DIVA MOLECULAR DETECTION OF SUSPECTED CASE OF NEWCASTLE AND ENCEPHALOMYLITIS DISEASE IN LAYERS

Amjed. H. Ulaiwi
Assist.Prof.
Coll. Vet. Med., University of Baghdad
E-mail: amjed.h@covm.uobaghdad.edu.iq

ABSTRACT

The aim of the study investigates the molecular diagnosis between (the Avian encephalomyelitis virus and Newcastle disease virus and differentiating the infected from vaccinated animals (DIVA) strategies. The 1st detection by RT-PCR for(PMV-1) as primary detection then, the samples were prepared as in FTA card Whatman® and sent to (AniCon Labs) Germany to detect by one step RT-PCR (DIVA technique) between NDV and AEV genotype. The result of the molecular investigation as primary detection revealed three samples were positive with Ct values (34.0, 26.6, and 35.8) respectively. Then, in the detection of AEV the result showed all samples were negative. finally, by using (DIVA) with two primers to detect general (PMV1-9) by (M-gene) all samples were positive at Ct values (20.8, 20.1, and 25.2) respectively, and then path type by (F gene) to differentiate infected from vaccinated samples also all samples were positive with Ct values (23.8, 23.3 and 23.1) respectively. the study concluded that the samples can differentiate between AEV and NDV by using DIVA strategies to find pathotypes and differentiating between infected and vaccinal isolate based on certain amino acids in the primers.

Key words: M gene, F gene, one-step RT-PCR, meso-velogenic ND.

الكشف الجزيئي بتقنية DIVA عن الحالات المشتبه فيها لمرض نيوكاسل والتهاب الدماغ والنخاع في الدجاج البياض

أمجد حسين عليوي
أستاذ مساعد
كلية الطب البيطري – جامعة بغداد

المستخلص

تهدف الدراسة إلى التحقق عن طريق التشخيص الجزيئي بين مرضي التهاب الدماغ والنخاع الطيري الفايروسي AEV ومرض نيوكاسل NDV الفايروسي للتفريق بين العزلات اللقاحية والممرضة(DIVA). اجري التشخيص الاولي بواسطة RT–PCR لفايروس نيوكاسل النوع الأول (PMV–1)، ثم تم تحضير العينات كما هو الحال في بطاقات FTA Whatman® Card وأرسلت إلى مختبرات(AniCon Labs) المانيا، من خلال استخدام تقنية 1–step RT–PCR (DIVA)وللتفريق بين فايروسات NDV و AEVالنمط الجيني. كانت نتائج الفحص الجزيئي كاكتشاف أولي، كشفت هذه النتائج عن ثلاث عينات كانت إيجابية بواسطة مع قيم Ct كانت (34 و 26, 6و38, 8) على التوالي. بعد ذلك، من خلال الكشف عن AEV أظهرت النتائج أن جميع العينات كانت سلبية. باستخدام تقنية (DIVA) مع اثنين من البادئات للكشف العام لفيروس مرض نيوكاسل ولجميع الأنواع المصلية (PMV1–9) للجينM كانت جميع العينات موجبة عند قيم Ct (20, 8 و20, 1 و25, 2) على التوالي، كما استخدم النمط الوراثي بواسطة جين F للتمييز بين العينات المصيبة واللقاحية وكانت النتائج Ct (23, 8و 23, 3 و23, 1) على التوالي.استنتجت الدراسة أن العينات يمكن أن تفرق بين AEV وNDV، باستخدام استراتيجيات DIVA للعثور على الأنماط المرضية والتمييز بين العزلات المصيبة واللقاحية على بعض الأحماض الأمينية في البادئات.

الكلمات المفتاحية: جين M، جين F، خطوة واحدة لتفاعل البوليمر المتسلسل، عترة متوسطة –ضاري لنيوكاسل

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INTRODUCTION

Newcastle disease (ND) and Avian encephalomyelitis disease (AE) revealed similar signs as well as with other nervous diseases in poultry clinically (18). In poultry, production (ND) is a serious viral disease with major economic losses and worldwide distribution (2,6). The isolates of (PMV) are categorized into nine serotypes (APMV-1 to APMV-9), especially (APMV-1) (31). The molecular detection of Newcastle disease virus (NDV) depends on six main proteins especially matrix protein (M) for general detection as (APMV) and (F protein) for velogenic NDV, with major RNA genome negative-sense, and single-stranded non-segmented (27,28,29). Experimentally, the virulence of NDV is classified according to pathogenicity: into (velogenic, mesogenic, lentogenic, and asymptomatic) forms, the velogenic NDV (vvNDV) produce neurological signs with hemorrhagic lesions and/ or respiratory signs (12). While(AE) is one of the important viral diseases many avian young species with an economic impact on poultry production (3,7,30). The disease has neurological signs especially, tremors and ataxia is transmitted vertically and horizontally, and also caused 40-60% morbidity and a 25% mortality rate (21,22). The AE virus belongs to the (family *Picornaviridae*, genus *Tremovirus*) the genome single-stranded RNA with a small positive sense (19,20). Molecular classification of virus contains four structural proteins (VP-1, VP-2, VP-3, and VP4) with seven non-structural proteins (15). Targeting of AEV detection by an (RT-PCR) method using primers depends mainly on the (VP2 gene) (32). DIVA strategies are the goal for differentiation between the diseases and eradication (23).

MATERIALS AND METHODS

Sampling: Brown table egg layer (70 days old), the number (50 000) chickens, the flock exhibits a sudden onset of nervous manifestation with mortality rate elevated reach to 5% of the flock, the preparation of samples as follows.

1-Six samples (Three Brain and three Trachea) detected in Iraq RT-PCR for(PMV-1)

2- the samples were prepared as in FTA card Whatman® FTA Cards for isolation, purification, and storage of genetics material for diagnostic and research applications

3- The samples were sent to (AniCon Labs) in Germany sponsored by (Boehringer Ingelheim) a financier and supporter of scientific research, to detect by one step RT-qPCR (DIVA technique) the presence of NDV and AEV and to genotype the existing virus.

Primers: Avian Encephalomyelitis Virus (AEV) : Method: Species-specific RT-PCR (Kylt® Avian Encephalomyelitis) (16).

Parameter: General NDV (aPMV-1) (**M-Gene**) Name of the oligonucleotide: PROBE: HEX APMV-1 (HEX / BHQ) Sequence (5, to 3,): GGGACRGCHTGCTATCC (Method: Serotype-specific RT-PCR) (Kylt® Paramyxovirus 1).

1- Parameter: NDV type 1 (aPMV-1) - DIVA - detection of velogenic strains (**F-protein**) Name of the oligonucleotide: PRIMER: APMV-1 F Sequence (5, to 3,): AGTGATGTGCTCGGACCTTC (F- Gene) Name of the oligonucleotide: PRIMER: (APMV-1 R Sequence 5, to 3,): CCTGAGGAGAGGCATTTGCTA Method: Pathotype-specific Real-Time RT-PCR (Kylt® aPMV-1 pathotype).The protocol of primers occurs according to GoTaq® 1-Step RT-PCR System: Based on certain amino acids in the matrix protein gene as general Paramyxovirus-1 (PMV-1). (F protein) the sequenced strain could be categorized to the velogenic (highly virulent) PMV-1 strains, meaning the strain carries amino acids typical for velogenic strains. The PMV-1 was classified on the pathogenicity site especially (F2/F1 cleavage). The (F protein) was based on (360 bp) represented (APMV-1).

RESULTS AND DISCUSSION

The result of clinical signs and Post mortem findings showed the flock exhibits a sudden onset of nervous manifestation (head trimming, star gazing, incoordination, imbalance, and unilateral paralysis) with fever, mortality rate elevated reach to (5%) of the flock. The Post mortem changes include congested carcass, some with petechial hemorrhages, congested liver, pale spleen, congested viscera and proventriculus, necrotizing and engorged payer's patches,

transparent intestine, with bile stained content and engorged cecal tonsils. The result of clinical signs and post-mortem findings agree with kommers *et. al.* showed the neurological NDV strains that were isolated in the flocks accompanied by nervous signs and visceral changed paralysis of wings or legs was (unilateral or bilateral), head or muscular tremors, torticollis with congestion of body organs and mortality rate reach to 50%. Also, the mesogenic strain of PMV-1 caused low mortality in chickens at age 4-week-old with nervous signs such as paralysis, torticollis and tremors of head (1). The post-mortem lesion (4) the outbreak of 2-week-old chickens with APMV-3 observed enlargement and congestion of the spleen and liver with focal necrosis in the pancreas. Also, the infection with APMV-5 showed splenomegaly with discoloration of the liver and hemorrhages in the proventriculus and small intestine (10). As well as the infection in doves with APMV-7 observed congestion and enlargement in the spleen and liver (11). Also, these signs and lesions could cause by Avian encephalomyelitis (epidemic tremor) AEV affecting the nervous system with the same rate of morbidity and mortality (14). The similarity between AEV and APMV in neurological symptoms as uni or bilateral paralysis, ataxia, and head or neck tremors (16,17). The molecular results revealed the 1st detection of PMV-1 in Iraq in six samples (3 Brain and 3 Trachea) revealed three samples were positive (2 Brain and. Trachea) with Ct values of (34.0, 26.6 and 35.8) respectively.

Table 1. Samples detected of APMV-1 by RT-PCR

NDV Site ID	Sample ID	Channel Result	Ct	EndPt
A9	**Trachea 1**	**NEG**	**0**	**9**
A10	**Brain 1**	**NEG**	**0**	**6**
A11	**Trachea 2**	**POS**	**34.0**	**19**
A12	**Brain 2**	**POS**	**26.6**	**37**
A13	**Trachea 3**	**NEG**	**0**	**9**
A14	**Brain 3**	**POS**	**35.8**	**16**

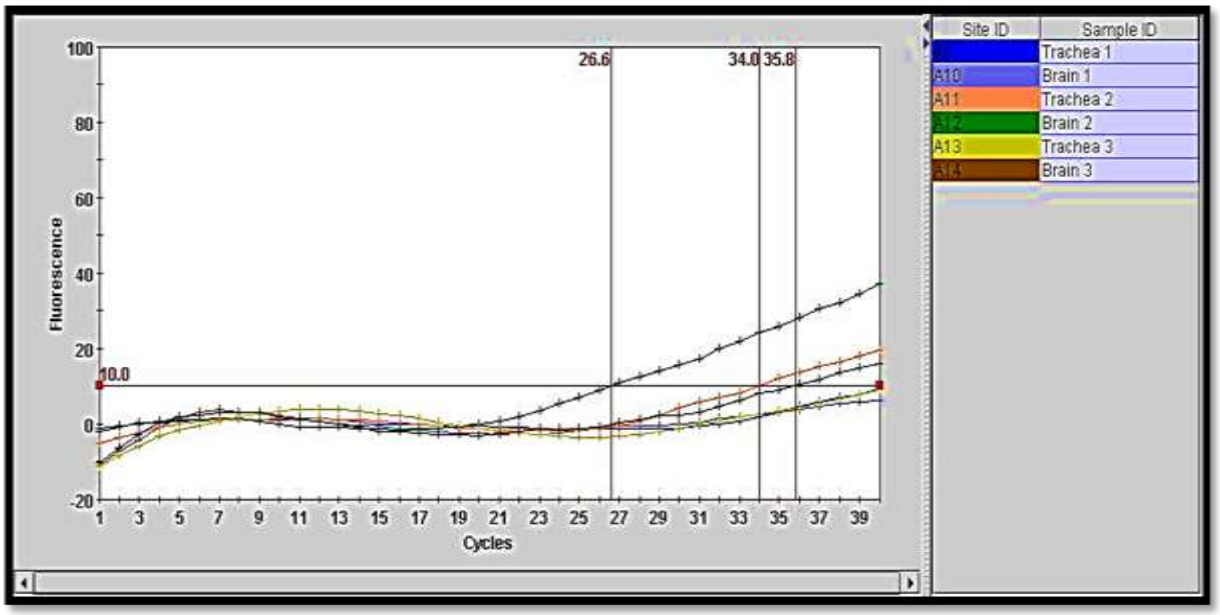

Figure 1. Samples detected by RT-PCR positive samples with Ct values

The molecular identification of PMV-1 was the best and most accurate method that agrees with sameera *et. al.* molecular detection of NDV was the sensitive method by using RT-PCR. Also, recently quicker and more sensitive (RT-PCR) for detection and genotyping of PMV-1 (24,25). As well as the using PCR assays specific for mesogenic/ velogenic PMV-1 with Ct values (23.4, 29.0, and 30.8) respectively in chicken had high analytical sensitivity (13). The results to AEV showed not detected by RT-PCR all samples were negative.

Table 2. Species-specific RT-PCR (Kylt® Avian Encephalomyelitis)

Sample No.	Sample Description	Result
A1804251.001	FTA-card (spot 1)	not detectable
A1804251.002	FTA-card (spot 2)	not detectable
A1804251.003	FTA-card (spot 3)	not detectable

Finally, the samples used one-step RT-PCR (DIVA) to differentiate infected from vaccinated animals. The result showed in 1st step of detection of general (PMV1-9) by matrix protein gene (M-gene) all samples were positive at Ct values (20.8, 20.1, and 25.2) respectively.

Table 3. Samples detection for (NDV, aPMV) RT-PCR (Kylt® PMV)

Sample No.	Sample Description	CT	Result
A1804251.001	FTA-card (spot 1)	20,8	positive
A1804251.002	FTA-card (spot 2)	20,1	positive
A1804251.003	FTA-card (spot 3)	25,2	positive

Then, the result in 2nd step of detection pathotype of APMV-1 to differentiate infected (meso-velogenic strain) from vaccinated samples (lentogenic strain). The result reveled all samples were infected (meso-velogenic) strain with Ct values (23.8, 23.3 and 23.1) respectively.

Table 4. Samples detection for serotype-1 (NDV, PMV-1) - DIVA - detection of velogenic strains, Pathotype-specific RT-PCR (Kylt® aPMV-1 pathotype).

Sample No.	Sample Description	CT lentogenic	CT velo-/meso genic	Result
A1804251.001	FTA-card (spot 1)	-	23,8	velogenic strain positive
A1804251.002	FTA-card (spot 2)	-	23,3	velogenic strain positive
A1804251.003	FTA-card (spot 2)	-	23,1	velogenic strain positive

The PMV-1 was detected on (360 bp) and for the pathogenicity site especially (F2/F1 cleavage). The (F protein) was based on represented (APMV-1). The results of one-step RT-PCR (DIVA) to differentiate infected (meso/velogenic) strains from vaccinated (lentogenic) strain for detection of general PMV and pathotype strains correspond to the following studies that showed the detection of NDV by (RT-PCR) assays is now extensively applied because this assay is less tired as well as faster and accurate through discovering important two proteins matrix protein (M), fusion protein (F) (8,9). Also The identity of the reference of general PMV can be detected by the rRT-PCR protocol by using specific primer and probes for the M gene (26). Then the molecule reaction by PCR assays specifically (DIVA) technique for differentiating between lentogenic (vaccinal) and mesogenic/ velogenic (infected) the detection of approximately 10 and 20 copies of strains had high analytical sensitivity (13). As well as the Iraqi isolates of NDV can be diagnosed by using specific primers designed through using the hemagglutinin-neuraminidase (HN) gene based on discovering the site of pathogenicity by using one-step RT-PCR (5).

CONCLUSION

The study was concluding that the samples were isolated and detected by RT-PCR through 1st detection of general PMV by using the M gene and using DIVA strategies to find pathotypes and differentiating between infected and vaccinal isolate based on certain amino acids in the (F protein) the sequenced strain could be categorized as the velogenic (highly virulent) PMV-1 strains, meaning the strain carries amino acids typical for velogenic strains.

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