Molecular Biological and Biochemical Studies on Avian Influenza Virus Receptors in Different Avian Species

Hussein I. El-Belbasi*; Mohamed F. Dowidar and Safaa I. Khater

Department of Biochemistry, Faculty of Veterinary Medicine, Zagazig University,Egypt
*h_elbelbasi2003@yahoo.com

Abstract: Avian influenza viruses are considered to be the key contributors to the emergence of human influenza pandemics. A major determinant of infection is the presence of virus receptors on susceptible cells to which the viral haemagglutinin is able to bind. Avian viruses preferentially bind to sialic acid α 2,3-galactose (SA α 2,3-Gal) linked receptors, whereas human strains bind to sialic acid α 2,6-galactose (SA α 2,6-Gal) linked receptors. Although ducks are the major reservoir for influenza viruses, they are typically resistant to the effects of viral infection, in contrast to the frequently severe disease observed in chickens In order to understand whether differences in receptors might contribute to this observation, we studied the expression of influenza receptors in upper and lower respiratory organs of ducks and chickens (expression of ST3Gal-III sialyltransferase and ST6Gal-I sialyltransferase genes) using semi quantitative RT-PCR. There was a marked difference in the expression of primary receptor type in the trachea of chickens and ducks. In chicken trachea, SA α 2,6-Gal was the dominant receptor type whereas in ducks SA α 2,3-Gal receptors were most abundant. This suggests that chickens could be more important as an intermediate host for the generation of influenza viruses with increased ability to bind to SA α 2,6-Gal receptors and thus greater potential for infection of humans. Chicken tracheal and intestinal epithelial cells also expressed a broader range of SA α 2,3-Gal receptors in contrast to ducks, which suggests that they may be able to support infection with a broader range of avian influenza viruses.

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1. Introduction

The influenza viruses are medium-sized, comprising enveloped and negative sense RNA viruses with a segmented genome. Taxonomically, they belong to the virus family Orthomyxoviridae. There are three genetically and antigenically distinct types of influenza viruses called A, B, and C. Type A viruses are further divided into subtypes according to the combination of two main envelope glycoproteins the hemagglutinin (HA)and neuraminidase (NA). To date, 16 HA subtype (H1–H16) and 9 NA subtypes (N1–N9) have been found (Ghaleb, 2009). Influenza A virus infects several hosts, including humans, birds, swine, and horses, but individual viruses are usually adapted to sustained infection in only one species. Viruses isolated from these different species bind sialic acid through their surface glycoprotein, hemagglutinin, and require this interaction for productive infection (Gambaryan et al., 2005). The first step in the virus infection process is the recognition of cellular structures that act as specific receptors. This determines the virus tissue tropism and is performed by viral adhesion proteins (Tardieu et al., 1982) .The viral attachment to the host cell is critical for tissue and species specificity of virus infections (Debby et al., 2007). Influenza virus initiates infection by binding of the viral hemagglutinin (HA) to sialic acid on the cell surface. (Stray and Air, 2001). The receptors for influenza viruses are sialic acids (SAs), which are usually formed 2,3 or 2,6 configuration linked to the cell-surface glycoproteins and glycolipids (Harduin-Lepers et al., 2005). Sialic acid is an essential component of cell surface receptors for a variety of microorganisms and microbial toxins (Mouricout, 1997). Sialic acid is added to the terminal sugar of glycoproteins and glycolipids by enzymes called sialyl transferases (Harduin-lepers et al., 2005).

Sialyl transferases (SiaTs) are required to synthesize all known sialyloligosaccharides (Shuichi, 1995). The ST3Gal III, preferentially transfers sialic acid in α 2,3 linkage to the Galß1-3GlcNAc disaccharidic sequence(Catherine et al.,1999). While ST6Gal-I generates an α 2-6 linkage of sialic acid to underlying N-acetyllactosamine (Weinstein et al.,1982). The differential expression of sialic acids in the mammalian respiratory tract may help to explain the low infectivity but high pathogenicity of some avian strains (Gambotto et al., 2008).

Influenza infection is initiated by virus attachment to sialic acid-containing cell-surface molecules traditionally called viral receptors. The spectrum of sialylglycoconjugates varies substantially between viral host species as well as target tissues and cell types of the same species leading to variations in the receptor-binding specificity of viruses circulating in these hosts. It is believed that a poor fit of avian viruses to receptors in humans limits the emergence of new pandemic strains (Matrosovich et al., 2008). Influenza A viruses attach to host cells by binding of the hemagglutinin (HA) protein to sialosaccharides on the host cell surface. The HAs of influenza A viruses from different host species differ in their specificity of binding. For example, HAs of human influenza A viruses preferentially recognize sialic acid (SA) α 2,6-Gal-terminated saccharides (α 2,6-SA), whereas HAs of avian influenza viruses preferentially recognize SA α 2,3-Gal-terminated saccharides (α 2,3-SA) (Connor et al.,1994). These differences generally correspond with the variation in the type of SAs expressed at important sites for influenza A virus replication in the respective host species. For example, human tracheal epithelium expresses mainly α 2,6-SA, whereas duck intestinal epithelium expresses mainly α 2,3-SA. Therefore, the type and distribution of SA is considered to be an important factor in the susceptibility of different host species to influenza A viruses (Suzuki et al., 2000). The SA recognized by influenza A virus is not only important in the host species range but also in its transmissibility (Tumpey et al., 2007). The HA protein mediates virus binding to sialic acid (SA)-containing host cell surface molecules and promotes the release of viral ribonucleoprotein complexes through membrane fusion.

Influenza virus infectivity is influenced by 2 entities:-

1- SA species (N-acetylneuraminic acid [NeuAc] and N- glycolylneuramic acid [NeuG].

2- The type of linkage to galactose (sialyloligosaccharides terminated by SA linked to galactose by an (alpha 2,6 linkage [Ac alpha 2,6Gal] or an alpha 2,3 linkage [Ac alpha 2,3Gal]) on the host cell surface (Rogers et al.,1985).

The host range selection of the receptor binding specificity of the influenza virus hemagglutinin occurs during maintenance of the virus in different host cells that express different receptor sialo-sugar chains (Yasuo, 2005). Ducks and chickens are important hosts of avian influenza virus (AIV) with distinctive responses to infection. Frequently, AIV infections in ducks are asymptomatic and long-lasting in contrast to the clinically apparent and transient infections observed in chickens. These differences may be due to the host response to AIV infection (Sean et al., 2009).

2. Materials and methods.

Bird selection and grouping:

Four groups of healthy, four weeks aged birds are classified as fellow:

1. group 1 : 5 chicken (Baladi).
2. group 2 : 5 chicken(Hubber).
3. group 3 : 5 duck(Baladi).
4. group 4 : 5 duck(Pekeni).

Tissue preparation:

- Birds were sacrified using highly sterilized scissors (180°Cfor 6 hours) to avoid RNA degradation by RNases and latex gloves weared to minimize RNase contamination.
- After excision of trachea and lung of tested birds, they were wrapped in aluminium foil and put immediately in liquid nitrogen container to make snap-freezing of tissue and minimize action of endogenous RNase.
- Samples were taken to detect the level of gene expression of ST3Gal-III(Galβ1-3(4)GlcNAcα2,3-sialyltransferase) and ST6Gal-I (Galβ1-4GlcNAcα2,6-sialyltransferase) in that organs.

Reverse transcriptase polymerase chain reaction(RT–PCR):

Using a semi-quantitative RT–PCR according to **(Mallet et al., 1995).**

A-Protocol of RNA extraction from tissue: total RNA was extracted with RNeasy Mini Kit (QIAGEN).

B-Protocol of reverse transcription polymerase chain reaction: (one step RT-PCR) by using Robus T 1 RT-PCR kit(FINNZYMES)

The protocol was as fellow:-

All components, reaction mixes and samples were kept on ice. And the following reaction component were added to a nuclease free tube placed on ice.

Table (1): Reaction set up:

RT-PCR mix component	volume
10x Robus T reaction buffer	5 µl
50 mM MgCl2	1.5 µl
dNTP mix(10mM each)	1 µl
Template RNA	5 µl
Down stream primer	10 pmol
Up stream primer	10pmol
AMV RT 5 U/ µl	1 µl
DyNAzyme EXT DNA polymerase 1U/ µl	2 µl
RNase free water	Add to 50 µl

Gently mix the components, cycling conditions have to be optimized for each amplicon. and was transferred to the thermal cycler.(2720 thermal cycler Applied Biosystems).

Cycling instructions:

1-For ST3Gal lll gene: the primer for ST3Gal lll was synthesized to amplify PCR products that cross introns to avoid confusion between mRNA transcript and genomic DNA.

The primers used to amplify this gene are:

Forward: 5- CGGATGGCTTCTGGAAATCTGT- 3

Reverse: 3- AGTTTCTCAGGACCTGCGTGTT-5 the product size was 300 bp.

Table (2)

Cycle step	Temp	time	Number of cycle
cDNA synthesis	48 °C	30 min	1
Inactivation of AMV reverse transcriptase and denaturation of the cDNA-RNA hybrid	94 °C	2 min	1
PCR amplification Denaturation Annealing extention	 94 °C 63 °C 72 °C	 30 sec 1 min 1.5 min	36 cycles
Final extention	72 °C	7 min	

2-For ST6 Gal I gene::- the primer for ST6 Gal I were synthesized to amplify PCR products that cross introns to avoid confusion between mRNA transcript and genomic DNA.

The primers used to amplify this gene are:

Forward: 5-TGGGCCTTGGCAGGTGTGCTGTTG-3

Reverse: 3-AGGCGAATGGTAGTTTTTGAGCCCACATC-5 the product size was 150 bp.

Table (3)

Cycle step	Temp	time	Number of cycle
cDNA synthesis	48 °C	30 min	1
Inactivation of AMV reverse transcriptase and denaturation of the cDNA-RNA hybrid	94 °C	2 min	1
PCR amplification Denaturation Annealing extention	 94 °C 50 °C 72 °C	 45sec 1 min 1 min	 35 cycles
Final extention	72 °C	7 min	

Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) were amplified parallely as internal control (481bp) and its sequence:

Forward: 5-ACTTGTGATCAATGGGCACGCCATC - 3

Reverse: 3-CTTCCCATTCAGCACAGGGATGAC-5

For the Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) amplified by 35 cycle using (2720 thermal cycler Applied Biosystems). Each cycle consist of :

Denaturation 94°C for 45 second

Annealing 62 °C for 30 second

Extention 72°C for 45 second

The PCR products were separated by agarose gel electrophoresis.

C-Agarose gel electrophoresis (Sambrook and Maniatis., 1989)

1- Run parameters :
   - Use 1-5 volts/ cm of the tank lenth.
   - Allow bromophenol blue to run 2/3 of the gel lenth before terminating the run.
2- stop the run and transfere the gel to a transilluminator, observe and photograph. Photographing using polarized camera and parameters are preferably 302 nm wave lenghth, 2500 uW / cm2, or more, and using 22 A filter.

10- Using 100 bp- DNA ladder for electrophoresis of PCR product of GAPDH,ST3Gal-III and ST6Gal-I (100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000bp) from Quiagen.

11-Analysis of PCR product using GEL pro-software to detect quantitation of bands for GAPDH, ST3Gal-III and ST6Gal-I genes.

3. Results and Discussion:

The host receptor distribution pattern in the chicken and duck upper and lower respiratory tract may be functionally significant for the evolution of viruses with a human like receptor specificity and thus for the transmission of influenza from birds and

mammals. In this work, we conducted an extensive examination on the level of expression of influenza virus Receptors in trachea and lung of two different breeds of each chickens and ducks. Their was no difference in the reported results observed due to the breed of animals, and the receptor expression was consistent between individual animals within each species. Using RT-PCR in the gene expression of ST3GAL III and ST6GALI which add sialic acids to the terminal sugar of glycoproteins and glycolipids, we found that the trachea of ducks (Baladi, Pekeni) show high expression level of ST3GAL III) while trachea of chickens(Baladi, Hubber) show law expression level. (Figure:1) but Chickens trachea (Baladi,Hubber) show very high expression level of ST6GAL I in comparson to that of ducks trachea(Baladi, Pekeni) that show lawer expression level of ST6GAL I. (Figure:3). These results were in agreement with (Suresh et al.,2009) who reported that The major species difference that they observed between chickens and ducks in the relative distribution of SA α 2-3 Gal and SA α 2-6 Gal receptors was along the tracheal epithelium. In chicken tracheal epithelium, SA α 2-6 Gal was the dominant receptor type, whereas in ducks the SA α 2-3Gal receptor was more abundant in the ciliated cells of the tracheal epithelium, it was found that the ratio of SA α 2-6 Gal to SA α 2-3 Gal in chickens trachea was approximately 10:1 whereas in duck the ratio was 1:20. The tracheal mucous glands of both chickens and ducks predominantly expressed SA α 2-6 Gal receptor type. The observed difference in dominant receptor type between chickens and ducks was confined to the upper airway (trachea). While the dominant SA α 2-6 Gal receptor expression pattern in chickens trachea was in contrast to a previous study (Wan and Perez, 2006) which, using lectin binding, found that 85% of the epithelial cells in chicken trachea were positive for SA α 2-3 Gal receptors, while only 10% were positive for SA α 2-6 Gal receptors.

Also we found that the lung of ducks (Baladi, Pekeni) showed high expression level of ST3GAL III .while lung of chickens(Baladi, Hubber) showed low expression level. but the difference between expression level of ducks trachea and lung tissues is high in case of trachea more than the lung tissue, but the expression level of chicken trachea is lower than that of lung tissue. (Figure:2). And Chickens lung (Baladi,Hubber) showed very high expression level of ST6GAL I in comparson to that of duck lung (Baladi, Pekeni) that show lower expression level of ST6GAL .

But the difference between expression level of ducks trachea and lung tissues is high in case of lung more than the trachea tissue, while the expression level of chickens lung is lower than that of trachea tissue. (Figure:4). The present results were also in agreement with the findings of Gambaryan et al (2002), who reported that human influenza viruses with SA α 2,6-Gal specificity bound to cell membranes isolated from chickens (but not ducks) tracheal cell membranes. Chicken alveolar cells expressed both receptor types. The difference in the predominant receptor across the tracheal epithelial lining in chickens and ducks could be an important contributing factor to influenza virus entry via the upper respiratory tract. In particular, such differences could impact on the susceptibility of each species to avian H5N1 influenza with its preferential tropism for infection of the respiratory tract rather than the intestines. The differences in receptor expression reported in the present study suggest that they may be responsible, at least in part, for some of the differences between ducks and chickens in the pattern of disease following influenza infection. While the presence of a virus receptor is clearly not sufficient to confirm that cells or tissue support efficient virus replication or transmission, the widespread replication of influenza virus in multiple organs has been reported in both chickens (Swayne, 1997) and ducks (Londt et al., 2008) following infection with highly pathogenic viruses.

This study suggests that some chickens and ducks tissues may facilitate entry of both human and avian viruses, with the ensuing danger of virus reassortment. However, further work is required to confirm that the tissues expressing both receptor types are able to support virus replication. The dominant presence of SA α 2-6 Gal receptor along the chicken tracheal epithelium shows some similarities to the prevalence of the receptor in mammals such as human and pig. This suggests that chickens may be important intermediate hosts for the transmission of influenza to humans, in particular for influenza viruses such as H5N1, which show a respiratory tropism in birds. Whilst much attention has been placed on the role of pigs as “mixing vessels”, the potential importance of chickens for the evolution of humanised influenza viruses should not be overlooked and, as such, wants further studies. Previous studies on role of sialic acid linkage (SA α 2,3 or SA α 2,6) during influenza virus infection have shown the importance of expression of these glycans in restricting infection by viruses in different hosts.

In this work, we found the presence of both S.A α 2-3 Gal receptor and SA α 2-6 Gal receptor in chikens trachea and lung due to the expression of the two genes suggest that they may be susceptible to infection with wider range of avian influenza viruses with broader receptor specificity.

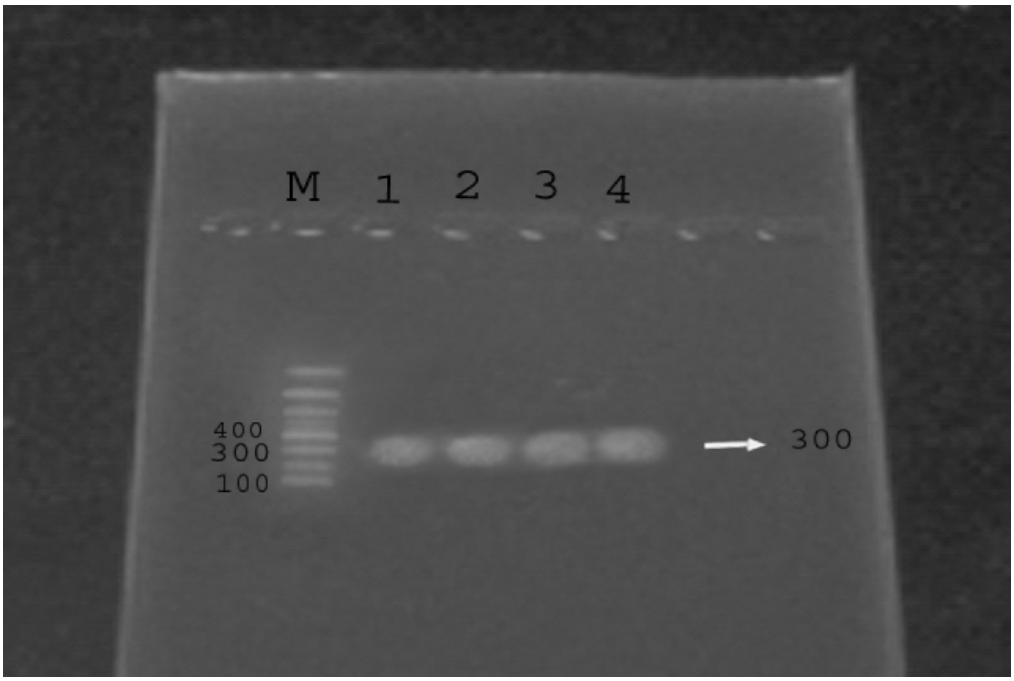


Figure (1): The elctrophoretic photograph showing the pattern of ST3GALIII mRNA expression in trachea of different birds

M: DNA ladder Lane 1: chicken Baladi trache Lane 2: chicken Hubber trachea
Lane 3: Duck Baladi trachea Lane 4: Duck Pekeni trachea

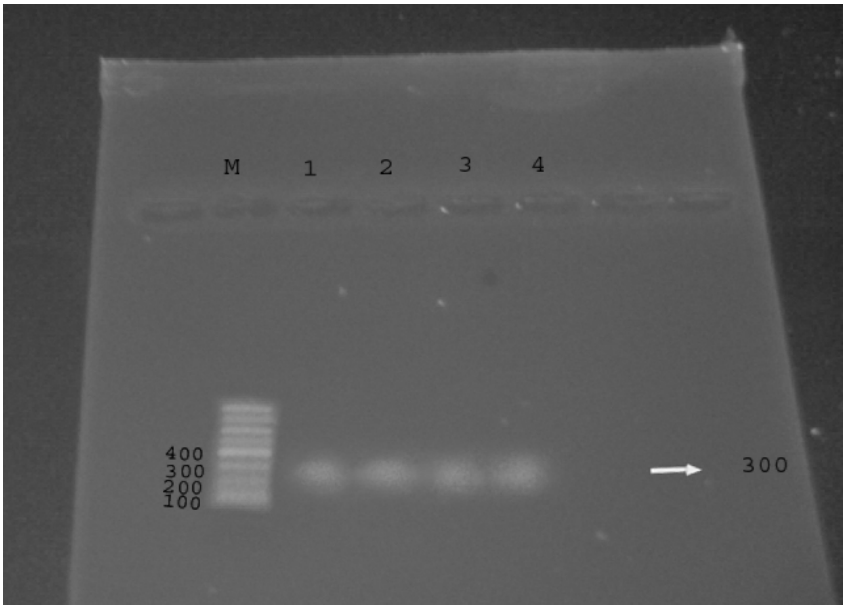


Figure (2): The elctrophoretic photograph showing the pattern of ST3GAL III mRNA expression in lung of different birds

M: DNA ladder Lane 1: chicken Baladi trachea Lane 2: chicken Hubber trachea
Lane 3: Duck Baladi trachea Lane 4: Duck Pekeni trachea

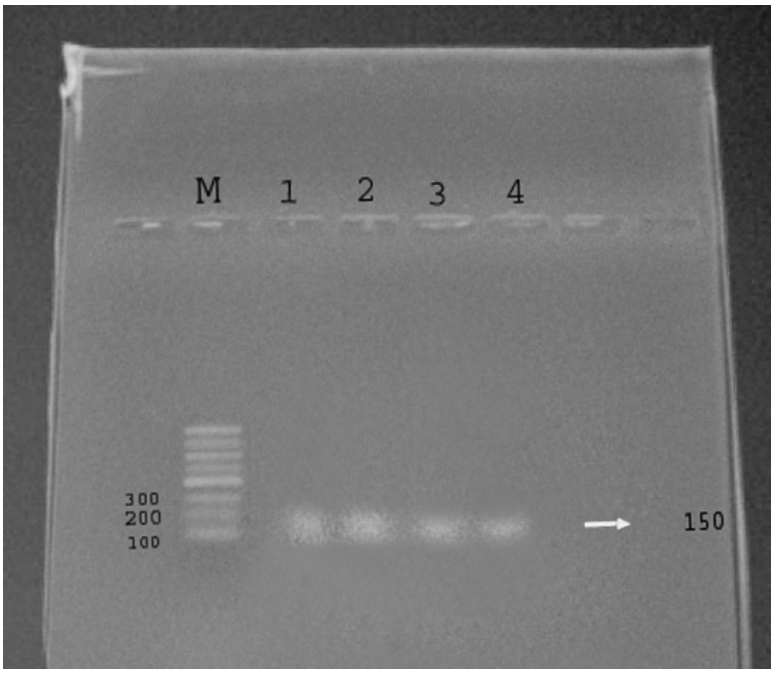
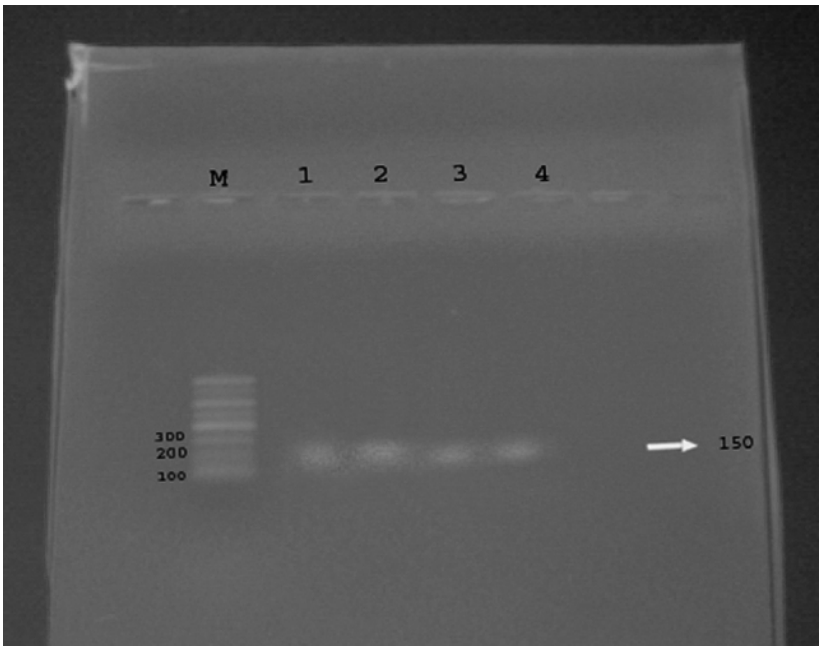


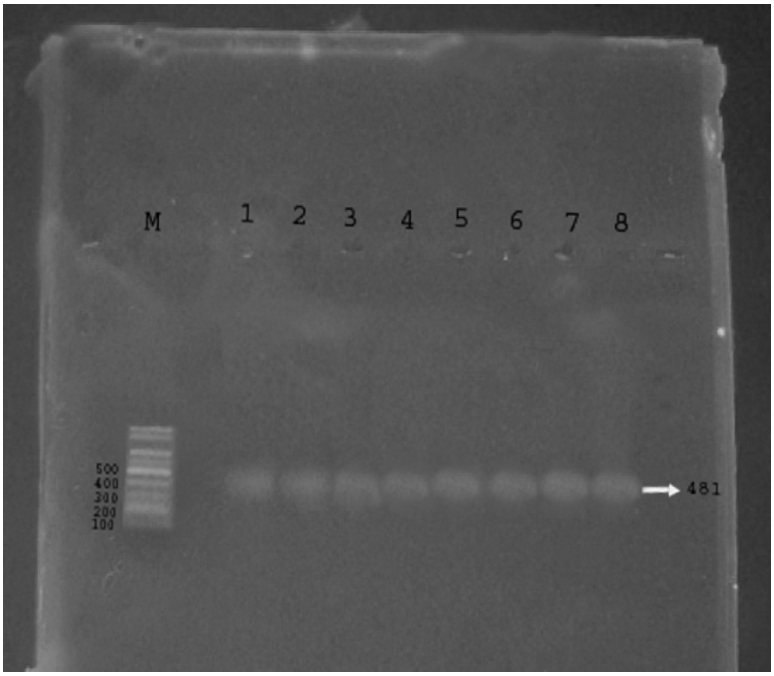
Figure (3):The elctrophoretic photograph showing the pattern of ST6GALI mRNA expression in trachea of different birds

M: DNA ladder Lane 1: chicken Baladi trachea Lane 2: chicken Hubber trachea
Lane 3: Duck Baladi trachea Lane 4: Duck Pekeni trachea



Figure(4): The elctrophoretic photograph showing the pattern of ST6GAL I mRNA expression in lung of different birds.

M: DNA ladder Lane 1: chicken Baladi trachea Lane 2: chicken Hubber trachea
Lane 3: Duck Baladi trachea Lane 4: Duck Pekeni trachea



Figure(5): The elctrophoretic photograph showing the pattern of glyceraldhyde 3 phosphate dehydrogenase (GAPDH) mRNA expression in trachea and lung of different birds.

M : DNA ladder Lane 1: chicken Baladi trachea Lane 2: chicken Hubber trachea
Lane 3: Duck Baladi trachea Lane 4: Duck Pekeni trachea Lane 5: chicken Baladi lung
Lane 6: chicken Hubber lung Lane 7: Duck Baladi lung Lane 8: in Duck Pekeni lung

Corresponding Author:
Hussein I. El-Belbasi
Department of Biochemistry, Faculty of Veterinary Medicine, Zagazig University,Egypt
h_elbelbasi2003@yahoo.com

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