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SURVEILLANCE ON BACTERIA ASSOCIATED WITH AVIAN INFLUENZA VIRUS OUTBREAKS IN COMMERCIAL BROILER FLOCKS IN EGYPT.

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ABSTRACT

Avian influenza (AI) (H5N1) outbreaks with very high mortality in twenty four (24) broiler flocks in Egypt provinces (El-Behira, El-Garbia, Kafer El-Sheikh, and Alexandria) were subjected to laboratory investigations in order to detect Enterobacteria associated with Avian influenza virus (AI) outbreaks in broiler flocks. Samples were collected from heart blood and liver for bacteriological examination. The samples for viral isolation were inoculated into specific pathogen free (SPF) embryonated chicken eggs (9-11 days old). The allantoic fluids (AF) were harvested 36 hours post incubation and were tested by using Haemagglutination (HA) test, Haemagglutination-inhibition (HI) test, Agar gel precipitation test (AGPT) and Reverse Transcription Polymerase Chain Reaction (RT-PCR). Escherichia coli and Salmonella were determined by using a specific media for isolation and identification of both enterobacteria isolates. Twenty isolates out of twenty four (20 / 24) flocks which represent 83.33% of examined outbreaks having haemagglutination-inhibition titer by using avian influenza (AI) common type A antibodies ranging from 2^4 to2^8 . The same isolates were also confirmed by Agar gel precipitation test **(**AGPT**) and Reverse Transcription Polymerase Chain Reaction (RT-PCR).**

A total of 16 samples out of 20 (16 / 20) (represented 80 %) examined samples from broiler flocks associated with Avian influenza virus outbreaks proved to be E.coli while a total of 4 samples(4 / 20) (represented 20 %) proved to be Salmonella enteritidis. The results of bacterial isolates were confirmed by biochemical test and serological identification.

INTRODUCTION

Avian influenza, sometimes avian flu, and commonly bird flu, refers to "influenza caused by viruses adapted to birds."

The first description of avian influenza (AI) dates back to 1878 in northern Italy, when ***Perroncito (1878)*** described a contagious disease of poultry associated with high mortality. The disease, termed "fowl plague", was initially confused with the acute septicemic form of fowl cholera. The term fowl plague was substituted by the more appropriate term highly

pathogenic avian influenza (HPAI) at the First International Symposium on Avian Influenza. ***Lupiani and Reddy (2009)*.**

All known viruses that cause influenza in birds belong to the species influenza A virus. Influenza virus A is a genus of the Orthomyxoviridae family.

Influenza A viruses are negative sense, single-stranded, segmented RNA viruses which has eight segments of its genome surrounded by a lipid envelope. A peculiar characteristic of the virus is that it contains rod-shaped and mushroom-shaped glycoproteins called haemagglutinin and neuraminidase respectively. ***Hirst (1941; and 1950).***

Both structural proteins are also important antigenic components of the virus. Avian influenza viruses are capable of agglutinating red blood cells of various animal species ***Buxton and Fraser (1977).***

Following the observation by ***Hirst (1941)*** that influenza viruses agglutinate chicken erythrocytes, it was found that several other viruses are capable of agglutinating erythrocytes from certain animal species ***Hallauer (1949).*** Consequently, the haemagglutination reaction became a much widely used technique for measuring either viral antigen or antibody concentrations. Both HA and HI tests are reliable, economical and time saving test for initial diagnosis and monitoring of AI outbreaks as both also needs ordinary used chemicals.

At the end of the 19th and early 20th Centuries 'fowl plague' was often reported in chickens and in several countries this disease was probably enzootic. However, in the second half of the 20th Century reports of influenza infections of chickens have been rare compared to infections of other domestic poultry despite the higher populations of chickens. Between 1964 and 1982 only three outbreaks in chickens were recorded. ***Pomeroy (1982).***

Investigated the presence of bacterial infections associated with Avian influenza virus (AI) in two farms of 200 km apart ***Kazeem et al. (2008).*** Haemolytic E.coli and unidentified Gram variable rod were isolated from the first farm, pasteurella haemolytica and haemolytic E.coli were isolated from the second farm. While ***King et al. (2009)*** mentioned that proteolytic cleavage of haemagglutinin is required for cell entry by receptor-mediated endocytosis and plays a key role in pathogenicity of the influenza virus and the presence of proteolytic bacteria in the intestinal tract of poultry suggests the possibility of yet-to-be-described role(s) in cleavage of haemagglutinin that may alter the pathogenicity of avian influenza viruses.

All Egyptian strains were very closely related and belonging to subclade 2.2 of the H5N1 virus of Eurasian origin, the same one circulating in the Middle East region and introduced into Africa at the beginning of 2006. The most obvious features of these outbreaks were severe clinical signs and high mortalities as well as very rapid and widespread occurrence within the country in a very short time. ***Aly et al. (2008).***

Since 2003 until July 2009, the highly pathogenic H5N1 virus has infected 433 people worldwide causing 263 fatalities. Other countries that have borne the brunt of the disease are Thailand with 25 cases with 17 deaths; China with 38 cases and 25 deaths; Vietnam with 111 cases and 56 deaths. ***May (2009).***

The application of control policies, ranging from stamping out to emergency and prophylactic vaccination, is discussed on the basis of data generated in recent outbreaks and in light of new regulations, also in view of the maintenance of animal welfare. ***Capua and Marangon (2007).***

The present paper is mainly concerned to a surveillance studies which was carried out on twenty four (24) commercially broiler flocks suffered from Avian influenza virus (AI) outbreaks in four governments (El-Behira, El-Garbia, Kafer El-Sheikh, and Alexandria) to detect Enterobacteria associated with Avian influenza virus (AI).

MATERIALS AND METHODS

A. MATERIALS

A.1. Samples:

A total of twenty four (24) commercial broiler farms of various age groups suffering from Avian influenza virus (AI) outbreaks at different localities (El-Behira, El-Garbia, Kafer El-Sheikh, and Alexandria) were surveyed for Enterobacteria associated with Avian influenza virus (AIV) outbreaks. All farms were showing signs of avian influenza virus (AI) infection.

A.2. Specific-Pathogen-Free Eggs (SPF):

Specific-Pathogen-Free Eggs (SPF) obtained from the SPF project of the ministry of Agriculture for isolation and propagation of Avian influenza virus (AIV) isolates from commercial broiler chickens.

A.3. Reference Avian Influenza (AI) Antisera:

Common group-A Avian Influenza (AI) antiserum was kindly provided by Dr. M. M. Aly, Animal Health Research Institute, Dokki, Cairo, Egypt.

A.4. Samples for virus isolation:

Specimens were collected from trachea, lung, air sac, spleen and liver from suspected diseased birds, 10% tissue suspensions were prepared from minced tissues and finally the inocula were obtained by centrifugation at 3000 rpm in cooling centrifuge for 15 minutes.

A.5. Chicken Red Blood Cells (R.B.Cs) Suspension:

Blood was collected after slaughtering of 4-5 weeks old chickens. Blood was received in sterile tubes containing 3.8% sodium citrate solution. Equal volume of phosphate buffer saline (PBS) was added and the erythrocytes were sedimented by centrifugation, after 3 wash cycles, for haemagglutination-inhibition (HI) test, the RBCs were used as 0.5% suspension in PBS, for haemagglutination (HA) test, the RBCs were used as 1% suspension in PBS, while for the slide haemagglutination (HA) test, the RBCs were used as 10% suspension in PBS.

A.6. Phosphate Buffer Saline (PBS):

PBS was prepared according to ***Oxoid Manual (1982)*.**

A.7. Antibiotic Mixture:

This was used to control bacterial and fungal contamination in inoculum suspension and consists of:

Penicillin-G sodium	100 IU / ml
Dihydrogen streptomycin	100 ug / ml
Gentamycin	50 units / ml
Mycostatin	100 units / ml

A.8. Agar Gel Medium For Precipitation Test:

The medium consist of 1.2% Noble agar (Difco), 8% sodium chloride and 7.5% glycerin in distilled water. These ingredients were dissolved as usual, PH adjusted to 7.2 and then autoclaved and cooled at 45° C before being distributed onto clean microscopic slides in 5 ml volumes ***Bauditz (1963)*.** After solidification of the agar layer the slides were kept in petridishes on water moistened filter paper at refrigerator temperature until used.

A.9. Reverse Transcription Polymerase Chain Reaction (RT-PCR) Test:

Used for serological identification of H5N1 avian influenza virus and to confirm the obtained results of HA and HI test. ***Lee et al. (2001).***

RT-PCR for one sample:

Component	**Volume**
2x reaction mix	12.5 ul
Template RNA	5.0 ul
Forward primer	0.1 ul
Reverse primer	0.1 ul
Super Script III RT/Platinum Taq Mix	0 ul
Distilled water	6.3 ul

Primers:

Forward primer:
YAGRTAYTGGGCHATAAGRAC

Reverse primer:
GCATTGTCTCCGAAGAAATAAG

A.10. Media used for isolation of Enterobacteriaceae:

Using of Nutrient broth as Liquid media and MacConkey's Agar Medium and SS Agar Medium as Solid media and this media used for isolation and identification of Enterobacteria which described in ***Oxoid Manual (1982)*.**

A.11. Reagents and solution used to biochemical identification of bacterial isolates:

For biochemical identification of enterobacteriaceae isolates (E.coli and Salmonella) use IMViC tests plus urease test according to ***Walter et al. (1970).***

A.12. Serological Identification of Enterobacteriaceae:

Suspected isolates were examined serologically according to ***Kauffman White Scheme (1972)*.**

B.METHODS

B.1. Virus isolation by chicken embryo inoculation:

The prepared inoculum were inoculated into 9 day old specific pathogen free (SPF) embryonated

chicken eggs via allantoic sac (AS), using 5 eggs and 0.2 ml as inoculum /egg. Inoculated eggs were incubated at 37 C for 5 days and candled daily for mortalities. Deaths recorded within the first 24 hours post-inoculation (PI) were excluded and considered as non-specific. Dead embryos and survivors that were killed at the end of the observation period by chilling for few hours in the refrigerator were examined for gross lesions and their allantoic fluids were harvested. The presence of haemagglutinating agent was checked by testing harvested fluids by rapid slide haemagglutination (HA) test.

B.2. Haemagglutination (HA) Test:

Slide HA test and standard quantitative plate method was carried out as reported by ***Laboratory Manual for the isolation and identification of avian pathogens (1989).***

This test was useful in the rapid detection of HA activity in harvested allantoic fluids from inoculated SPF embryonated chicken egg (ECE).

B.2.a. Slide Haemagglutination (HA) Test

This test was used for the detection of haemagglutinating agents in harvesting allantoic fluid of inoculated SPF embryonated chicken eggs.One drop of 10% chicken red blood cell (RBCs) suspension in sterile saline (0.8% sodium chloride) was placed onto a microscopic slide, mixed with one drop of allantoic fluid to be tested and the result was read within one minute.

B.2.b. Microtiter Plate Haemagglutination (HA) Test

1- 0.025 µl of PBS were dispensed into each well of a plastic U-shape bottomed microtiter plate.

2- 0.025 µl of suspected sample (antigen) were placed in the first well, make double fold serial dilution.

3- 0.025 µl of 1% chicken red blood cell (RBCs) were dispensed to each well.

4- Mix by trapping the plate gently and then allow the RBCs to settle for about 20-30 minutes at room temperature.

5- HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of RBCs.

6- The HA unite (HA titer) of each isolates consider the last dilution giving haemagglution activities (Rosita-Shape).

B.3. Haemagglutination-inhibition (HI) Test:

1- The β procedure of the (HI) test in microtiter plates as described by ***Charles w. Beard in A***

Laboratory Manual for the isolation and identification of avian pathogens (1989).

2- The HI titer is the highest dilution of antigen causing complete inhibition to common group

A avian influenza antiserum.

3- Four HA units of each virus are used in the test.

4- The agglutination is assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the control wells (containing

RBCs and PBS only) should be considered to show inhibition.

5- The HI unite (HI titer log2) of each isolates was consider the last dilution of antigen giving haemagglutination inhibition (end point).

B.4. Agar Gel Precipitation Test (AGPT):

This was carried out according to the method of ***Chubb and Churchill (1968)*** using the microtechnique described by ***Bauditz (1963)*** wells, 2 mm in diameter, were cut into the agar medium with a special kit which produced a pattern of six outer wells surrounding a central one. Four groups of such pattern could be made on one slide. Avian Influenza (AI) antiserum (common group-A) was placed into the central well, while the antigens to be tested were placed into the peripheral wells.

After filling the wells using Pasteur pipettes, the slides were labeled and incubated in humid chamber at room temperature over night. Reading was made on the next and subsequent day with the aid of an indirect light and final readings of the second day were recorded as follows: ─ = negative and + = clear, sharply defined precipitation line.

B.5. Reverse Transcription Polymerase Chain Reaction (RT-PCR) for AIV detection:

HA-positive allantoic fluids from the virus cultures were also tested for the H5 gene by RT-PCR.

Method of RT-PCR for one sample

1- In tube the distilled water was put

2- The 2x reaction mix was added

3- The forward and reverse primer were added

4- The template RNA from each sample was put in separated RT-PCR tube

5- The Super III enzyme was added to the master mix

6- 20 ul from master mix was added to the template RNA

7- The thermocycler was adjusted as following:

45° C for 60 min----------one cycle

55° C for 15 min----------one cycle

94° C for 3 min----------one cycle

94° C for 30 sec----------40 cycle

50° C for 30 sec ----------40 cycle

68° C for 30 sec ----------40 cycle

68° C for 5 min ----------one cycle

04° C for 24 hr ----------one cycle

Agarose Gel Electrophoresis

RT-PCR product was separated at 100V for 2 min. followed by 50V for 30 – 50 min. in 0.8% agarose gel immersed in 1x TAE buffer stained with ethidium bromide.

Purification of Viral RNA

1- 560 µl of prepared Buffer AVL containing carrier RNA were pipetted into a 1.5 ml microcentrifuge tube.

2- 140 µl positive HA allantoic fluids in 1st embryonated chicken egg passage were added to the Buffer AVL- carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 sec.

3- At room temperature (15-25° C) were incubated for 10 min.

4- The tube was briefly centrifuged to remove drops from the inside of the lid.

5- 560 µl of ethanol (96-100%) were added to the sample, and mix by pulse-vortexing for 15 sec. after mixing, briefly centrifuge the tube to remove drops from inside of the lid.

6- 630 µl of the solution from step 5 were carefully applied to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. The cap was closed, and centrifuged at 6000 x g (8000 rpm) for 1 min. the QIAamp Mini column was placed into a clean 2 ml collection tube, and discard the tube containing the filtrate.

7- The QIAamp Mini column was carefully opened, and repeat step 6.

8- The QIAamp Mini column was carefully opened, and adds 500 µl of Buffer AW1. The cap was closed, and centrifuge at 6000 x g (8000 rpm) for 1 min. the QIAamp Mini column was placed into a clean2 ml collection tube (provided), and discard the tube containing the filtrate.

9- The QIAamp Mini column was carefully opened, and adds 500 µl of Buffer AW2. The cap was closed, and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min. continue directly with step 11, or to eliminate any chance of possible Buffer AW2 carryover, perform step 10, and then continue with step 11.

10- Recommended: the QIAamp Mini column was placed in a new 2 ml collection tube (not

provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

11- The QIAamp Mini column was placed in a clean 1.5 ml microcentrifuge tube (not provided). The old collection tube containing the filtrate was discarded. The QIAamp Mini column was carefully opened, and adds 60 µl of Buffer AVE equilibrated to room temperature. The cap was close, and incubates at room temperature for 1 min. centrifuge at 6000 x g (8000 rpm) for 1 min.

B.6. Bacteriological isolation of Enterobacteriaceae:

Samples collected were inoculated into broth and incubated at 37 C for 24 – 48 hours. Then inoculated media were subcultured onto selective media ***Oxoid Manual (1982)***. These cultures were incubated at 37 C for 24 hours. Suspected colonies were biochemically identified according to ***Cruickshank et al. (1975).***

B.7. Escherichia coli and Salmonella antisera identification:

Biochemically identified cultures were examined using Escherichia coli and salmonella antisera. A loopful was taken from culture and mixed with a test serum on a clean dry glass slide to form homogenous suspension. The slide was rotated in a swirling movement. The result is considered positive if agglutination was visible by the naked eye.

RESULTS

Clinical Signs, and pathological finding in naturally infected birds on visited farms

Surveying avian influenza (AI) vaccinated and/or unvaccinated infected commercial broiler flocks of 32-41 days of age from 24 flocks located at various districts for AIV infection revealed that most of infected flocks showed respiratory distress, cyanosis of comb and wattles, edema and swelling of the head, locomotory disturbance, incoordination or stilted gait. In addition to general signs of depression, ruffled feathers, loss of appetite and weakness was noticed besides the elevation of daily mortality percent ranged between 0.54% and 33.01% from the onset of the disease in different commercial broiler flocks. (Table 1).

Postmortem examination revealed that chickens had general congestion in most veins of the abdominal viscera. The tracheal mucosa was edematous, hemorrhagic with mucopurulent exudates. Lungs were congested and/or had hemorrhages with edema. The liver samples of the birds were severely enlarged and very friable. Petechial hemorrhages were found on epicardium, coronary fat, adipose tissues of the abdomen, and in mucosa of proventriculus and duodenum. White necrotic foci with hemorrhages were seen in the spleen, and hemorrhagic patches on shank bone.

Isolation of AIV from commercial broiler chicken flocks

Results of inoculated samples prepared from minced tissues of infected birds collected from 24 commercial broiler flocks via allantoic-sac route in SPF embryonated chicken eggs (ECE) are presented in (Table 2 and 3).

Inoculated SPF eggs for 3 blind passages yielded 20 haemagglutinating agents from the 1st passage, 2 haemagglutinating agents from the 2nd passage and anther 2 haemagglutinating agents from the 3rd passage. (Table 2).

The embryo mortalities pattern of the inoculated samples via allantoic-sac inoculation of the 1st passage is shown in (Table 3) nearly all embryo mortalities were during 3rd to 4th day post inoculation (PI).

The haemagglutination titer of the inoculated sample by using microtiter plate haemagglutination (HA test) showing different titer ranged from 2^4 to 2^7 . (Table 4).

Identification of the virus isolates

Haemagglutination-Inhibition (HI) test:

The haemagglutination-inhibition titer of the haemagglutination (HA) positive in the 1st chicken embryo passage by using microtiter technique (haemagglutination-inhibition test) showing different pattern of haemagglutination-inhibition titer ranging from 2^4 to 2^8 while for the 2 haemagglutinating agents from the 2nd passage and anther 2 haemagglutinating agents from the 3rd passage were 2^0 . (Table 4).

Serological identification using Haemagglutination-inhibition (HI) test (β procedure) by using avian influenza (AI) common type A antiserum revealed that all isolates were avian influenza

virus except isolates number 5, 8, 16 and 19 were haemagglutinating viruses other than avian influenza virus (AIV) (Table 4).

Agar Gel Precipitation Test (AGPT) and Reverse Transcription Polymerase Chain Reaction (RT-PCR) Test:

For more confirmation of the HI result the samples were examined by AGPT and RT-PCR test which gaves the same results. (Table 5) and (Figs. 1, and 2).

Isolation and Identification of bacteria associated with AIV from commercial broiler chicken flocks

E.coli Isolation:

The culture of collected swabs from suspected organs of diseased birds (heart blood and liver) on MacConkey's agar and incubated over night at 37° C revealed that sixteen samples (80%) were positive for E.coli and four samples were negative (data presented in Table 6).

Identification of E.coli isolates:

The suspected colonies on MacConkey's agar media were smooth, circular, convex, bright pink and often with a reddish bile zone precipitate surrounding colonies.

Biochemical identification of E.coli isolates were positive for Indol and Methyle red (MR) test and negative for Voges proskauer (VP), citrate and Urease test (Table 7).

Salmonella Isolation:

The culture of collected swabs from suspected organs of diseased birds (heart blood and liver) on MacConkey's agar and SS agar medium incubated over night at 37° C revealed that four samples (20%) were positive for Salmonella and sixteen samples were negative (data presented in Table 6).

Identification of Salmonella isolates:

The suspected colonies were growing on MacConkey's agar media but does not ferment lactose as colonies appear pinpoint light yellow or colorless, smooth, circular.

The suspected colonies on SS agar medium were colorless, usually with or without black center.

Biochemical identification of Salmonella isolates were positive for Methyle red (MR) and citrate test and negative for Indol, Voges proskauer (VP) and Urease test (Table 7).

Serological Identification of Bacteria

Identification of E.coli by specific antisera:

By slide agglutination test technique confirm that all isolates except isolates number 2, 3, 4 and 15 were E.coli (Table 8).

Identification of Salmonella antisera:

By slide agglutination test technique confirm that isolates number 2, 3, 4 and 15 were salmonella (Table 8).

DISCUSSION

The highly pathogenic influenza viruses (H5N1) infected chickens in Egypt from February to December 2006 were characterized at

immunologic and molecular levels. ***Bahgat et al. (2009).***

The clinical signs and postmortem examination of 24 outbreaks in broiler flocks in Egyptian provinces (El-Behira, El-Garbia, Kafer El-Sheikh, and Alexandria) were similar to those AIV infection as described by ***(Swayne and Halvorson, 2003; Kobayashi et al., 1996)*.**

Avian influenza (AI) H5N1 outbreaks were accompanied with high mortality rate. There a difference in mortality rate between 24 broiler flocks (Table 1) and that may be due to different hygienic control measures among farms. This was supported by ***WHO (2006)*** which reported that biosecurity consists of a set of management practices which, when followed, collectively reduces the potential for the transmission and spread of disease causing organisms - such as the Avian Influenza virus -onto and between sites, animals and humans.

There was difference in mortality rate and mortality percent among 24 different broiler breed flocks as it was very high in non vaccinated which ranged between 8.96 % to 33.01 % and this agreement with ***Busani et al. (2009)*.** So mortality percent is very high in non vaccinated groups, followed by groups vaccinated by heterologous neuraminidase vaccines (H5N2) followed by groups vaccinated by homologous neuraminidase vaccines (H5N1). And this was similar to studies of ***Lee et al. (2007)*** which mentioned that one dose of 128 haemagglutinin (HA) homologous H5N1 vaccine induced 100% protection in mortality and prevented viral shedding completely after lethal dose virus challenge, whereas one dose of 64 HA unit of heterologous H5N3 vaccine only induced 50% protection in mortality, and it did not prevent viral shedding.

Most of the fatal avian viruses are known to have haemagglutinating activity with chicken erythrocytes ***Sarker (2006).*** As such this property is being used for the preliminary screening of the poultry farm for the presence of any such devastating viruses such as Avian influenza, Newcastle and Egg drop syndrome etc.

Haemagglutination-inhibition (HI) test by using avian influenza (AI) common type A antibodies revealed that all isolates were avian influenza virus except isolates number 5, 8, 16 and 19 were haemagglutinating viruses other than avian influenza virus (AIV) as this four isolates have the same clinical signs and postmortem lesions as avian influenza virus (AIV) which may lead to miss diagnosis with avian influenza virus (AIV) in the field (Table 4). This similar to studies of ***Shotridge et al. (1977).*** These four isolates may be Newcastle viruses.

Infected chickens can yield positive haemagglutination inhibition (HI) antibody as early as 3 to 4 days after the appearance of first disease signs, HI- test can be useful as a serological test for diagnosis of the disease and evaluation of immune response of vaccinated chickens with inactivated vaccine as recommended by ***(Allan, 1981; Beck and Swayne, 1997; OIE, 2004).*** HI result was interoperated as recommended by ***(CEC, 1992; OIE, 2005).***

Table 3 shows embryo mortalities pattern of the inoculated samples via allantoic sac (AS) [1st Passage] as each sample inoculated in 5 specific pathogen free (SPF) embryonated chicken eggs. There were no mortalities in 1st day as if death occur at 1st day the egg will be discarded as it consider non specific death.

Cloacal and faecal dropping swabs for surveillance testing of chicken sheds were also tested for presence of the HA gene by the PCR test described by ***Spackman et al . (2002).*** HA-positive allantoic fluids from the virus cultures were also tested for the H5 gene by PCR. This is presented in (Table 5) and (Figs. 1, and 2) for more confirmation of the result serological examination of serum samples by AGPT test and RT-PCR test revealed that the same results as HI test that all isolates were positive AI by AGPT and RT-PCR test except isolates number 5, 8, 16, 19 were negative

It is generally known that secondary or concurrent bacterial infections tend to intensify the clinical pictures and other effects of primary viral diseases. ***Swayne (2003)*.**

Raffaello et al. (1996) discovered that a selective-differential medium MCPB was proposed for the preliminary recognition of Gram-negative Enterobacteriaceae on the primary isolation plates.

By culturing of collected swabs from suspected organs of diseased birds (heart blood and liver) to isolate of Enterobacteriaceae by using MacConkey's agar and incubated over night at 37° C revealed that sixteen samples were positive for E.coli and four samples were positive for Salmonella (data presented in Table 6).

Isolates were examined with a series of biochemical tests. The data were analyzed with respect to the use of economical and practical procedures for the accurate identification of lactose-fermenting and-non fermenting Enterobacteriaceae within 24 hr after isolation. ***Walter et al. (1970).***

For biochemical identification E.coli isolates were positive for Indol and Methyle red (MR) test and negative for Voges proskauer (VP), citrate and Urease test while Salmonella isolates were positive for Methyle red (MR) and citrate test and negative for Indol, Voges proskauer (VP) and Urease test (Table 7). These results were similar to those results described by many authors ***(Ramirez, 1968; Zabransky et al., 1969).***

CONCLUSION

Finally, we can conclude that avian influenza is highly contagious viral infection. Outbreaks of avian influenza in poultry industry cause devastating economic losses.

Isolates number 5, 8, 16 and 19 were haemagglutinating viruses other than avian influenza virus (AIV) have the same clinical signs and postmortem lesions as avian influenza virus (AIV) which may lead to miss diagnosis with avian influenza virus (AIV) in the field. They might be Newcastle viruses.

Twenty isolates out of twenty four (20／24) which represent 83.33% of examined outbreaks having haemagglutination-inhibition titer by using avian influenza (AI) common type A antibodies ranging from 2^4 to2^8 .

A total of 16 samples out of 20 (16 ⁄ 20) (represented 80 %) examined samples from broiler flocks associated with Avian influenza virus outbreaks proved to be E.coli while a total of 4 samples(4 ⁄ 20) (represented 20 %) proved to be Salmonella enteritidis.

Special attention to enterobacteria associated with avian influenza virus (AIV) as this bacteria can increase complications like mortality rate, morbidity rate, clinical signs and postmortem lesions that lead to great economic losses in the poultry industry.

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Table (1) : History of The Examined Broiler Flocks for Bacteria Associated With Avian Influenza Outbreaks

Serial NO.	Flock NO.	Locality	Total NO./Flock	Broiler Breed	Total Age / Day	Age of Onset of Disease	Vaccination		Mortality Rat from Onset of Outbreaks					Total Mortality	*Mortality Percent %
							Yes / No	Type of Vaccine	1st Day	2nd Day	3rd Day	4th Day	5th Day		
1	1	Hosh Issa El-Behira	8000	Cubb	38	33	Yes	H5N1	13	15	28	28	20	104	1.30 %
2	2	Shobrakhet El-Behira	9600	Avian 48	36	31	Yes	H5N1	15	25	38	62	84	224	2.33 %
3	3	Shobrakhet El-Behira	10000	Cubb	34	29	Yes	H5N2	65	120	134	84	160	563	5.63 %
4	4	Shobrakhet El-Behira	3000	Cubb	38	33	Yes	H5N2	18	32	16	22	18	106	3.53 %
5	5	Kom Hamada - El-Behira	8500	Hubbard	36	31	Yes	H5N1	4	7	10	17	8	46	0.54 %
6	6	Abu El Matamer El-Behira	3500	Cubb	36	31	Yes	H5N1	6	22	18	18	20	84	2.40 %
7	7	Shobrakhet El-Behira	10000	Ross	38	33	No	—	318	620	813	750	800	3301	33.01 %
8	8	Shobrakhet El-Behira	12000	Cubb	40	35	Yes	H5N1	17	272	17	32	48	386	3.22 %
9	9	Basyoun El-Garbia	8500	Cubb	40	35	Yes	H5N1	27	28	48	32	28	163	1.92 %
10	10	Basyoun El-Garbia	10000	Cubb	38	33	Yes	H5N1	18	18	24	36	48	144	1.44 %
11	11	Bernia El-Garbia	25000	Cubb	36	31	Yes	H5N1	65	45	40	73	66	289	1.16 %
12	12	Bernia El-Garbia	8000	Cubb	34	29	Yes	H5N2	63	73	83	83	110	412	5.15 %

*** Mortality Percent % was calculated from the onset of outbreaks till the time of sampling.**

Table (1) Continuation:

Serial NO.	Flock NO.	Locality	Total NO./Flock	Broiler Breed	Total Age / Day	Age of Onset of Disease	Vaccination		Mortality Rat from Onset of Outbreaks					Total Mortality	*Mortality Percent %
							Yes / No	Type of Vaccine	1st Day	2nd Day	3rd Day	4th Day	5th Day		
13	13	Berma El-Garbia	12000	Cubb	32	27	No	—	68	148	311	248	300	1075	8.96 %
14	14	Basyoun El-Garbia	10000	Arboacre Plus	40	35	No	—	280	360	420	348	618	2026	20.26 %
15	15	Desouk Kafer El-Sheikh	7500	Cubb	38	33	Yes	H5N2	18	32	26	43	52	171	2.28 %
16	16	Desouk Kafer El-Sheikh	10000	Cubb	36	31	Yes	H5N2	31	15	35	63	68	212	2.12 %
17	17	Fouha Kafer El-Sheikh	15000	Avian 48	36	31	Yes	H5N1	22	18	32	36	36	144	0.96 %
18	18	Desouk Kafer El-Sheikh	8000	Avian 48	36	31	Yes	H5N1	48	63	52	66	48	277	3.46 %
19	19	Kom Demes Kafer El-Sheikh	3000	Ross	40	35	Yes	H5N1	7	17	17	25	38	104	3.47 %
20	20	Alexandria	10000	Cubb	38	33	Yes	H5N1	17	33	36	43	62	191	1.91 %
21	21	Alexandria	15000	Ross	38	33	Yes	H5N2	37	84	102	117	89	429	2.86 %
22	22	Alexandria	15000	Ross	38	33	Yes	H5N2	64	86	107	184	210	651	4.34 %
23	23	Alexandria	12000	Cubb	41	36	Yes	H5N2	124	218	163	155	222	882	7.35 %
24	24	Alexandria	9500	Cubb	34	29	Yes	H5N2	133	128	228	165	210	864	9.09 %

*** Mortality Percent % was calculated from the onset of outbreaks till the time of sampling.**

Table (2) : Haemagglutination Activities (HA) *[Slide Haemagglutination Test] of The Inoculated Samples Via Allantoic Sac (AS) Inoculation in Embryonated Chicken Eggs (ECEs)

Serial NO.	Flock NO.	** Inoculation Via Allantoic Sac (AS)		
		*** 1st Passage	2nd Passage	3rd Passage
1	1	+ve	**** nd	nd
2	2	+ve	nd	nd
3	3	+ve	nd	nd
4	4	+ve	nd	nd
5	5	-ve	+ve	nd
6	6	+ve	nd	nd
7	7	+ve	nd	nd
8	8	-ve	-ve	+ve
9	9	+ve	nd	nd
10	10	+ve	nd	nd
11	11	+ve	nd	nd
12	12	+ve	nd	nd
13	13	+ve	nd	nd
14	14	+ve	nd	nd
15	15	+ve	nd	nd
16	16	-ve	-ve	+ve
17	17	+ve	nd	nd
18	18	+ve	nd	nd
19	19	-ve	+ve	nd
20	20	+ve	nd	nd
21	21	+ve	nd	nd
22	22	+ve	nd	nd
23	23	+ve	nd	nd
24	24	+ve	nd	nd

* Slide Haemagglutination Test using 10 % washed chicken RBCs.

** Allantoic Sac (AS) Inoculation was done at 9 - day - old chicken embryos.

*** Detection of Haemagglutination activities by slid Haemagglutination test.

**** nd = Not Done.

Table (3) : Embryos Mortalities Pattern of The Inoculated Samples via Allantoic Sac (AS) [1st Passage]

Serial NO.	Sample NO.	*Embryos Mortalities Post-Inoculation						Total Embryos Mortalities
		1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	
1	1	0/5	1/5	2/5	2/5	—	—	5/5
2	2	0/5	0/5	3/5	2/5	—	—	5/5
3	3	0/5	3/5	1/5	0/5	1/5	—	5/5
4	4	0/5	0/5	3/5	2/5	—	—	5/5
5	5	0/5	0/5	2/5	2/5	1/5	—	5/5
6	6	0/5	0/5	4/5	1/5	—	—	5/5
7	7	0/5	1/5	1/5	2/5	1/5	—	5/5
8	8	0/5	2/5	1/5	2/5	—	—	5/5
9	9	0/5	1/5	1/5	2/5	0/5	1/5	5/5
10	10	0/5	2/5	2/5	0/5	1/5	—	5/5
11	11	0/5	0/5	3/5	0/5	2/5	—	5/5
12	12	0/5	1/5	2/5	1/5	1/5	—	5/5
13	13	0/5	0/5	3/5	2/5	—	—	5/5
14	14	0/5	2/5	3/5	—	—	—	5/5
15	15	0/5	1/5	2/5	1/5	1/5	—	5/5
16	16	0/5	1/5	1/5	1/5	2/5	—	5/5
17	17	0/5	0/5	0/5	2/5	1/5	2/5	5/5
18	18	0/5	1/5	2/5	2/5	—	—	5/5
19	19	0/5	2/5	2/5	0/5	1/5	—	5/5
20	20	0/5	1/5	2/5	2/5	—	—	5/5
21	21	0/5	2/5	3/5	—	—	—	5/5
22	22	0/5	3/5	2/5	—	—	—	5/5
23	23	0/5	3/5	2/5	—	—	—	5/5
24	24	0/5	4/5	1/5	—	—	—	5/5

* Each Sample Inoculated in 5 Embryonated Chicken Eggs (ECE).

Table (4) : Haemagglutination (HA) and Haemagglutination-Inhibition (HI) Titer By Using Avian Influenza (AI) Common Type A Antibodies

Serial NO.	Flock NO.	Log 2 HA Test	Log 2 HI Test by Using Common Type A Antibodies
1	1	2^5	2^5
2	2	2^6	2^4
3	3	2^7	2^6
4	4	2^5	2^7
5	5	2^5	2^0
6	6	2^5	2^6
7	7	2^6	2^7
8	8	2^6	2^0
9	9	2^6	2^5
10	10	2^7	2^6
11	11	2^7	2^7
12	12	2^6	2^8
13	13	2^5	2^7
14	14	2^4	2^7
15	15	2^5	2^6
16	16	2^5	2^0
17	17	2^6	2^6
18	18	2^5	2^7
19	19	2^5	2^0
20	20	2^6	2^6
21	21	2^6	2^8
22	22	2^5	2^8
23	23	2^7	2^7
24	24	2^6	2^6

Table (5) : Results of Agar Gel Precipitation Test (AGPT) and RT-PCR Test for Detection of H5N1 Avian Influenza Virus from Examined Broiler Flocks

Serial NO.	Flock NO.	* Agar Gel Precipitation Test (AGPT)	RT-PCR Test Result
1	1	+ve	+ve
2	2	+ve	+ve
3	3	+ve	+ve
4	4	+ve	+ve
5	5	-ve	-ve
6	6	+ve	+ve
7	7	+ve	+ve
8	8	-ve	-ve
9	9	+ve	+ve
10	10	+ve	+ve
11	11	+ve	+ve
12	12	+ve	+ve
13	13	+ve	+ve
14	14	+ve	+ve
15	15	+ve	+ve
16	16	-ve	-ve
17	17	+ve	+ve
18	18	+ve	+ve
19	19	-ve	-ve
20	20	+ve	+ve
21	21	+ve	+ve
22	22	+ve	+ve
23	23	+ve	+ve
24	24	+ve	+ve

* Agar Gel Precipitation Test (AGPT) Were Carried Out By Using Group A Avian Influenza Antisera.

Table (6) : Bacterial Isolates " Entero Bacteria " from Examined Broiler Flocks Associated With Avian Influenza (AI) Outbreaks on Agar Medium

Serial NO.	Flock NO.	Escherichia Coli *	Salmonella *
1	1	+ve	-ve
2	2	-ve	+ve
3	3	-ve	+ve
4	4	-ve	+ve
5	6	+ve	-ve
6	7	+ve	-ve
7	9	+ve	-ve
8	10	+ve	-ve
9	11	+ve	-ve
10	12	+ve	-ve
11	13	+ve	-ve
12	14	+ve	-ve
13	15	-ve	+ve
14	17	+ve	-ve
15	18	+ve	-ve
16	20	+ve	-ve
17	21	+ve	-ve
18	22	+ve	-ve
19	23	+ve	-ve
20	24	+ve	-ve

* Identification of Colonies Depend Upon Morphlogical Character of The Isolated Colonies.
+Ve = Positive.
-Ve = Negative.

Table (7) : Result of Biochemical Tests of Isolated Bacteria " Entero Bacteria " Associated With Avian Influenza Outbreaks

Serial NO.	Flock NO.	Simmon's Citrate Test	Indol Test	Voges Proskauer (VP) Test	Methyle Rred (MR) Test	Urease Test	Type of Microorganies
1	1	-ve	+ve	-ve	+ve	-ve	Escherichia coli
2	2	+ve	-ve	-ve	+ve	-ve	Salmonella
3	3	+ve	-ve	-ve	+ve	-ve	Salmonella
4	4	+ve	-ve	-ve	+ve	-ve	Salmonella
5	6	-ve	+ve	-ve	+ve	-ve	Escherichia coli
6	7	-ve	+ve	-ve	+ve	-ve	Escherichia coli
7	9	-ve	+ve	-ve	+ve	-ve	Escherichia coli
8	10	-ve	+ve	-ve	+ve	-ve	Escherichia coli
9	11	-ve	+ve	-ve	+ve	-ve	Escherichia coli
10	12	-ve	+ve	-ve	+ve	-ve	Escherichia coli
11	13	-ve	+ve	-ve	+ve	-ve	Escherichia coli
12	14	-ve	+ve	-ve	+ve	-ve	Escherichia coli
13	15	+ve	-ve	-ve	+ve	-ve	Salmonella
14	17	-ve	+ve	-ve	+ve	-ve	Escherichia coli
15	18	-ve	+ve	-ve	+ve	-ve	Escherichia coli
16	20	-ve	+ve	-ve	+ve	-ve	Escherichia coli
17	21	-ve	+ve	-ve	+ve	-ve	Escherichia coli
18	22	-ve	+ve	-ve	+ve	-ve	Escherichia coli
19	23	-ve	+ve	-ve	+ve	-ve	Escherichia coli
20	24	-ve	+ve	-ve	+ve	-ve	Escherichia coli

Table (8) : Serological Identification of Isolated Bacteria Associated With Avian Influenza Outbreaks

Serial NO.	Flock NO.	* Slide Agglutination Test	
		Escherichia Coli	Salmonella
1	1	+ve	nd**
2	2	nd	+ve
3	3	nd	+ve
4	4	nd	+ve
5	6	+ve	nd
6	7	+ve	nd
7	9	+ve	nd
8	10	+ve	nd
9	11	+ve	nd
10	12	+ve	nd
11	13	+ve	nd
12	14	+ve	nd
13	15	nd	+ve
14	17	+ve	nd
15	18	+ve	nd
16	20	+ve	nd
17	21	+ve	nd
18	22	+ve	nd
19	23	+ve	nd
20	24	+ve	nd

* Using Polyvalent Antiserum.

** nd = Not Done.

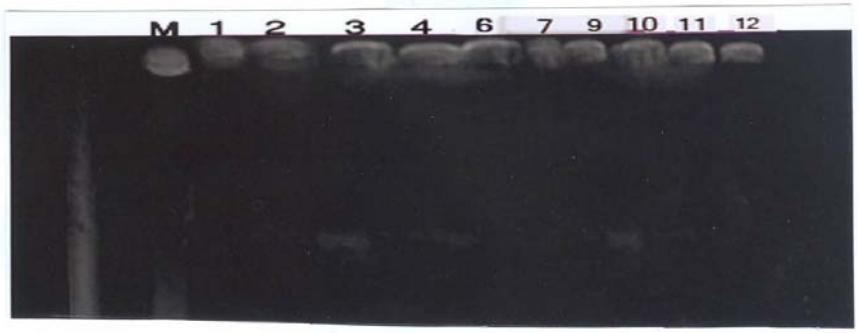

Fig. 1: Screening by specific RT-PCR product on 0.8% agarose gel of the 500 bP, M-marker, 1-12 inoculated samples.

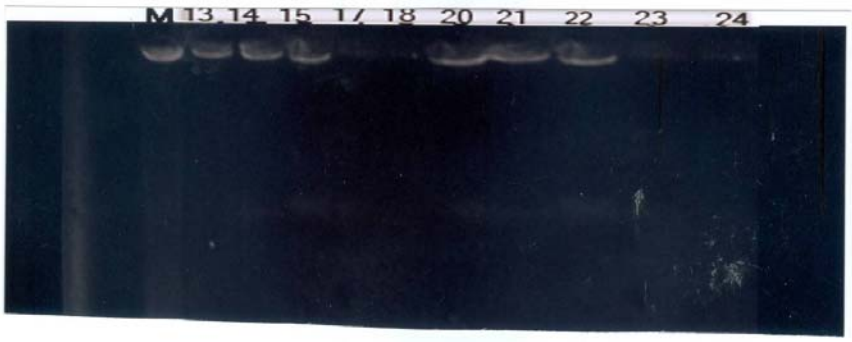

Fig. 2: Screening by specific RT-PCR product on 0.8% agarose gel of the 500 bP, M-marker, 13-24 inoculated samples.

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