Molecular Diagnosis of Adeno Virus Associated with Hydropericardium Hepatitis Syndrome of the Broiler Chickens in Nineveh Province, Iraq

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Introduction

Fowl adenoviruses (FAdV) are members of the genus Aviadenovirus and the family Adenoviridae, which is divided into three divisions. According to serum cross-neutralization tests and restriction enzyme digest patterns, Group 1 is split into 12 serotypes (FAdV-1 8a and 8b - 11) and 5 species (FAdV A, B, C, D, and E). FAdV are linked to a variety of avian disorders, such as inclusion body hepatitis, hydropericardium hepatitis syndrome (HHS), and Egg Drop Syndrome EDS. FAdV subtypes 4 are the most common cause of these diseases in the chicken [1-3]. FAdV4 is the etiological agent for HHS which is a linear double stranded DNA virus. The genome is approximately 43 to 46 kb, non-enveloped and icosahedral in shape. Hexon, Penton, and the fiber protein are three structural proteins that are encoded by the FAdV genome [4, 5].

HHS was first observed in 1987 at Angara Goth, Pakistan and therefore it was named as Angara disease. Later, the disease was reported in other countries including India where the disease was first detected in Jammu followed by Punjab and Delhi in 1994 [6] and the disease was first report in Iraq in 1991[7].

Broiler chicken of 3-6 weeks of age are mostly affected by HHS [8, 9]. There are 12 serotypes belonging to the first group of poultry adenoviruses, which are widespread in various parts of the world, and the serotype 4 has been isolated from most of the cases of HHS, as it was isolated in Ecuador, Chile, Japan, Mexico and...
Pakistan[10,11]. The infected birds don’t exhibit any obvious signs, though a sudden death was noticed between 2-5 days. Birds with sporadic cases may exhibit various postures, grow dull and dejected in the final stages, and gather in a corner. The eyelids were closed, and the beak and chest were resting on the ground. The gross lesions in 90% of the affected birds, are the pericardium accumulates fluids with a green tint or fluid that is colorless, watery to jellylike, heart malformation with floating in the pericardial sac’s apex and petechial hemorrhage. The liver’s alterations include ecchymotic or petechial hemorrhage, extensive areas of mottled focal necrosis, friable swelling, and yellow, paleness. Lung congestion is present [6,7,11,12].

The risk of HHS outbreaks could be minimized by adopting proper management and biosecurity measures [13] in addition to this, vaccination might play a key role in preventing HHS. However, there is still dire need of a vaccine with higher efficacy and fewer sides to counter FAdVs in poultry [13]. For that, this study aimed to survey diagnosis for the HHS virus in broiler chicken farms in the six areas of Nineveh Province, namely Mosul, Tal Afar, Hamdaniya, Bartella, Al-Baaj and Qayyarah for the period October 2021 to March 2022 by molecular diagnosis of the adeno virus that causes HHS in broilers by PCR technique, as well as gene sequencing to determine the strain of the virus.

**Material and Methods**

**Sample collection**

A poultry population of total 24 samples from 12 broiler farms of six areas (2 farms for each area): Mosul, Tal Afar, Baaj, Bartela, Hamdanya and Qayara in Nineveh Province visited regularly to study outbreak of HHS during the period from October 2021 to march 2022. Suspected flocks were clinically examined, clinical sign and symptoms were observed. A total of 24 samples of dead chickens were collected after necropsy examination observed, liver samples and stored in (-20) degree for molecular diagnosis by polymerase chain reaction PCR.

**Diagnosis of hydropericarditis and hepatitis by polymerase chain reaction PCR**

The first step was relied upon extraction of DNA from chicken liver tissue using the analysis kit supplied by Geneaid, Taiwan, then preparation of agarose gel and DNA electrophoresis: was done for DNA transfer and detection, 1% agarose gel was prepared. Then the gel solution was added in the basin of the Tray of the transfer device after the special comb was fixed to form the Wells at the edges of the gel. Then the Tray was placed in an electric relay tank containing an appropriate amount of X1 TBE solution, after which we lifted the comb softly. The gel was photographed under ultraviolet rays using a gel documentation device to be able to see the DNA bundles as well as the PCR reaction product.

In the PCR reactions the concentration of DNA in all samples was adjusted by dilution with TE solution to obtain the concentration required to perform the PCR reactions, and it was 50 ng/microliter for each sample. The presence of the amplified region was detected, as 4 μl (100 nanogram) of template DNA, and 1 μl (10 picomol) of each gene-specific primer were added to the contents of the master mix (Table 1).

<table>
<thead>
<tr>
<th>No.</th>
<th>Stage</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycle number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Initial denaturation</td>
<td>95</td>
<td>6 min.</td>
<td>1</td>
</tr>
<tr>
<td>2.</td>
<td>Denaturation</td>
<td>95</td>
<td>1.30 min.</td>
<td>35</td>
</tr>
<tr>
<td>3.</td>
<td>Annealing</td>
<td>46</td>
<td>1.30 min.</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Extension</td>
<td>72</td>
<td>2 min.</td>
<td></td>
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<tr>
<td>5.</td>
<td>Final extension</td>
<td>72</td>
<td>5 min.</td>
<td>1</td>
</tr>
</tbody>
</table>

**TABLE 1. The sequence of hexon in DNA**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Hexon-A</td>
<td>5’- CAARTTCAGRCAGACGGT -3’</td>
</tr>
<tr>
<td>Hexon-B</td>
<td>5’- TAGTGATGMCGGACGATCAT -3’</td>
</tr>
</tbody>
</table>

**TABLE 2. The special program for the PCR method**

reaction using the special program for the reaction as shown in the following (Table 2).

**DNA sequencing analysis**

The DNA sequencing technique is the basis for identifying and detecting genetic mutations, SNP variations and the strain of virus. Usually, the output of the PCR reaction is used to determine the amplified segment sequences in which genetic differences are required.

The sequence of nitrogenous bases of the amplified DNA pieces of the chicken liver tissue was determined. The PCR reaction products of the aforementioned samples were sent with the primers of the resulting package. The sequence of genes was read based on the 3130 Genetic Analyzer device supplied by the Hitachi Co., Japan. Gene-specific sequences were matched with those documented in the National Center for Biotechnology Information (NCBI) and the results were analyzed using BLAST.

**Result**

**Diagnosis the adenovirus by PCR**

All results showed that the PCR of liver samples for 12 broiler farms of the six regions in Nineveh Province, namely: Mosul, Talafar, Hamdaniya, Bartella, Al-Baaj and Qayyarah with a positive results of HHS adenovirus and the result was 897bp (Fig. 1), in the period (October 2021 - March 2022).

**Molecular diagnosis of adenovirus by DNA sequencing**

The result of molecular diagnosis of adenovirus by DNA sequencing revealed that the pathogenic HHS adenovirus causative agent of the six areas in Nineveh Province was adenovirus serotype 4 of one strain E1B for all of them according to the gene sequencing below and corresponding with sequencing ID: XM_040669733.2 of National Center for Biotechnology Information NCBI (Fig. 2).

A G C G G G G A G G C G G G G G G G C -
C A T G C G G G G G G C G G G A C G A C G -
G C T C T G G T G G A G G T G C G C G A C C C C -
G C G C C C C T T T C T G C C A C C G A C C G -
G A C C G G A T G C T G T G G A G G C G A C T G -
G A G C G A C G C G C G A C G G G G C -
G C T G C C G G C G G C G C C C C C C C C C C -
G G G A C G G A A G A C G C C G C A A A G G T -
G A G C G A G A G C T C C C G C C C G G C C C -
G G C C C C G C A A C C C C C C G C G G C -
G G G A A G G A G C C C G C C A G T C C C C G T G -
G A G C C C C C C G C C C G C G A G A C G C C C -
C A G G A G C G G A G C G A C G A G C G C C -
G T C C G C C C G C A G C C G C C A C G G A G C T -
G C G C G G A G C G C C G C C G G G A G A C C -
C A C C T C C C C T T C T C G C T G T C C C C C C C -
C C C G G T G C G G T G G G G C G T G C G G A C G -
G G C G G T G T G C T G A G G A A G A G G G C -
G T T C A G C T C C G A G C T G C T G C T G C T G -
G T C A T C C C C C T G C T G C T C A G C C A C -
G T C T G C T C A C C T G G G G C T G G G G G A T C T A -
C A T C G G G A A G C G C T G G C G G C T C C T C G -
G C A A A C C C G C T G T G A G C G C G C G G C G C G -
G G G G A G C G G C C T C C C T G C T G C T G C T G -
G G G G G G T G C A A G C G G G G T G C T C A G C G C -
G G C C G T C T G T C C C G C G A C C C C G C G G C -
G T G G C A C G C A G C G G C G T C C C C C C T C C -

Fig. 1. PCR Result of Liver Tissue Samples Containing HHs Adenovirus 897 bp
Discussion

Although the disease has been reported in almost all the areas of Iraq, the objective of the current study was to accurately diagnose HHS fowl adenovirus in the broilers farms of six regions in Nineveh Province, namely: Mosul, Talafar, Hamdaniya, Bartella, Al-Baaj and Qayyarah by PCR technique and DNA gene sequencing to detect the accurate strain of adenovirus. Adenoviruses are among the viruses that are widely spread in all types of birds, as shown by numerous studies, where the presence of antibodies to adenoviruses was observed in healthy chickens and these viruses were isolated from uninfected natural birds as well [14].

Two significant illnesses known as inclusion body hepatitis IBH and hydropercardium hepatitis are caused by chicken adenoviruses. Although in certain situations, each disease is seen separately, the two cases have been consistently seen together, hence this pathological condition is known as hydropercardium hepatitis syndrome.

This sickness affects young chickens and is a severe illness that is accompanied by anaemia, and fluid accumulation surround the heart (Hydropercardium) [15, 16].

There are 12 different avian adenovirus serotypes, but the majority of the viruses found in cases of hydropercardium hepatitis belong to the serotypes 4 and 8 because which they have the ability to cause disease without the immunosuppression caused by some viruses, for example, infectious bursal disease IBD virus or any other factor that suppresses the immunity of chickens, but coincidence with infection with viruses that cause immunosuppression such as IBD and Chicken infectious anemia CIA results in severe concurrent diseases in broiler chickens [17]. Although, some strains of fowl adenovirus may produce a mild infection [17, 18].

The result of PCR as an accurate diagnosis for HHS fowl adenovirus FAdV Hexon gene in the broilers farms of six regions in Nineveh Province, was 897bp which is positive for all liver
tissues of broilers farms and is consistent and compatible with what was previously diagnosed by PCR technique targeting the FAAdV Hexon gene. Interestingly, hexone protein and fiber proteins were described to play a major role in the pathogenicity of serotype 4 of chicken adenovirus, specifically because of the amino acid residues. At position 188 of the hexone protein is responsible for the pathogenicity of serotype 4 of chicken adenovirus [19, 20].

In recent years, the results presented by the DNA Sequencing technology be highly accurate in identifying genetic mutations [21], so the result of the molecular diagnosis by genetic sequencing of the DNA amplified pieces of adenovirus from chicken livers, showed that the causative agent of the disease in the six regions was serotype 4 of one strain E1B for all of them according to the XM_040669733.2 gene sequence from NCBI. The serotype 4 considered the dominant serotype associated with the HHS and IBH in Iraq in particular and the Middle East in general, as it was observed that strains of chicken adenovirus FAdV in the Emirates clustered together with the same serotype of the virus and spread in Saudi Arabia (KY606586.1), Pakistan (MH151202.1 and EU931693.1), Nepal (MN604721.1), and China (MK629523.1, KY426988.1 and MH006602.1) (22, 23), this is consistent with what was stated with the study of Abdulrahman et al.[22], where isolates of chicken adenovirus FAAdV in the years 2013-2021 in Iraqi Kurdistan that there are two different types of the virus, and both genotypes have a different genetic evolution, namely FAAdV-D and FAAdV-E. This is agree with other studies in Korea during the outbreak of chicken adenovirus [24].

It is necessary to continue investigating the spread of diseases caused by the chicken adenovirus FAdV and to understand the genetic epidemiology of the viruses associated with it, as co-infections from multiple serotypes have been observed in other regions of the world, such as China [14].

Acknowledgment

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Conflict of interest

There is no conflict of interest with personal financial statement.

References


هدفت هذه الدراسة إلى التشخيص الجزيئي لمتلازمة موه التامور والتهاب الكبد في فروج اللحم في محافظة نينوى، العراق في HHS. تهدف الدراسة إلى تحديد وتشخيص الفيروسات المسببة لمتلازمة موه التامور والتهاب الكبد في فروج اللحم في ست مناطق في محافظة نينوى، العراق وهي: الموصل، تلعفر، البعاج، بينص الديك، البرطلة، الحمدانية، وبعض المناطق الأخرى خلال الفترة من تشرين الأول (أكتوبر) 2021 إلى آذار (مارس) 2022. تم التحري وملاحظة المرض في الحقول المذكورة. تم جمع عينات أكياس لحم فروج اللحم بما مجموعه 24 عينة بواقع 12 عينة لجهاز فروج لحم في المناطق الستة (حبلين لكل منطقة) بواسطة تقنية تفاعل البلمرة المتسلسل PCR للفيروسات وال налогов Hexon للفيروسات الدواجن. من أجل التشخيص الدقيق، تمت تحليل وتحليل DNA sequencing التسلسل الجيني لعينات آكياس لحم فروج اللحم في المناطق الست في محافظة نينوى، العراق باستخدام تفاعل البلمرة المتسلسل PCR. أظهرت النتائج التحليل الجيني PCR أن عينة آكياس لحم فروج اللحم في منطقة الديك، حيال 897bp، كانت موجبة للفيروسات الدواجن. كذلك، تم تحديد شكل الفيروسات الدواجن المسبب لمتلازمة موه التامور والتهاب الكبد في الحقول المذكورة. تم استخدام برنامج NCBI في تحليل وتحليل وتحديد نوع الفيروسات الدواجن المسبب لمتلازمة موه التامور والتهاب الكبد في فروج اللحم في محافظة نينوى، العراق.

الكلمات المفاتيح: متلازمة موه التامور والتهاب الكبد، فروج اللحم، التحصين الجزيئي، فروج اللحم، محافظة نينوى.