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EFFECT OF FORMALIN AND -PROPIOLACTONE ON PATHOGENICITY AND ANTIGENIC PROPERTIES OF H5N1 AVIAN INFLUENZA VIRUS

BY

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ABSTARCT

Twenty two isolates of influenza virus isolated from various 22 poultry farms at different districts in Dakahlia and Damietta provinces. Three isolates of influenza virus from two different sub-clades were inactivated by - Propiolactone (BPL) and formalin (F). Inactivated isolates were used for preparation of hyperimmune serum in three week old chickens. Cross hemagglutination inhibition (HI) test was performed for prepared serum against the homologous and heterologous antigens to determine the specificity of the antibodies produced. Obtained results showed that the highest cross reactivity was between the serum and its corresponding virus and the serum produced against BPL inactivated viruses give higher cross reactivity with the three viruses than that of formalin inactivated viruses.

INTRODUCTION

Avian influenza has become a disease of great importance for animal and human health. Influenza viruses are segmented, negative-strand RNA viruses that are belonged to family Orthomyxoviridae which divided into 3 genera: Influenza A, B,C. Influenza A viruses are the only type reported to cause natural infections of birds and are further divided into subtypes according to antigenic characteristics of surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) **(Ilaria Capua and Stefano Marongon, 2006)**, HA has 18 subtypes (H1–H18) and NA has 11 subtypes (N1-N11) **(Suxiang Tong et al., 2013).**

All birds are susceptible to the infection with AI. Some species are more susceptible to infection than others. AI viruses are classified as being highly pathogenic AI viruses (HPAIVs) or low pathogenic AI viruses (LPAIVs) **(Sohail et.al, 2008).**

The primary characteristic of the HPAI virus in chickens and turkeys is rapid, high mortality, which can reach 100% within 36-48 hours post infection. Virulence can vary by virus strain and is affected by numerous host factors, but it is common for chickens and turkeys to die soon enough post infection that gross lesions are absent and clinical signs are observable for only a very short period before death. Clinical signs consist of severe depression and/or neurological signs **(Swayne, E. and Suarez, D. L.2000),** while LPAIV is caused by viruses other than the HPAIVs. Although LPAIVs can cause secondary infections with other pathogens and lead to economic losses, such as losses attributed to an egg-drop syndrome in chickens, LPAIVs do not usually produce obvious clinical signs in infected birds. However, LPAIVs of H5 and H7 subtypes have the potential to evolve into HPAIVs **(M.Horie et al., 2009).**

In Egypt, The first HPAI outbreak was announced on 17th February 2006 sever outbreaks of HPAI (H5N1) have emerged in several governorates and were associated with drastic mortality up to 100% in infected chickens **(Aly et al., 2007).** The disease is now endemic and outbreaks are detected from north to south of the country **(Samaha, 2007).**

The aim of the present work is:

1- Genetic characterization of isolated strains of AIV by polymerase chain reaction (PCR) and sequencing.

2- Preparation of hyper-immune serum against isolated strains of avian influenza virus and cross matching between them.

MATERIALS AND METHODS

A total of freshly dead or euthanized chickens were collected from various 22 poultry farms at different districts in Dakahlia and Damietta provinces from clinically diseased birds showed high mortality and respiratory signs, suspected to be infected with AIV. Samples include (liver, spleen, larynx and trachea samples) were collected from poultry of different breeds (12chicken broilers, 9 chicken breeders and 1 chicken layers). Samples were prepared **According to OIE 2012,** collected AAF were tested by

slide and plate hemagglutination test, rapid antigen chromatographic detection kits and submitted friendly to National Reference Laboratory for Newcastle Disease and Avian Influenza Istituto Zooprofilattico Sperimentale delle Venezie Legnaro, Padua, Italy for RT-PCR and Phylogenetic analysis. Three isolates from two different sub-clades including: V9= (A/chicken/Egypt/3982-43/2010) in sub- clade B1, V10= (A/chicken/Egypt/3982-44/2010) and V112= (A/poultry/ Egypt /3982-55/2010) in sub-clade A1 were inactivated by BPL and formalin according to **Lee et.al, 2006 and Sohail et.al, 2008** in brief, The virus-AAF material (the antigen) divided into two groups (group B & group F) for each isolates. Group B inactivated with BPL with a final BPL concentration of 0.1% in the AAF for 2 hours at 37 C and group F inactivated with formalin with a final formalin concentration of 0.1% in the AAF for 16 hours at 37^{O}C.

Inactivation of Isolates tested by inoculating five ECEs with 0.2 mL of the BPL and formalin treated AAF preparation. Then were tested for virus growth with HA assay. Antiserum were prepared in 3 week old chickens using 6 groups of 5 birds for each inactivated isolate (groups B9,B10 and B112 for isolates 9,10 and 112 which inactivated by BPL respectively and groups F9,F10 and F112 for isolates 9,10 and 112 which inactivated by formalin respectively) with a negative control group. They were kept strictly isolated in units. Chickens were inoculated S/C with 0.25 ml of inactivated isolates three times with two weeks interval. Sera were collected two weeks after the last booster inoculation. **(Madeley et.al, 1971).**

Prepared serum was tested for presence of AI virus antibodies by HI test against Isolates 9,10 and 112. According to **Lee et. al, (2006),** atypical procedure (diluted serum constant virus) of HI test was performed in 96 well microtiter plate using prepared hyperimmune serum.

RESULTS

Obtained results of sequencing of H5 gene showed that the six isolates belonged to the 2.2.1 clade according to WHO/FAO/OIE nomenclature. Also sequence analysis of HA gene segment of six isolates revealed that two main distinct subclades named A and B fitting the specific clade definition criteria described by WHO/OIE/FAO H5N1 Evolution Working Group have co- circulated in domestic poultry since late 2007 **(Fig. 1).**

The isolates 20= (A/ Chicken/ Egypt/3982/52/2010), 9=(A/ Chicken/ Egypt/3982/43/2010) and 18= (A/ Chicken/ Egypt/3982/50/2010) were belonging to subclade B1 and the isolates112=(A/ Poultry/ Egypt/3982/55/2010), 111= (A/ Poultry/ Egypt/3982/56/2010) and 10= (A/ Chicken/ Egypt/3982/44/2010) were belonging to subclade A1. The isolate (A/ Chicken/ Egypt/3982/50/2010) has about 80% similarity percent with the other two isolates in subclade B1 ((A/ Chicken/ Egypt/3982/52/2010) and (A/ Chicken/ Egypt/3982/43/2010)). The isolates (A/ Chicken/ Egypt/3982/52/2010) and (A/ Chicken/ Egypt/3982/43/2010) have about 99% similarity percent. The isolate (A/ Chicken/ Egypt/3982/44/2010) has about 79% similarity percent with the other two isolates in subclade A1; (A/ Poultry/ Egypt/3982/55/2010) and (A/ Poultry/ Egypt/3982/56/2010). The isolates (A/ Poultry/ Egypt/3982/55/2010) and (A/ Poultry/ Egypt/3982/56/2010) have about 100% similarity percent

Obtained cross HI test results showed that the highest cross reactivity was between the serum and its corresponding virus, for example: F9 & B9 give the

highest antibody titer with V9. (Table 1).Serum produced against BPL inactivated viruses (B9, B10 and B112) give higher cross reactivity with three viruses (V9, V10 and V112) than that produced against formalin inactivated viruses (F9, F10 and F112).

Table 1. Results of Cross-HI test:

Antigen	Antiserum titer					
	B9	B10	B112	F9	F10	F112
9= (A/chicken/Egypt/3982-43/2010)	512	64	32	256	64	32
10=(A/chicken/Egypt/3982-44/2010)	256	1024	128	256	512	64
112=(A/poultry/Egypt/3982-55/2010)	256	64	512	32	64	256

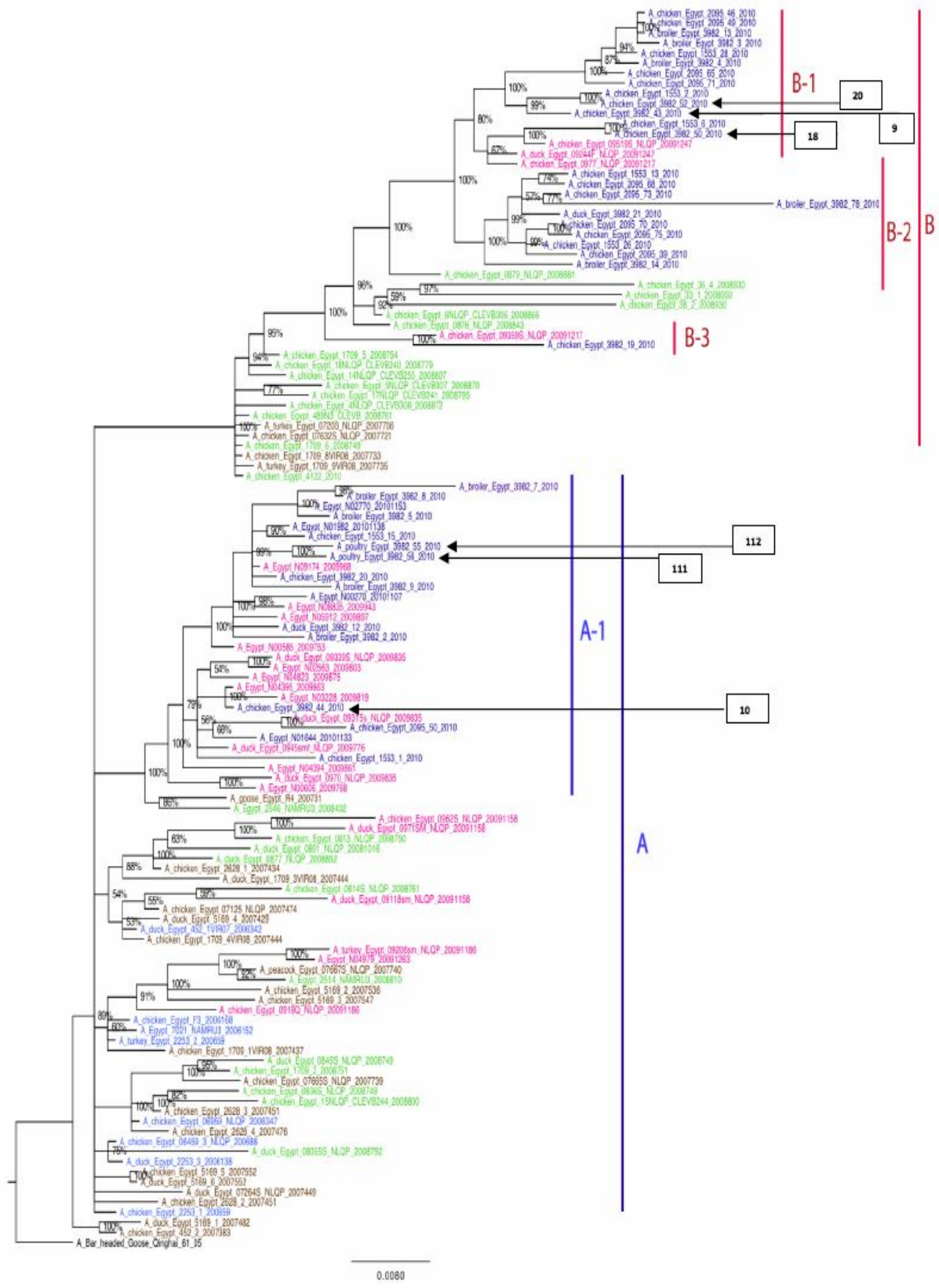

Fig. 1. Phylogenetic analysis of HA gene forAIV

DISCUSSION

Trials for avian influenza virus isolation from chickens were carried out on specimens that were collected from various 22 poultry farms at different districts in Dakahlia and Damietta governorates from clinically diseased birds suspected to be infected with AIV. Diseased chickens showed classical signs of AI including high mortalities, respiratory signs, recumbence, edema and cyanosis of comb and wattles, hemorrhagic lesion on shank, swelling of the face,watery greenish diarrhea and sever drop in egg production in layer and breeder birds (50%- 70%). These finding were in agreement with that described by **(Hinshaw et al., 1983, Mohan et al., 1981)** who mentioned that the most common symptoms of AI are mild to severe respiratory disease. For layer flocks or breeder flocks, drops in egg production can also be observed. The drops in egg production can be severe with the flocks never returning to full production. Also our results were in similar to that obtained by **Elbers, et al. 2004** who reported that chickens infected with AIV have ruffled feathers and swelling of the head, face, upper neck, leg shanks, and feet from subcutaneous edema and may have accompanying petechial-to-ecchymotic subcutaneous hemorrhages, especially of the non-feathered skin. Some viruses produce cyanosis of the wattles, combs, and snood.

Out of 22 isolates, 6 isolates (9, 10, 18, 20, 111 and 112) were subjected to sequencing and phylogenetic analysis.

Asian lineage HPAI (H5N1) is divided into two antigenic clades. Clade 1 includes human and bird isolates from Vietnam, Thailand, and Cambodia and bird isolates from Laos and Malaysia. Clade 2 viruses were first identified in bird isolates from China, Indonisia, Japan, and South Korea before spreading westward to the Middle East, Europe, and Africa. The Clade 2 viruses have been primarily responsible for human H5N1 infections that have occurred during late 2005 and 2006, according to **WHO**.

In this study, Sequencing and phylogenetic tree of six AI viruses isolated during 2010 were done and the Phylogenetic tree showed that all HA gene sequences belonged to the 2.2.1 clade according to WHO/FAO/OIE nomenclature. These results mean that there are great genetic differences and variations between the isolates of 2010 which

presented in different emergent clade (clade 2.2.1) rather than clade 2.2. This agreed with **Arafa et al. (2010)** who concluded that H5N1 is continuing to mutate with multiple heterogenic strains persisting in Egypt. The data from their study identifies distinct genetic markers in both HA and NA genes and suggests grouping Egyptian isolates into two major HA isolate sublineages from 2006 to 2008 and into three smaller, emergent subgroups. The different subgroups did not appear to segregate by relation to the date of isolation, to the species of origin, nor to the geographic location of the viruses.

Also sequence analysis of HA gene segment of six isolates revealed that two main distinct subclades named A and B fitting the specific clade definition criteria described by WHO/OIE/FAO H5N1 Evolution Working Group **(WHO/OIE/FAO; 2008)** have co-circulated in domestic poultry since late 2007. These results agreed with **Cattoli et al., 2011** who mentioned that; although viruses belonging to both subclades were collected from the same country and during the same time period, they exhibited different selection profiles and rates of nucleotide substitution, suggesting that they are subject to differing evolutionary pressures. This could reflect the circulation of viruses belonging to each of these subclades in distinct host environments such as separate poultry sectors (e.g. backyard versus commercial flocks) .

Obtained cross HI results showed that the highest cross reactivity was between the serum and its corresponding virus, for example: F9 & B9 give the highest antibody titer with V9.

Serum produced against BPL inactivated viruses (B9, B10 and B112) give higher cross reactivity with three viruses (V9, V19 and V112) than that produced against formalin inactivated viruses (F9, F10 and F112).

Our results were similar to that obtained by **Polly and Guerin, 1957** who mentioned that BPL treatment did not affect HA or antigenicity of viruses. On other hand **Jonges et al, 2010** mentioned that; BPL inactivation reduced the hemagglutination titer and NA activity of the human influenza virus 10-fold or more, BPL treatment resulted in complete inactivation of influenza virus, it negatively affected both HA and NA functions of the tested virus. They also mentioned that formalin treatment appeared to

be superior to the other treatments for antigenic characterization and measurement of B- and T-cell responses. Also **Goldstein and Tauraso, 1970** find that BPL adversely affected the HA and antigenic properties of influenza virus. Incontrast, the other methods of inactivation (i.e.,Formalin, Merthiolate, and UV light) resulted in an increase in HA activity and no apparent change in antigenicity. The more severe action of BPL was also reflected in its ability to reduce infectivity quickly and markedly.

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الملخص العربي

تأثير الفورمالين والبيتابروبيولاكتون على العدوى والخواص الأنتيجينية لفيروس انفلونزا الطيور إتش ٥ إن ١

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* قسم أمراض الدواجن والارانب كلية الطب البيطري جامعة المنصورة .

تمت هذه الدراسة علي ٢٢ مزرعة دواجن يشتبه اصابتها بمرض انفلونزا الطيور وتم تجميع عينات من الكبد والحنجرة و القصبة الهوائية والطحال .تم تجميع العينات من مناطق وسلالات مختلفة من محافظتى الدقهلية ودمياط. وأخذت هذه العينات من طيور تعانى من أعراض أكلينيكية مختلفة لمرض انفلونزا الطيور فى شكل إرتفاع شديد فى معدلات النفوق (تصل إلى ١٠٠ ٪) – إنخفاض حاد فى إنتاج البيض فى قطعان أمهات التسمين وقطعان البياض - أعراض تنفسية - زرقان وتورم فى العرف والدلايات- أنزفة على الأرجل –إسهالات مائية خضراء .

تم عزل الفيروس فى بيض مخصب خالى من المسببات المرضية عمر ٩-١١ يوم و تم التعرف على الفيروس باستخدام الإختبارات التشخيصية المختلفة بما فيها اختبار التلازن الدموى وإختبار الكروموتوجرافى السريع للإنفلونزا . و تم إرسال العينات للمعمل المرجعي لامراض النيوكاسيل وانفلونزا الطيور بايطاليا لعمل إختبار تفاعل انزيم البلمرة المتسلسل العكسي لتأكيد وجود الفيروس وتم تحليل التتابع الجيني وشجرة الجين المسئول عن التلازن الدموى لست عينات.

تم إختيار ٣ معزولات ثم تمت معاملتها بكلا من الفورمالين وبيتابروبيولاكتون ثم حقنت هذة المعزولات في دجاج عمر ثلاثة اسابيع لتحضير اجسام مضادة للفيروس عن طريق حقن الدجاج ثلاث مرات بفارق اسبوعن وتم اختبار السيرم باستخدام اختبار مانع التلازن الدموي مع نفس المعزولة والمعزولات الاخري وباستخدام اختبار مانع التلازن الدموي لوحظ ان اعلي معدل للسيرم كانت مع الفيروس المقابل له (نفس المعزولة) وقد لوحظ ايضا ان السيرم المحضر ضد المعزولات التي تمت معاملتها بالبيتابروبيولاكتون اعطت نتائج اعلي من الثلاث معزولات المستخدمة من السيرم المحضر ضد المعزولات التي تمت معاملتها بالفورمالين.