

Preparation of a hemagglutinating antigen for the IBV Sharkia variant designated

(IBV-EG/ SHARKIA –F629-2015).

Bayoumie, H.A.A.

Senior Researcher, Animal health Res. Inst. Zagazig (Poult. Dis. Dept.)

Received in 11/1/2016

Accepted in 22/2/2016

Abstract

In the present study the haemagglutinating antigen for the IBV isolate designated **(IBV-EG/ SHARKIA – F-629-2015)** was successfully prepared for amore precise laboratory diagnostic purposes.

The afore mentioned isolate was propagated in SAN eggs., the allantoic fluid was collected after the second passage and concentrated by dialysis or by high speed concentration at 30000 rpm . The concentrated AF was either treated with phospholipase or by neuraminidase to convert the virus into a hemagglutinating virus. One HA unit was reached at dilution 1: 1024 when centrifuged at 30000 rpm and treated with neuraminidase enzyme.

The specificity and the sensitivity of the hemagglutination inhibition test were compared to the serum neutralization test upon the same serum samples using the aforementioned viral isolate and they yield comparable results.

Abbreviations: AF = allantoic fluid; WCRBC = washed chicken red blood cells; HA = hemagglutination; IBV = infectious bronchitis; HI= hemagglutination inhibition test; ELISA=Enzyme linked immunesorbant assay. MDA= maternally derived antibodies; SAN= specific antibody negative; ECE = Embryonated chicken eggs; SNT=serum neutralization test.

Key words: Hemagglutination assay, hemagglutination inhibition, IBV, neuraminidase treatment, Phospholipase, SNT.

Introduction

Variation exists among members of the family Coronaviridae in their ability to agglutinate red blood cells. Although members of group II are efficient hemagglutinating agents, the others in groups I or III (where IBV is classified) are not **Schultze et al., (1991)** and **Holmes and Lai (1996)**. Several attempts have been made to convert IBV into a hemagglutinating virus that is capable of agglutinating WCRBCS (**Bingham et al., 1975, Schultze et al., 1992, and Schultze et al., 1994**). IBV acquires hemagglutinating activity after treatment with phospholipase C (**Bingham et al., 1975, King 1984, Lashgari and Newman 1982 and Muneer et al., 1988**). This activity could be inhibited by

specific antiserum (**Bingham et al., 1975**). Neuraminidase was successfully introduced to remove sialic acid residues from the spike glycol protein of the IBV envelope to induce its HA activity (**Schultze et al.,1991, 1992, 1994 and Schultze and Herrler 1992**).

Schultze et al. (1992) interpreted how IBV acquires the hemagglutinating activity after treatment with neuraminidase. They mentioned that neuraminidase treatment removes the alpha 2, 3-linked N-acetylneuraminic acid from the surface of the virus. And they mentioned that this property is unique for IBV only.

HI test and ELISA were compared in their abilities to detect IBV antibodies for IBV at different circumstances such as (post vaccina-

tion, during infection, and MDA determination) **Bayoumie and Hikal (2015)**. HI test for IBV was performed using the hemagglutinating antigen of the (Mass- 41. and 4/91serotypes). ELISA was performed at two dilutions (1/100, 1/ 1000) which is nearly the reciprocal of dilution of (7 and 10) in HI test in a trial to set two points for comparing the obtained results of the two tests. ELISA test showed 100% sensitivity and specificity at dilution 1/100 and showed 80.96%, 95.5% respectively at 1/1000 dilution. The sensitivity and specificity of HI test were 80.91%, 95% respectively when Mass-41 antigen was used and was 73.91%, 62% when 4/91 antigen was used. The difference in sensitivity and specificity of HI reflects its selectivity during serotyping and this picture will necessarily differ if samples were tested against other antigens like (D- 274, 1466,.....etc.). **Bayoumie and Hikal (2015)** found that ELISA titers are misleading in the prediction of the suitable time for vaccination.

HI test against IBV was used in a serosurveillance in Sharkia governorate at year 2014 using a variable IBV HA antigens (Mass-41, 4/91, and d-274) (**Bayoumie and Hassanen, 2015**) recorded Seropositive titer for IBV-D274 in a totally non vaccinated flocks against such antigens., which refer to the IBV circulating in the poultry premises. The fore mentioned results was fortified by the results of **Bayoumie et al., (2015)** when they successfully isolated the IBV circulating in Sharkia designated (**IBV-EG/ SHARKIA-F629 2015**) and this isolate had showed (86%) similarity to the IBV-CR88121vaccinal strain, (85.3%) similarity to IBV-4-91 vaccinal strain and(82.9 %) similarity to Dutch variants D-274 vaccinal strain. These results highlights the diagnostic and serotyping value of the HI test for IBV and this was helpful for proper selection of the suitable protective vaccinal strain.

The present study aims to prepare a haemagglutinating antigen from the IBV isolate designated (**IBV-EG/ SHARKIA – F629 2015**) for a more precise laboratory diagnostic purposes and compare its results to SNT.

Materials and Methods.

Material

1- Field IBV sample.

An IBV variant 2 isolate., that was isolated and molecularly confirmed by **Bayoumie et al., (2015)**, this isolate was designated (**IBV-EG/ SHARKIA –F629 2015**).

2- ECE

SAN-ECE from a small native breeder flock that received AIV vaccination only were used for viral assays and SNT.

3- Chicks

Ten day old chicks hatched from fertile native Saso eggs, were used for preparing hyper immune serum needed for SNT.

4- Sera

4-1. Negative control serum.

It was obtained from a sacrificed day old SPF chicks (Prof. Dr. S.AL- Assely. Poult. Dis. Abbassia serum and vaccine production Inst. NRC.)

4-2. Hyper immune sera

Collected from chick that was inoculated at day old with (**IBV-EG/ SHARKIA –F-629 2015**).

4-3. Serum from IBV vaccinated flocks

Sera samples from 35 day old Commercial-broiler flock that was vaccinated against IBV.

5- Membrane filters.

Syringe membrane filter 450 nm Thermo scientific Nalgene. Cat. no. 190-2545 (8-0404-40493).

6-Washed chicken RBCS (WCRBCS).

WCRBCS were obtained from 28dayold specific antibody negative chicken (SAN) raised for this purpose.

7-Dialysis hollow fiber role.

Visking dialysis role. SERVA electrophoresis GmbH. 21 mm diameter lot.120573 with 1 nm pores.

8-Polyethylene glycol.

Polyethylene glycol powder 6000 (**Alpha Chemika**) Serial. no. (AL 3120) Batch. no. (p 20911) mf g (2/ 2011), exp. (2/2016)

9-Buffers.

9-1. Saline

9-2. Hepes buffer:

It was prepared as described by **Villegas**

(1998).

10-Enzymes.

10-1. Phospholipase-C enzyme.

phospholipase C type 1 (Sigma, St. Louis, MO) Cat. No. P 7633 -25 UN., Lot # 061M8623V, P code 1002101759 was used.

10-2. Neuraminidase enzyme.

Neuraminidase enzyme type V from *Clostridium perfringens* (Sigma, St. Louis, MO) Cat. No. N 2876 – 10 un., Lot # SLBD9831 V, P code 1001685488, was used.

Methods

1-Sample preparation for ECE inoculation.

The IBV field isolate under investigation was diluted to make 1/10 suspension in saline then filtrated through a 450 nm syringe membrane filter (Thermo scientific Nalgene). 0.2ml of the filtered material was inoculated according to **Momayez et al., (2002)**. In order to be sure that the sample was not contaminated with hemagglutinating viruses. The harvested AFs were tested for the lack of positive HA activity due to any other hemagglutinating virus before enzymatic treatment.

2-Virus concentration

2-1. Ultracentrifugation

The harvested allantoic fluid of the second passage from the inoculated ECE was centrifuged at 30 000 g for 90 min **Villegas (1998)** using (**MSE*60000** cooling centrifuge) Zagazig vet. Coll. Virus pellet was Re-suspended in HEPES buffer at pH 6.5 by adding 1 ml of buffer per each 100 ml of the initial AS used prior to centrifugation. (100 x concentration) **Villegas (1998)**.

2-2. Dialyses hollow fiber concentration.

The harvested allantoic fluids (AFs) of the second passage from the inoculated ECE were placed in the dialyses hollow fiber role and le-gated then covered for overnight with Polyethylene glycol powder at 4°C for virus concentration (**Trudel and Payment, 1980 and Bayoumie et al., (2015)**). The concentrated AF was preserved until treated with phospholipase or neuraminidase enzymes.

3-Enzymatic treatment

3-1. Neuraminidase enzyme treatment.

A working solution 1 U/ml of neuraminidase was prepared from the vial containing (10 U/ml) using saline (pH7.2) as diluent. 1ml of the working solution was mixed with 1ml of the dialysed or ultracentrifuged AFs, and held at 37°C for 30 min, and then were placed at 4°C for 5 min according to **Momayez et al., (2002)**.

3-2. Phospholipase enzyme treatment.

Harvested allantoic fluid was treated with phospholipase C type 1 (PLC), at a final concentration of 1unit/ml, for two hours at 37°C. **DE WIT (2015)** personal communication.

4- Quantitative Micro Hemagglutination Test (HA)

25µl of both enzyme treated AFs were mixed with 25µl of saline and diluted serially. Then 25µl of .8% WCRBCS was added for each individual well. HA reaction was read within 20min. (**Villegas, 1998**). One HA unit is the well with the highest dilution, where there is hemagglutination (No button).

5-HI for IBV

HI tests, for the fore mentioned antigen were performed as described by **Villegas (1998)**.

6-Viral titration

Viral titration was performed according to **Villegas (1998)**. Titer calculation was performed according to the method of Spearman – Karperin (**Brian and Kangro, 1996**).

7-Serum neutralization (SNT)

SNT was performed according to **Thayer and Beard (1998)**. Specific mortality in ECE due to IBV were confirmed after treatment of the AF from inoculated embryos with neuraminidase and obtaining a positive hemmagglutination (**Rauno et al., 2000 and Bayoumie et al., 2015**).

8-Preparation of hyperimmune sera

prepared by inoculating day old chick repeatedly every 7 days with (**IBV-EG/ SHARKIA – F629 2015**) until the age of 70 day (**Bayoumie, 2004**).

Table (1). Comparison of a direct micro HA after neuraminidase or phospholipase treatment using different concentration methods

Virus concentration tool	Micro HA activity		AF = allantoic fluid
	Neuraminidase treatment	Phospholipase treatment	
Dialysed AF	1:128	1:64	
Ultracentrifuged AF	1:1024	1:512	

Table (2). Viral propagation, titration, serum neutralization and HI.

Items	Sub item	Embryonic mortality at the tested dilutions								A G P T	Virus titer	NI	HI titer*
		-1 5.3	-2 4.3	-3 3.3	-4 2.3	-5 1.3	-6 0.3	-7	-8				
Viral propagation	1 st passage	A	0/5							-	-		
	2 nd passage	B	2/5							-	-		
	3 rd passage	C	3/5							+	-		
Viral titration	D	5/5	5/5	5/5	5/5	4/5	3/5	2/5		+	10 ^{6.3} EID ₅₀ / 0.1 ml		
V.+ negative serum	E1					5/5	2/5	1/5			10 ^{6.1} EID ₅₀ / 0.1 ml		
V.+ positive serum	E2	5/5	5/5	0/5	0/5	0/5	0/5				10 ^{2.5} EID ₅₀ / 0.1 ml		
V.+ serum of IBV vaccinated	T.S ₁	a		5/5	3/5	0/5	0/5				10 ^{4.1} EID ₅₀ / 0.1 ml	3.6	4
	T.S ₂	b		2/5	0/5	0/5	0/5				10 ^{3.1} EID ₅₀ / 0.1 ml	3	6
	T.S ₃	c		4/5	3/5	0/5	0/5				10 ^{3.9} EID ₅₀ / 0.1 ml	2.2	5
	T.S ₄	d		5/5	5/5	5/5	3/5				10 ^{6.1} EID ₅₀ / 0.1 ml	0	2
	T.S ₅	e		3/5	2/5	0/5	0/5				10 ^{3.5} EID ₅₀ / 0.1 ml	2.6	6
	T.S ₆	f		1/5	0/5	0/5	0/5				10 ^{2.7} EID ₅₀ / 0.1 ml	3.4	7
	T.S ₇	g		5/5	5/5	4/5	3/5				10 ^{3.9} EID ₅₀ / 0.1 ml	0.2	2
	T.S ₈	h		2/5	0/5	0/5	0/5				10 ^{2.9} EID ₅₀ / 0.1 ml	3.2	7
Mean ± SE												2.2±0.5	4.37±0.72
SD												1.41	2.03
Specificity												25%	25%
Sensitivity												75%	75%

T.S1= tested sample 1*= Serum dilutions ranged from 1:2 to 1:1024. All HI titers were expressed as log2 of the reciprocal of the highest serum dilution showing complete HI, * HI titers below the threshold of cut (3) are calculated as zero.

Specific mortality in ECE due to IBV were confirmed after treatment of the AF from inoculated embryos with neuraminidase and obtaining a positive hemmagglutination Bayoumie et al., (2015)

Results and Discussion

Results of the present work are illustrated in tables (1, 2). The following manuscript is a pilot trial to examine Results the preparation of a hemagglutinating antigen of the IBV local viral isolate designated (IBV-EG/ SHARKIA-F629-2015) and it was not intended for commercial antigen production purpose.

In the present study during performing SNT (table -2), the AFs from inoculated eggs were harvested, treated with neuraminidase enzyme and examined for HA activity to avoid the laborious work needed to examine them individually in AGPT to confirm their mortality due to IBV. This procedure also saved us the calculation of mortality based on embryonic PM lesion which is not consistent before viral adaptation and appearance of curling and dwarfing known for IBV. According to **Rauno et al., (2000)** rapid-plate hemagglutination (HA) test to detect infectious bronchitis virus (IBV) in AF of ECE after enzymatic treatment was introduced into routine procedures for IBV identification. This system was tested in 468 diagnostic cases received by the Poultry Diagnostic and Research Center at the Univ. of Georgia. AF from inoculated embryos were harvested and treated with commercially available neuraminidase enzyme. IBV in neuraminidase-treated allantoic fluid was identified by clear and consistent HA of chicken red blood cells within 1 min of incubation. The specificity of the neuraminidase rapid-plate HA assay was examined with other avian viruses (**Bayoumie et al., 2015**). **Schultze et al., (1992)** mentioned that this property is unique for IBV only. Sensitivity of this test was compared with embryo lesions and reverse transcriptase-polymerase chain reaction (RT-PCR). The rapid-plate HA assay of neuraminidase treated AF correlated with the RT-PCR during the early stages of IBV detection, identification, and isolation in ECE by **Bayoumie et al., (2015)**. The identification of IBV by the rapid plate HA test eliminates any guesswork in confirming IBV embryonic lesions (Table -2). Titrations of both vaccine and field strains of IBV can be per-

formed relatively quickly in chicken embryos because of the detection of HA activity of IBV in 48 hr as opposed to conventional titration methods that rely on visual inspection of embryo lesions 7 days post inoculation. Vaccine titrations can also be performed reliably with the rapid-plate HA assay with little or no fluctuation in final IBV vaccine titer. The establishment of the rapid-plate HA test was shown to be as sensitive as RT-PCR for the identification of IBV in field samples inoculated into chicken embryos (**Rauno et al., 2000** and **Bayoumie et al., 2015**). This sensitivity was consistent throughout several passages in chorio - allantoic sac the HA for IBV has become a method of choice because of its rapidity, accuracy, and most importantly its ability to identify the serotype of IBV isolates in micro HI using specific serum. However, RT-PCR remains expensive and is limited to the adequately equipped diagnostic laboratories. In addition, an accurate RT-PCR is often dependent on diagnostic sample propagation through the AF of ECE for viral enhancement and efficient viral RNA extraction (**Kown et al., 1993**, **Keeler et al., 1998**), these data points to value of HA, HI in serotyping of IBV in laboratories that is not holding RP- PCR facility .

These results also led us to try the preparation of concentrated IBV hemagglutinating antigen from the field isolate circulating in sharkia governorate termed (IBV-EG/ SHARKIA – F629-2015) to be used in the HI assays for its value in serotyping as mentioned by **Auvigne et al., (2013)**.

In the present study we were able to detect the presence of IBV after the second passage in ECE. **Rauno et al., (2000)** found that a total of 65% of diagnostic field samples were positive for IBV after the first passage in chicken embryos, 30% required a second passage, and 5%, a third passage. No differences were observed when the rapid-plate HA test and RT-PCR sensitivities were compared and this was in agreement with the work of **Rauno et al., (2000)** and **Bayoumie et al., (2015)**. **Rauno et al., (2000)** mentioned that propagation of IBV

through additional passage in ECE enhances RT-PCR results. On the other hand the rapid-plate HA test had significantly reduced the cost of complete IBV identification and serotype classification. The rapid-plate HA test accurately identifies the presence of IBV from the second passage post inoculation in chicken embryos and eliminates any unnecessary and additional blind passages in embryos.

In the present study during the preparation of concentrated IBV working antigen from the sharkia variant designated (IBV-EG/ SHARKIA – F629-2015) intended for use in HI assays (table -1) we had used the recipes of **Rauno et al., (2000)** and **DE WIT (2015)** for enzymatic treatment of the AFs and we used two methods for virus concentrations. **DE WIT (2015)** Studied Various aspects of production, storage and stability of the (HA) activity of IBV. From the results obtained, a standard procedure for the production of IBV, HA antigen to be used in the haemagglutination inhibition (HI) tests is suggested. The main points of the suggested procedure are (the virus should be concentrated but not purified; the virus should be treated with phospholipase C type 1 (PLC), at a final concentration of one unit/ml, for two hours at 37°C and the virus should be stored at 4°C after PLC treatment.

In the present study one HA unit was reached at the dilution of 1:1024 after neuraminidase treatment and ultracentrifugation while phospholipase treatment after ultra centrifugation resulted in a one HA unit at dilution 1:512 (table-1) **Rauno et al., (2000)** mentioned that neuraminidase was shown to be a strong and specific inducer of HA activity for IBV in embryos inoculated with IBV.

Rauno et al., (2000) mentioned that after Neuraminidase treatment., differentiation of IBV from either NDV or AI is easily accomplished.

In conclusion these results show that the rapid plate HA assay is specific, very sensitive, accurate, highly reproducible, simple, and economical.

References

- Auvigne, V.; Gibaud, S.; Leger, L.; Malher, X.; Currie, R. and Riggi, A. (2013).** A longitudinal study of the incidence of avian infectious bronchitis in France using strain-specific haemagglutination inhibition tests and cluster analysis. *Revue Med. Vet.*, 164: 417-424.
- Bayoumie, H.A.A. (2004).** Studies on Reovirus infection in chickens. A Phd thesis submitted for Poult. And rabbit Dis. Dept. Facult. of vet. Med Zagazig Univ.
- Bayoumie, H.A.A. and Hassaneen, O.A. (2015).** Epidemiological highlights on some viruses associated with increased mortality in chickens bred in sharkia governorate. *Assiut Vet. Med. J.* 61: 95-111.
- Bayoumie, H.A.A. and Hikal, M.A.G. (2015).** Comparison between HI and Elisa in detecting immune titer following IBV vaccination. *Assiut Vet. Med. J. Vol.*, 61: 46-55.
- Bayoumie, H.A.A.; El Nile, M.B.; Abd El-Samie, L.K. and Mha, M. El Deib (2015).** Chemical compositions of chicken eggs following infection with IBV. *Egypt J. Chem. Environ. Health*, 1: 821-836.
- Bingham, R.W.; Madge, M.H. and Tyrell, D.A.J. (1975).** Haemagglutination by avian infectious bronchitis virus-a Coronavirus. *J. Gen. Virol.* 28: 381- 390.
- Brian, W.J. and Kangro, H.O. (1996).** Virology methods manual. S. Academic Press, Inc., San Diego.
- DE WIT. (2015).** Personal communication through Sande, Huub van de. Product specialist (Diagnostica, PTS), in GD, Arnsbergstraat 7, 7418 EZ Deventer, the Netherlands, GD, PO Box 9, 7400 AA Deventer, the Netherlands,
- Holmes, K.V. and Lai, M.M.C. (1996).** Corona-viridae: the viruses and their replication. In: Fundamentals virology, 3rd ed. B. N. Fields, ed. Lippincott- Raven, Philadelphia. *J. Virol.* 65: 6232-6237.
- Keeler, C.L.; Reed, K.L.; Nix, W.A. and Gelb, J. (1998).** Serotype identification of avian infectious bronchitis virus by RT-PCR. *Avian Dis.* 42: 275-284.

- King DJ. (1984).** Observations on the preparation and stability of infectious bronchitis virus hemagglutination antigen from virus propagated in chicken embryos and chicken kidney cell cultures. *Avian Dis.* 28: 504-513.
- Kown, H.M.; Jackwood, M.W.; Brown, T.P. and Hilt, D.A. (1993).** Polymerase chain reaction and a biotin-labeled DNA probe for detection of IBV in chickens. *Avian Dis.* 37: 149-156.
- Lashgari, M.S. and Newman, J.A. (1982).** Preparing hemagglutinating antigen from isolates of infectious bronchitis virus. *Avian Dis.* 26: 508-519.
- Momayez, R.; Pourbakhsh, S.A.; Khodashenas, M. and Banani, M. (2002).** Isolation and identification of IBV from commercial chickens. *Archives of Razi Institute* 53: 1-9.
- Muneer, M.A.; Newman, J.A.; Sivanandan, V. and Goyal, S.A. (1988).** Growth, morphology and hemagglutination activity of infectious bronchitis virus. *Zootec. Int. April*: 55-58.
- Ruano, M.; Jhon El-atrache, and Villegas, P. (2000).** A Rapid-plate Hemagglutination Assay for the detection of infectious bronchitis virus. *Avian diseases* 44:99-104.
- Schultze B. and Herrler G. (1992).** Bovine corona-virus uses N-acetyl-9-O-acetylneuraminic acid as a receptor determinant to initiate the infection of cultured cells. *J. Gen. Virol.* 73: 901-906.
- Schultze, B.; Cavanagh, D. and Herrler, G. (1992).** Neuraminidase treatment of IBV coronavirus reveals a hemagglutinating activity that is dependent on sialic acid-containing receptors on erythrocytes. *Virology* 189: 792-794.
- Schultze, B.; Enjuanes, L.; Cavanagh, D. and Herrler, G. (1994).** N-acetylneuraminic acid plays a critical role for the haemagglutinating activity of IBV and porcine transmissible gastroenteritis virus. In: *Coronaviruses*. H. Laude and J. E Vaut-herot, eds. Plenum Press, NY. pp. 305-310.
- Schultze, B.; Gross, H.J.; Brossmer, R. and Herrler, G. (1991).** The S protein of bovine coronavirus is a hemagglutinin recognizing 9-O-acetylated sialic acid as a receptor determinant., *J. Virol*, 65: 6232-6237.
- Schultze, B.; Wahn, K.; Klenk, H.D. and Herrler, G. (1991).** Isolated HE-protein from hemagglutinating encephalomyelitis virus and bovine coronavirus has receptor destroying and receptor-binding activity. *Virology* 180: 221-228.
- Thayer, S.G. and Beard, C.W. (1998).** Serologic procedure in: *A lab.manual for the isolation and identification of avian pathogens*. 4th Ed., Pp. 255. Edited by Swayne, D.E.; Glisson, J.R.; Jackwood, M.W.; Pearson, J.E. and Reed, W.M. AAAP Univ. of Pennsylvania New Balton Center Kennett square PA. 19348-1692.
- Trudel, M. and Payment, P. (1980).** Concentration and purification of rubella virus haemagglutinin by hollow fiber ultra-filtration and sucrose density centrifugation. *Can. J. Microbiol.* 26: 1334- 1339.
- Villegas, P. (1998).** Titration of biological suspensions. In: *A laboratory manual for the isolation and identification of avian pathogens*, 4th ed. D. E. Swayne, J. R. Glisson, M. W. Jackwood, J. E. Pearson, and W. M. Reed, eds. Univ. Pennsylvania, Kennett Square, PA. pp. 248-253.

تحضير الانتجين الملزن لكرات الدم الحمراء من عترة الالتهاب الشعبي المغيرة المعزولة من الشرقية (IBV-EG/ SHARKIA –F629-2015)

د. هشام أحمد عبد البديع محمد بيومي

باحث أول امراض الدواجن الفيروسية . معمل بحوث صحة الحيوان بالزقازيق

المخلص العربي

في هذه الدراسة امكن تحضير الانتجين الملزن لكرات الدم الحمراء من معزولة الالتهاب الشعبي (IBV-EG/ SHARKIA –F629-2015) وهي معزولة حقلية مغيرة امكن عزلها من محافظة الشرقية عام ٢٠١٥ في دراسة سابقة تم دراسة التتابع النيكلوتيدي لها .

تم حقن المعزولة في بيض الدجاج المخصب و تم تجميع السوائل الجنينية من البيض المحقون بعد التمريرة الثانية و تم تركيز المعزولة بطريقة الدبلة و بطريقة التدوير بالطرد المركزي عند سرعة ٣٠٠٠ لفة / دقيقة . تمت معاملة نواتج التركيز الفيروسي بالطرق سابقة الذكر بإنزيم الفسفوليبيز و انزيم النيورامينيداز و ذلك في محاولة لإكساب الفيروس خاصية التلازن الدموي . من نتائج الدراسة وجدنا ان تركيز الفيروس بطريقة الطرد المركزي و المعاملة بإنزيم النيورامينيداز قد ادي الي الحصول علي معزولة ملزنة لكرات الدم الحمراء و كانت الوحدة الملزنة لكرات الدم الحمراء موجودة عند التركيز ١:١٠٢٤ . وتكمن القيمة الحقلية لهذا العمل في استعمال هذه المعزولة في اختبارات مانع التلازن الدموي لزيادة الدقة التشخيصية عند زيادة درجة القرابة بين العترة المشخصة و العترات الموجودة حقلياً بمحافظه الشرقية .

عند مقارنة نتائج اختبار مانع التلازن الدموي مع اختبار التعادل المصلي وجدنا ان نتائج اختبار مانع التلازن الدموي قارب اختبار التعادل المصلي في الحساسية و التخصصية . و كذلك يمكن استعمال اختبار التلازن الدموي و اختبار مانع التلازن الدموي في تأكيد الإصابة بمرض الالتهاب الشعبي و في معايرة الفيروس و في معايرة اللقاحات في المعامل التي لا يتوفر لها إمكانيات اختبار RT-PCR