suspension (0.5 Mac Farland scale). And incubated for 24h at 35°C, inhibition zones diameter was examined and measured in millimeters. The results were interpreted according to CLSI (2017).

RESULTS

Clinical Picture and Post-mortem Findings

283 broiler samples were thoroughly examined revealing: congestion in trachea, lungs and upper respiratory tract, and congestion in other internal organs including the kidneys that were highly suggestive for infection by AI, IB and ND. While intramuscular and subcutaneous hemorrhages, atrophy of the thymus and pale bone marrows instead of red characteristic of normal bone marrow was highly suggestive for infection by CIA.

Results for Detection of Viral Pathogens using PCR

Trachea, lung and kidney samples were examined for viral pathogens according to Table 1 using RT-PCR and PCR, results showed that IB is the major detected viral pathogen 50/70 by 71.4% followed by CIA in 100/163 by 61.3% respectively. On the other hand, AI was detected in 15/79 by 19% as displayed in Table 5.

165 out of 283 investigated samples were positive for infection by a viral pathogen with a prevalence rate of 58.5% of viral disease among the tested broilers flocks. All the 165 liver and intestine samples positive for viral pathogens were submitted for bacteriological isolation to detect possible complication with *E. coli* & salmonella. All samples submitted to the bacteriological isolation were positive to at least one bacterial pathogen, the results revealed that 153 (92.7%) of samples were positive for *E. coli* as following: 142(86%) were positive for *E. coli* only while 11 (6.7%) were positive to *E. coli* and salmonella while salmonella was detected in 12(7.3%) of samples alone.

The bacteriological examination showed a different ratio for detection of the bacterial pathogen in different organs as followed: 111(78.2%) intestine samples positive for *E. coli* and 31(21.8%) liver samples positive for *E. coli*. On the other hand, salmonella was isolated from 12(52.1%) from intestine and 11(47.9%) from liver samples and 2(8.7%) from liver and intestine.

Serological typing of a randomly selected isolate from each flock as representative for the bacterial pathogen among the affected broiler showing that 10 *E. coli* isolates belonged to 3 different serogroups: O_{125}, O_{158} and O_{111}. 5 salmonella isolates were identified into: *S. entraitidis*, *S. gaille* and *S. altona* as in Table 6.

Detection of the antimicrobial pattern expressed a high prevalence of MDR pathogen as followed: one isolate (10%) of *E. coli* isolates was resistant to at least seven anti-microbial agents, 2 isolate (20%) resistant to nine, ten and eleven anti-microbial agents equally while 3 isolates (30%) resistant to eight antimicrobial agents. Salmonella isolates also showed a much similar MDR pattern with 2 isolates (20%) resistant to eight antimicrobial agent and 4 isolates (40%) resistant to nine and ten anti-microbial agents equally.

Table 7 shows that all the *E. coli* and Salmonella isolates were totally resistant to sulfamethazine, nalidixic acid and oxytetracycline. *E. coli* isolates: 9 isolates were resistant to chloramphenicol and trimethoprim, followed by 8 isolates to tetracycline, 7 isolates resistant to streptomycin, neomycin, norfloxacin and doxycycline, finally 6 isolates were resistant to gentamycin.

While salmonella isolates were: 5 isolates resistant to norfloxacin, trimethoprim, tetracycline and streptomycin, 4 isolates resistant to chloramphenicol and neomycin, 2 isolates resistant to doxycycline, one isolate resistant to gentamycin.

Table 1: Different organs were examined as following

Tested organs	Single viral detection				Mixed viral detection				Total
	AI	IB	ND	CIV	AI, IB	AI, ND	ND, IB	AI, IB, ND	
Trachea	2	10	24	-	40	24	7	13	120
Lung	2	10	24	-	40	24	7	13	120
Kidney	2	10	-	163	40	24	7	13	259
Total tested bird	2	10	24	163	40	24	7	13	283

(Kidney samples were not tested for ND)

Table 2: Primers and probes used in multiplex RT- PCR for the detection AI H5 and H9

Primerandprobe	Sequence	Reference
H5LH1	ACATATGACTAC CCACARTATTCA G	Löndt et al. 2008
H5RH1	AGACCAGCT AYC ATGATTGC	
H5PRO	[FAM]TCWACAGTGGCGAGTTCCCTAGCA[TAMRA]	
H9F	GGAAGAATTAATTATTATTGGTCGGTAC	Ben Shabat et al. 2010
H9R	GCCACCTTTTTCAGTCTGACATT	
H9 Probe	[FAM]AACCAGGCCAGACATTGCGAGTAAGATCC[BHQ]	

Table 3: Primers and probes used in RT-PCR for the detection IB and ND

Primerandprobe	Sequence	Reference
IB-F	ATGCTCAACCTTGTCCCTAGCA	Meir et al. 2010
IB-R	TCAAACTGCGGATCATCACGT	
IB PRO	[FAM]TTGGAAGTAGAGTGACGCCCAAACTTCA [TAMRA]	
ND F+4839	TCCGGAGGATACAAGGGTCT	Wise et al. 2004
ND F-4939	AGCTGTTGCAACCCCAAG	
ND Probe	[FAM]AAGCGTTTCTGTCTCCTTCCTCCA[TAMRA]	

Table 4: Primers used in PCR for the detection CIA.

Primerandprobe	Sequence	Segment size	Reference
CIV –F	CTAAGATCTGCAACTGCGGA	418bp	Hussein et al. 2002
CIV –R	CCTTGGAAG CGGATAGTCAT		

Table 5: Results for detection of viral pathogens.

Positive samples for	Single viral detection				mixed viral detection					
	AI	IB	ND	CIA	AI, IB	AI, ND	ND, IB	AI, IB, ND	Total	%
AI	-	-	-	-	-	15	-	-	15/79	19
IB	-	10	-	-	20	-	7	13	50/70	71.4
ND	-	-	0	-	-	0	0	0	0/69	0
CIV				100	-	-	-	-	100/163	61.3

(% the percentage of positive samples to the total number of examined samples for the disease)

Table 6: The serological identification of *E. coli* and salmonella isolates

Bacterial isolate	Serotype	No. of each isolate	Percentage	Total
E. coli	O_{125}	7	70	10
	O_{158}	2	20	
	O_{111}	1	10	
Salmonella	*S. altona*	1	20	5
	S. gaille	2	40	
	S. entraitidis	2	40	

Table 7: The MDR pattern of both *E.coli* and Salmonella isolates

Bacterial pathogen	Antimicrobial drug											
	SMZ 100mg	CN 10mg	NA 30mg	FOS 200mg	C 30mg	S 10mg	N 30mg	TE 30mg	OT 30mg	NX 10mg	TMP 5mg	DO
E. coli	10/10	6/10	10/10	0/10	9/10	7/10	7/10	8/10	10/10	7/10	9/10	7/10
Salmonella	5/5	1/5	5/5	0/5	4/5	5/5	4/5	5/5	5/5	5/5	5/5	2/5

DISCUSSION

The increasing alertness of the one health call for optimal health for people, animals, and environment requires proper diagnosis of the circulating pathogens, and the awareness of the MDR pattern of the existing bacterial serotypes. Therefore, the study was conducted on 282 broiler samples from Giza and El-Kalubia Province to detect the circulating pathogens in the region revealing a high incidence of IB 71.4% that goes with findings of Kamel et al. (2010) who found that IB was 66.6% prevalent in different broilers farms in Egypt.

Followed by 61.3% incidence rate of CIA virus, an emerging viral avian disease circulating in the African poultry producing countries at the last thirty years. The disease causes a serious disease in young age and immune suppression, the findings agree with Gholami-Ahangaran et al. (2013) who detected CIA infection in chickens in Iran (58.4%), while Mohamed (2010) found that 26.6% of tested broiler flocks were positive for detection of CIA using PCR in Assuit, Upper Egypt. AI virus was found in 19% of examined samples agreeing with Haji-Abdolvahab et al. (2019) declaring that 21.9% of tested farms were infected by AI.

Although the ND is one of the major viral avian diseases in all over the world as well as Egypt, but during this study all the 69 samples that were examined for ND virus were negative for ND by RT-PCR, these results may be attributed to 65.2% of the tested samples were positive for other pathogens and low percent of circulating ND strains in the region during this period with a good vaccination program.

All samples submitted to the bacteriological isolation were positive to at least one bacterial pathogen, indicating low biosecurity practices implemented at the small poultry flocks, and limited access of veterinary services. Also a standing prove that the poultry industry is not only threaten by viral pathogens only but also bacterial pathogens causing much worst complications, and providing a permanent threat for the man, animal and environment safety.

The results showed a high prevalence of *E. coli* among the virally infected broilers 153(92.7%) either alone 142(86%) or with salmonella 11(6.7%), the results emphasis that *E. coli* remains one of the greatest challenges that threaten the poultry production, these results agree with the findings of Halfaoui et al. (2017) who found that *E. coli* represents 86.66% of the pathological specimens and disagree with Ibrahim et al. (2019) who isolated *E.coli* from 53.4% of sick chickens in northern Jordan.

Salmonella was detected in 23 (13.9%) either 12 (7.3%) salmonella alone or 11 (6.7%) with *E. coli* agreeing

with the findings of a study conducted on broilers farms at El-Gharbia and El-Menofia Governorates during November 2015 to November 2016 that state on prevalence of salmonella in 15.4% of the bacteriologically tested farms by Sultan et al. (2018).

E. coli was isolated mainly from 122 (79.7%) intestine samples and 31 liver samples indicating that *E. coli* is a natural inhabitant in the broilers gut under stress (viral infection and in appropriate husbandry) is upgraded into a pathogenic, *E. coli* exaggerating the hazard that challenge the production. While salmonella was isolated from intestine 12 (52.1%), 11 (47.9%) from liver samples and 2(8.7%) from both liver and intestine samples. The results recommend the use of the intestine as the organ of choice for the bacteriological isolation of both *E. coli* and salmonella.

Biochemical identification and the serogrouping of *E. coli* isolates showed that the isolates belonged to serogroups O_{125}, O_{158} and O_{111} and these serotypes are usually associated with colibacillosis in poultry that agrees with Roshdy et al. (2012) who detected the following serogroups in diseased chickens O_{44}, O_{158}, O_{114}, O_{91}, O_{111}, O_{125}, O_{103}, O_{142}, O_{26}, O_{78}, O_{127} and O_{164}.

Salmonella isolates were serogrouped into: *S. entraitidis*, S. gaille that came first and *S. altona* which emphasize the fact that *S. entraitidis* usually circulates in the broilers farms agreeing with Rabie et al. (2012) and Ammar et al. (2016) in Egypt.

A further investigation was applied to detect the MDR pattern of the bacterial pathogen for a proper comprehension of the situation using Disc diffusion method by12 different antimicrobial discs of different antimicrobial groups, the results showed a complete resistance pattern of all the bacterial isolates to sulfamethazine, nalidixic acid and oxytetracycline. Harmonizing with Hamed et al. (2021) who found that the greatest resistance from the salmonella and *E. coli* isolates collected from poultry farms in Egypt was to these antibiotics among others.

E. coli isolates showed a high resistance pattern to chloramphenicol, trimethoprim, tetracycline, streptomycin, neomycin, norfloxacin and doxycycline that ranged from 90 to 70% which agrees with previous studies (Ibrahim et al. 2019; Jahantigh et al. 2020; Rafique et al. 2020) who found a high resistance pattern to doxycycline, streptomycin, tetracycline, and trimethoprim in Egypt, Iran, and Pakistan, respectively, but Gentamycin expressed a low resistance 60% agreeing with Amer et al. (2018) who declared that 55% of the *E. coli* isolates from the diseased broilers in Egypt were resistant to gentamicin.

Salmonella isolates showed a complete resistance to norfloxacin, trimethoprim, tetracycline and streptomycin supported by Bedekelabou et al. (2020) who found a complete resistance of salmonella isolates to trimethoprim, tetracycline and disagree with Mendonça et al. (2019) who found low levels of resistance to tetracycline (15.4%), streptomycin (7.7%), norfloxacin (3.3%) and trimethoprim (3.3%) from salmonella isolates isolated from broilers.

Salmonella expressed 80% resistance against chloramphenicol and neomycin disagreeing with Sohail et al. (2021) who found a low resistance to these antibiotics by salmonella isolates in Afghanistan, this may be explained by high use level of these antibiotics in Egypt, this study found a low resistance against gentamycin (20%) disagreeing with Mohammed et al. (2020) and agreeing with Belachew et al. (2021) who stated that salmonella isolated were sensitive to Gentamycin.

The previous findings can be concluded into IB, CIA and AI are circulating in many Egyptian broiler flocks as well as *E. coli* and salmonella specially O_{125}, *S.* Enteritidis. These bacterial isolates also expressed a high MDR pattern resulting in complicated problems in poultry flocks with high morbidity, mortality, loss of productivity and public health significance, this requires proper disease control through appropriate vaccination programs, good sanitary and hygienic measures, control of antibiotics use and implementing more effort in looking for other ways to control the microbial infection.

Conclusion

Proper hygienic measures are highly required in the small poultry flocks as it's the first shield against avian diseases, as well as implementing an appropriate restriction measures on the antimicrobial agent usage in these flocks either as a growth promoter or as treatment protocols in order to overcome the MDR dilemma.

Author's Contribution

Hanaa, AA. Ahmed shared in data collection, laboratory work, resources, writing, editing and submission of the manuscript. Ashraf, A. Abd El Tawab shared in design, analysis. Fatma, I. El Hofy shared in design. Wafaa M. M. Hassan shared in laboratory work. Manar E. El-khayat shared in reviewing the manuscript. All authors approved the final version.

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