

## **PREPARATION AND EVALUATION OF DIAGNOSTIC KITS OF NEWCASTLE DISEASE: INFECTIOUS BRONCHITIS AND INFECTIOUS BURSAL DISEASE**

Noura, F. Alkhalefa<sup>\*</sup>; Albagory, G.F. <sup>\*\*</sup>; Mansour, A.A. <sup>\*</sup> and Khodeir, M.H. <sup>\*\*\*</sup>

<sup>\*</sup> Faculty of Veterinary Medicine, Kafrelsheikh University

<sup>\*\*</sup> Faculty of Veterinary Medicine, Banha University

<sup>\*\*\*</sup> Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo

### **ABSTRACT**

*The present study was designed to prepare ND, IB and IBD anti sera conjugated with fluoresceine isothiocyanate and horse radish peroxidase to be available on request as local products saving time and cost. The obtained results indicated that the prepared Newcastle Disease (NDV); Infectious Bronchitis (IBV) and Infectious Bursal Disease (IBDV) antisera either in simple forms or conjugated forms with fluoresceine isothiocyanate and horse radish peroxidase are of high quality providing local kits of low cost and on time of request for diagnostic and confirmatory serological tests.*

*In addition, preparation of antisera in goats, which need less husbandry requirements than those required for chicken or rabbits, provide huge amounts of such kits in comparison with those prepared in other hosts, the thing which helps to decrease the cost.*

### **INTRODUCTION**

It is well known that successful control and eradication of infectious diseases depend mainly on accurate diagnosis that depends on application of sensitive, accurate and rapid serological tests. These tests often need specific anti-sera (hyper immune sera containing the specific antibodies)

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either in necked form (for agar gel precipitation test and virus neutralization test) or in conjugated forms with flourescine isothiocyanate (for fluorescent antibody technique) or with horse radish peroxidase (for enzyme linked immune sorbent assay).

Antibodies are useful for specifically recognizing antigens and the detection of antigen-antibody complex could be easy when precipitation occurs. Labeling of antibodies make antigen identification easier.

Specific, rapid and sensitive serological tests like fluorescent antibody technique (FAT) and enzyme linked immune sorbent assay (ELISA) are required for accurate diagnosis of infectious agents to reach to well applicable control measures. FAT depends mainly on antigen-antibody reaction in the presence of a fluorescent dye (flourescin isothiocyanate "FITC") which irradiates with ultraviolet light emitting apple green flourescence (*Tizard, 1996*). The technique depends on the presence of specific antiserum conjugated with FITC. FAT has gained wide acceptance in virology serving for many purposes such as the detection of cellular localization of viral antigens (*Spendlove et al, 1963*); establishment of temporal sequence after infection appearance (**Breitenfeld and Schafer, 1957**); and detection of virus infected cells which fail to produce viral progeny and still synthesize virus coded antigens which continue to harbor viral genetic material (*Tevethia et al, 1965*).

The present study was designed to prepare specific hyper-immune sera against NDV; IBV and IBDV in necked forms and in forms conjugated with flourescine isothiocyanate and horse radish peroxidase to be available on request as local kits avoiding the high cost and long time needed to provide the imported kits. It was taken in consideration to

prepare such anti-sera in different hosts (rabbits, chickens, ducks and goats) to choose and determine the most suitable host (from the quality and economic point of view). The obtained anti-sera (either in necked or conjugated forms) are evaluated through the application of HI; AGPT; SNT; FAT and ELISA.

## MATERIAL AND METHODS

### 1- Vaccines:

Inactivated oil adjuvant Newcastle disease (ND) vaccine (Lasota strain of a titer  $10^9$  EID<sub>50</sub>/ml); infectious bronchitis (IB) live attenuated vaccine (Massachusetts H<sub>120</sub> strain of a titer  $10^{8.5}$  EID<sub>50</sub>/ml) and infectious bursal disease (IBD) vaccine (D78 strain of a titer  $10^{8.5}$  EID<sub>50</sub>/ml) were supplied kindly by Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo. These vaccines were used for preparation of ND; IB and IBD anti-sera in different hosts.

### 2- Viruses:

Vero cell culture adapted strains of ND, IB and IBD viruses were kindly supplied by Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo. These viruses had titers of  $10^{6.5}$ ;  $10^6$  and  $10^7$  TCID<sub>50</sub>/ml respectively. They were used in serum neutralization test (SNT) and in preparation of viral antigens to be used in ELISA.

### 3- Infected cell cultures:

Vero cells were cultured on cover slips in Leighton's tubes in four groups; the first one was infected with NDV and the second one was infected with IBV while the third group was infected with IBDV. The fourth group of cell cultured cover slips was kept without infection as control. On the 2<sup>nd</sup> day post infection these cell cultures were fixed with

child acetone and subjected to direct fluorescent antibody technique using the corresponding prepared conjugated antiserum

#### **4- Virus antigens:**

ND, IB and IBD virus antigens were prepared in vero cell cultures according to *Lefevre and Diallo (1990)* to be used for the evaluation of the prepared anti-sera conjugated with horse radish peroxidase using ELISA.

#### **5- Experimental animals:**

Fifteen of each healthy Bosket rabbits of about 3 kg body weights; SPF chickens of about 2 Kg body weights; ducks of about 2.5-3 Kg body weights and three goats were used for preparation of NDV, IBV and IBDV anti-sera. In addition three of each species were kept as test control. These animals were housed under hygienic measures in separate isolates receiving balanced ration and adequate water. Five hosts of each species were used for preparation of each antiserum.

#### **6- Chicken RBCs:**

Chicken red blood corpuscles were obtained from health chickens and diluted to be 1% to be used in HI according to *Allan et al (1978)*.

#### **7- Cell culture:**

African green monkey kidney cell line (vero) established by *Yasumara and Kaowatika (1963)* were used in serum neutralization test and preparation of virus antigens. These cell cultures were supplied by the department of Pet Animal Vaccine Research, Vet. Ser. Vac. Res. Inst. Abbasia, Cairo, Egypt.

**8- Chemicals used for conjugation of the prepared anti-sera with Fluorescein isothiocyanate (C<sub>4</sub>H<sub>11</sub>NO<sub>5</sub>S) E:**

(C<sub>4</sub> H<sub>11</sub> NO<sub>5</sub> S) E was supplied by Merck, Darmstadt for Microscopy (M.Gew.389.39).

**9- Chemicals and reagents used for conjugation of the prepared antisera with horse radish peroxidase (HRP):**

A- HRP product number p-8375 type VI, lot 25C-9510 was supplied by Sigma Chemical Company. It had an activity of 365 purogallin units/mg.

B- Sodium borohydride (NaBH<sub>4</sub>) was supplied by S.D. fine chemical LTD Company; Chemical Manufacturing Division Fair, Lawn, New Jersey. It had a molecular weight of 105.99.

C- Sodium periodate (NaIO<sub>4</sub>).

**10- Standard anti-sera conjugated with hors radish peroxidase and fluorescein isothiocyanate:**

These conjugates were supplied by the Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, and used in a comparison with the prepared conjugates to evaluate their efficacies.

**11- Preparation of ND, IB and IBD hyper-immune sera:**

Hyper-immune sera against ND, IB and IBD viruses were prepared using the different vaccines according to the method described by *Ihemelanadu et al (1985)*.

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**12- Serum neutralization test (SNT):**

Both qualitative and quantitative SNT were carried out using the micro titer technique according to **Rossiter et al (1985)** and the titer of serum neutralizing antibodies was calculated as the reciprocal of the final serum dilution which neutralized and inhibited the CPE of 100-200 TCID<sub>50</sub> of the used virus according to **Singh, et al (1967)**.

**13- Haemagglutination (HA) and Haemagglutination inhibition (HI) tests:**

HA and HI tests were carried out according to *Allan et al (1978)*.

**14- Agar gel precipitation test (AGPT):**

The test was carried out according to *Cowan and Graves (1966)*.

**15- Precipitation of the immunoglobulin of the prepared antiserum:**

It was carried out using ammonium sulphate according to *Narin and Marrack (1964)*. The globulin content was estimated according to *Weichselbaum (1946)* and adjusted to be 20mg/ml using phosphate buffer saline.

**16- Conjugation of the prepared anti-sera with Fluorescein isothiocyanate:**

It was done according to the method of **Narin (1969)**.

**17- Conjugation of the prepared ND, IB and IBD antisera with HRP:**

It was carried out following up the method described by **Tijssen and Kurstak (1984)**.

### **18- Preparation of viral antigen:**

Preparation of ND, IB and IBD viral antigens was carried out according to *Brian and Hiller (1996)* from infected Vero cells. These antigens were used in ELISA to evaluate the prepared conjugates with horse radish peroxides.

### **19- Direct fluorescent antibody technique (FAT):**

Direct FAT was carried out on infected Vero cells with ND, IB and IBD viruses to evaluate the prepared antisera conjugated with fluoresceine isothiocyanate. The technique was carried out according to *Soliman et al (1989)*.

### **20- Checkboard and Direct ELISA:**

To determine the amount of antigen added during the coating procedure which uptake specific antibodies, a modified checkerboard titration was used according to the comminuted methods of *Hubschle et al (1981)* and direct ELISA was carried out according to *Voller et al (1976)*.

## **RESULTS AND DISCUSSION**

The obtained results could be summarized in the following tables and figures.

**Table (1):** NDV antibody titer in prepared anti-sera.

Prepared antiserum in	ND antibody titer (log <sub>2</sub> /ml) using		
	HI test	SNT	AGPT
Chickens	9	8	8
Ducks	8	7	8
Rabbits	8	7	7
Goats	9	9	7

**Table (2):** IBV antibody titer in prepared anti-sera.

Prepared antiserum in	IB antibody titer (log2/ml) using	
	SNT	AGPT
Chickens	9	8
Ducks	4	3
Rabbits	8	6
Goats	7	6

**Table (3):** IBDV antibody titer in prepared anti-sera.

Prepared antiserum in	IBD antibody titer (log2/ml) using	
	SNT	AGPT
Chickens	8	8
Ducks	7	6
Rabbits	8	8
Goats	7	7

**Table (4):** Evaluation of the prepared anti ND virus hyper-immune serum conjugated with FITC.

Tested conjugate dilution	Tested conjugate prepared in							
	Chickens		Ducks		Rabbits		Goats	
	A*	IC**	A	IC	A	IC	A	IC
1:10	4+	4+	4+	4+	4+	4+	4+	4+
1:100	4+	4+	4+	4+	4+	4+	4+	4+
1:1000	4+	4+	3+	3+	4+	4+	4+	4+
1:10000	3+	3+	2+	2+	3+	3+	4+	4+
1:100000	-	-	-	-	2+	2+	3+	3+
1:1000000	-	-	-	-	-	-	-	-

\*A = antigen

\*\*IC = infected cell culture

4+ = Strong positive reactions.

2, 3+ = Moderate positive reactions.

+ = Weak positive reactions.

- = Negative reactions.

**Table (5):** Evaluation of the prepared anti IB virus hyper-immune serum conjugated with FITC

Tested conjugate dilution	Tested conjugate prepared in							
	Chickens		Ducks		Rabbits		Goats	
	A*	IC**	A	IC	A	IC	A	IC
1:10	4+	4+	4+	4+	4+	4+	4+	4+
1:100	4+	4+	4+	4+	4+	4+	4+	4+
1:1000	3+	3+	2+	2+	4+	4+	4+	4+
1:10000	2+	+	-	-	3+	3+	4+	4+
1:100000	-	-	-	-	+	+	2+	2+
1:1000000	-	-	-	-	-	-	-	-

\*A = antigen

\*\*IC = infected cell culture

4+ = Strong positive reactions.

2, 3+= Moderate positive reactions.

+= Weak positive reactions.

- = Negative reactions.

**Table (6):** Evaluation of the prepared anti IBD virus hyper-immune serum conjugated with FITC.

Tested conjugate dilution	Tested conjugate prepared in							
	Chickens		Ducks		Rabbits		Goats	
	A*	IC**	A	IC	A	IC	A	IC
1:10	4+	4+	4+	4+	4+	4+	4+	4+
1:100	4+	4+	4+	4+	4+	4+	4+	4+
1:1000	4+	4+	4+	4+	4+	4+	4+	4+
1:10000	3+	3+	2+	2+	4+	4+	4+	4+
1:100000	2+	2+	+	+	2+	2+	3+	+
1:1000000	-	-	-	-	-	-	+	+

\*A = antigen

\*\*IC = infected cell culture

4+ = Strong positive reactions.

2,3+= Moderate positive reactions.

+= Weak positive reactions.

- = Negative reactions.

**Table (7):** Check board ELISA.

Antigen dilution	Tested antigen		
	ND	IB	IBD
1:1000	+	+	+
1:2000	+	+	+
1:3000	+	+	+
1:4000	+	+	+
1:5000	+	-	+
1:6000	-	-	-

**Table (8):** Titer of the prepared anti ND virus hyper-immune serum conjugated with horse radish peroxidase using direct ELISA

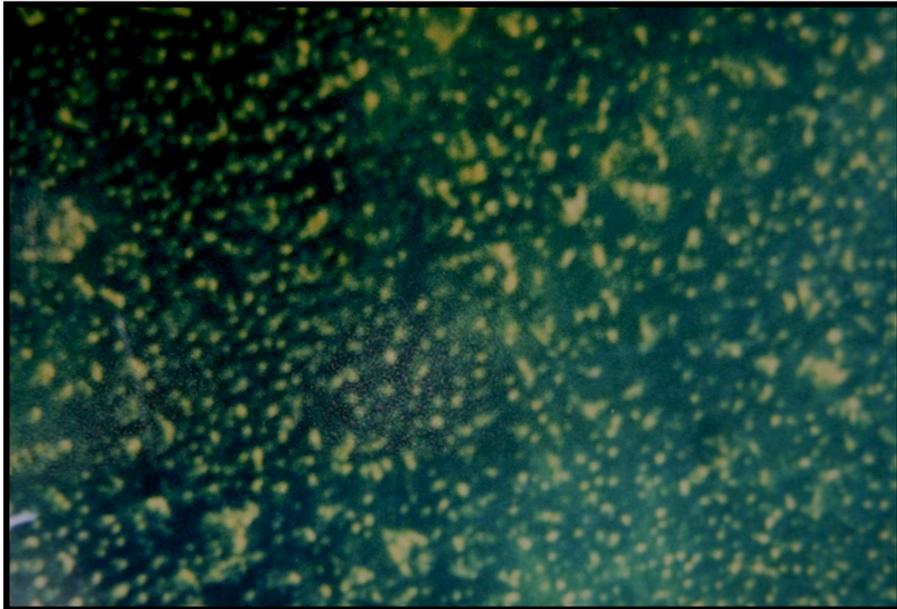
Conjugate Dilution	Conjugate serum prepared in			
	Chickens	Ducks	Rabbits	Goats
1: 100	+	+	+	+
1: 200	+	+	+	+
1: 300	+	+	+	+
1: 400	+	+	+	+
1: 500	+	-	+	-
1: 600	-	-	-	-

**Table (9):** Titer of the prepared anti IB virus hyper-immune serum conjugated with horse radish peroxidase using direct ELISA.

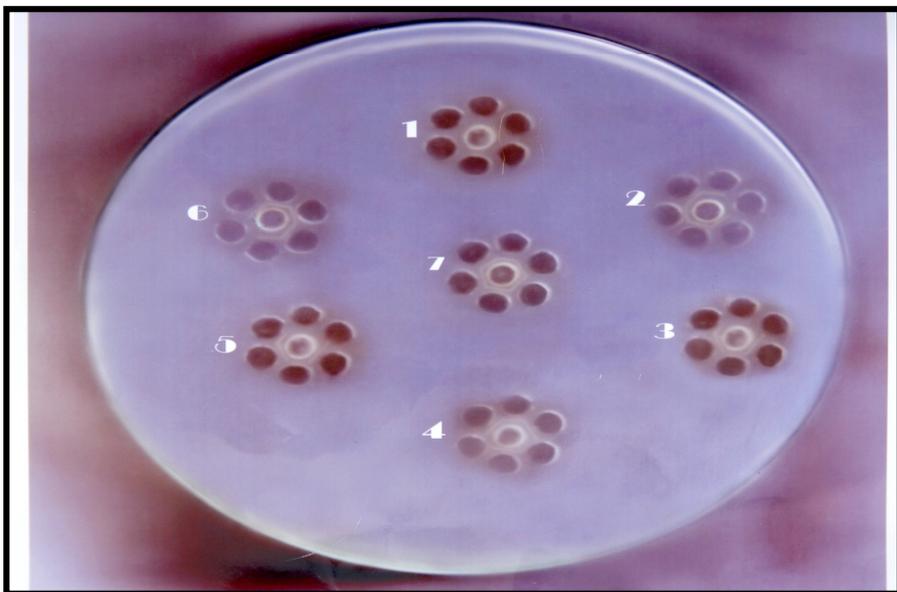
Conjugate Dilution	Conjugate serum prepared in			
	Chickens	Ducks	Rabbits	Goats
1: 100	+	+	+	+
1: 200	+	+	+	+
1: 300	+	+	+	+
1: 400	+	+	+	+
1: 500	+	-	+	-
1: 600	-	-	-	-

**Table (10):** Titer of the prepared anti IBD virus hyper-immune serum conjugated with horse radish peroxidase using direct ELISA.

Conjugate Dilution	Conjugate serum prepared in			
	Chickens	Ducks	Rabbits	Goats
1: 100	+	+	+	+
1: 200	+	+	+	+
1: 300	+	+	+	+
1: 400	+	+	+	+
1: 500	+	-	+	-
1: 600	-	-	-	-



**Fig(1):** Positive FAT applied on viral antigens (4+) showing strong apple green fluorescent reaction (100x).



**Fig (2):** AGPT showing clear precipitation lines with positive sera.

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During the present work different hosts were used for preparation of NDV; IBV and IBDV antisera in order to choose the best one from the quality and economic view.

The obtained results revealed that the prepared NDV antiserum had antibody titers of 9 log<sub>2</sub>; 8log<sub>2</sub> and 8log<sub>2</sub> in chicken serum; 8log<sub>2</sub>; 7log<sub>2</sub> and 8log<sub>2</sub> in duck serum; 8log<sub>2</sub>, 7log<sub>2</sub> and 7log<sub>2</sub> in rabbit serum and 9log<sub>2</sub>, 9log<sub>2</sub> and 7log<sub>2</sub> in goat serum as estimated by HI, SNT and AGPT respectively showing that the highest titers were detected in chicken and goat sera as shown in (Table-1). These titers appear to be of high levels and approach to those of *Box and Furamengen (1975)* and supported by the findings of *Ali and Abdul Aziz (1991)* who showed that the fourth vaccination with ND vaccine increased the antibody titers.

Regarding IBV antibody titers were found to be 9log<sub>2</sub> and 8log<sub>2</sub> in chicken serum; 4log<sub>2</sub> and 3log<sub>2</sub> in duck serum; 8log<sub>2</sub> and 6log<sub>2</sub> in rabbit serum and 7log<sub>2</sub> and 6log<sub>2</sub> in goat serum as shown in (Table-2) as estimated by SNT and AGPT respectively, and this appeared to agree with *Chubb and Cumming (1971)* who recorded that Precipitin appeared regularly in infected birds and persisted for at least 3 months.

Concerning the IBD antiserum showed antibody titers of 8log<sub>2</sub> in chicken serum; 7log<sub>2</sub> and 6log<sub>2</sub> in duck serum; 8log<sub>2</sub> in rabbit serum and 7log<sub>2</sub> in goat serum tabulated in (Table-3) by SNT and AGPT respectively. These results come in parallel with those reported by *Hiari and Shamakura (1972)* who recorded that. The precipitating antibodies were first detected 2 weeks after infection and stronger precipitin reaction was seen at 4 weeks after infection and similar to that reported by *Abd El- Wanis and Khodeir (2004)* who recorded that IBDV neutralizing antibody titers in the prepared hyper immune sera in

different hosts (chicken, duck, and rabbit) were 1024, 256, and 128, respectively and higher than that recorded by *Oladele et al (2004)* who determined the presence of infectious bursal disease virus (IBDV) antibodies in village chickens in Oyo state, Nigeria, out of 392 sera tested, 248 (63.3%) samples were positive with a titer rang of log<sub>2</sub> to 4 log<sub>2</sub>. This difference in the titers could be attributed to increase antibodies formation in response to repeated vaccination.

In order to evaluate the prepared FITC conjugated antisera; the direct fluorescent antibody technique was carried on virus antigens and infected Vero cells. *NDV-FITC conjugated antisera* showed positive fluorescent apple green reactions up to a dilution of 1:10000 for chicken and duck conjugates and 1:100000 for rabbit and goat conjugates as shown in (Table-4). These results appeared to be higher than that reported by *Gervelmeyer et al (1998)* who reported that In the direct fluorescent antibody test the 1:4 or 1:8 in phosphate buffer saline diluted conjugates reacted specifically with their homologous antigen in the impression smear prepared from organs of experimentally infected chickens and in cover slip cell cultures when used vaccines containing inactivated Newcastle disease (ND), Infectious bronchitis (IB) and Infectious bursal disease (IBD) viruses. This disagreement could be attributed to repeated vaccination.

On the other hand the *FITC conjugated anti-IBV sera* showed positive reactions up to 1:10000 for chicken conjugate; 1:1000 for duck conjugate and 1:100000 for rabbit and goat conjugates as shown in (Table-5) . These results appeared to be higher than that recorded by *Jones (1975)* who mentioned that IFA titers were detectable as early as one week after IBV infection and persisted for at least 6 weeks. Titers

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never exceeded 1:64. This disagreement could be due to higher antibody levels in the prepared sera lead to use of high dilution of conjugated anti sera.

Regarding to *the prepared anti IBD sera conjugated with FITC* revealed positive reactions up to a dilution of 1:100000 for chicken; ducks and rabbit conjugates and 1:1000000 for goat conjugate as it is clear from (Table-6). Similar findings were described by ***Abd El-Wanis and Khodeir (2004)*** who found that the prepared conjugates with (FITC) were able to detect the IBDV in different sites of infected eggs when they were diluted up to 1:105 giving strong, moderate or weak positive FAT according to the virus concentration in the tested sites.

Among the evaluation results of the prepared anti ND virus hyper immune serum conjugated with horse radish peroxidase using direct ELISA, it was found that positive results were obtained with conjugate dilution of 1: 500; 1:400; 1:500 and 1:400 for chicken; duck; rabbit and goat conjugates respectively , as shown in (Table-7).

IBV antisera conjugated with horse radish peroxidase showed positive results up to dilutions of 1:500 for chicken and rabbit conjugates and 1:400 for duck and goat conjugates as shown in (Table-8).

It was found that horse radish peroxidase Conjugated IBDV antisera showed positive results up to dilutions of 1:500 for chicken and rabbit sera and 1:400 for duck and goat sera, as shown in (Table-9). These results were lower than that reported by ***Marguardt et al (1979)*** who reported that the chromatographically purified rabbit anti chicken immunoglobulin-G conjugated with horse radish peroxidase was used optimally at dilution of 1:3000.

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## تحضير وتقييم كواشف تشخيصية لأمراض النيوكاسل والإنتهاب الشعبي المعدى والتهاب غدة فابريشس

د/نورا فيصل الخليفة\* ، أ/د/ جبر فكرى الباجورى\*\*

أ/د/ على عبد القادر منصور\* ، أ/د/ محمد حسن خضير\*\*\*

\* كلية الطب البيطري - جامعة كفر الشيخ - \*\* كلية الطب البيطري - جامعة بنها

\*\*\* معهد بحوث الأمصال واللقاحات البيطرية - العباسية - القاهرة

صممت الدراسة الحالية لتحضير أمصال نوعية مضادة لكل من فيروسات النيوكاسل والإنتهاب الشعبي المعدى والجمبورو (مرض غدة فابريشس المعدى) فى صور مفردة وصور مقترنة بمادة الفلوريسين أيزوثيوسينات والهورس راديش بيروكسيديز لزوم التعريف بمعزولات الفيروسات الثلاثة سيولوجيا بتطبيق أختبارات الفيروس المتعادل والترسيب المناعى فى الأجار والوميض الفلوريسنتى المناعى المباشر والأنزيم المرتبط الممدص المناعى على أن تحضر هذه الأمصال فى عوائل مختلفة لاختيار الأنسب منها من حيث القيمة العيارية والتكلفة الاقتصادية لتكون متاحة وقتما يتطلب الأمر توفيراً للوقت الذى قد يطول عند إستيراد مثل هذه المواد إضافة إلى توفير المال فى الغالب ما يكون المثل المستورد ذو قيمة عالية.

هذا وقد أوضحت النتائج أن المصل المضاد لفيروس النيوكاسل وجد أنه ذو معيار 9لو2 ، 8 لو2 للمصل المحضر فى الدجاج باختبار منع التلزن الدموى وأختبارى الترسيب فى الآجار والمصل المتعادل على التوالى. بينما كانت هذه القيم 8لو2، 7لو2 و8لو2 على التوالى للمصل المحضر فى البط وكانت مثيلاتها 8لو2، 7لو2 و7لو2 فى المصل الأرنبى و 9لو2، 9لو2 و7لو2 فى المصل الماعزى على التوالى. أما الأمصال المضادة للإنتهاب الشعبى المعدى فقد أظهرت المعايير 9لو2 و8لو2 فى

مصل الدجاج باختبار المصل المتعادل والترسيب فى الآجار على التوالى، 4لو2 و3لو2 بنفس الأختبارين على التوالى فى المصل المحضر فى البط، 8لو2 و6لو2 للمصل المتعادل والترسيب فى الآجار تواليا للمصل الأرنبى، 7لو2 و6لو2 على التوالى للمصل الماعزى. كما كان للمصل المضاد للجمبورو معايير باختبار المصل المتعادل والترسيب فى الآجار 8لو2 بكلا الأختبارين للمصل الدجاجى، 7لو2 و6لو2 على التوالى للمصل المحضر فى البط، 8لو2 و7لو2 بكلا الأختبارين للمصل الأرنبى والماعزى على التوالى.

عند إجراء أختبار الوميض الفلوريسنتى المناعى على خلايا الزرع النسيجى المعدية بالفيروسات قيد التجربة أو أنتيجيناتها، أعطت الأمصال المضادة للنيوكاسل والمقترنة بالفلوريسين أيزوثيوسينات نتائج إيجابية حتى تخفيف 1: 10000 للأمصال المحضرة فى الدجاج والبط وحتى تخفيف 1: 100000 للأمصال المحضرة فى الأرناب والماعز وأظهرت الأمصال المضادة للالتهاب الشعبى المعدى والمقترنة بنفس المادة نتائج إيجابية حتى تخفيف 1: 10000 للأمصال الدجاج 1: 1000 للمصل البط، 1: 100000 للأمصال الأرناب والماعز 0 كذلك تم الحصول على نتائج إيجابية للأمصال المقترنة المضادة للجمبورو حتى تخفيف 1: 100000 للأمصال الدجاج والبط وحتى تخفيف 1: 1000000 للأمصال الأرناب والماعز.

بتقييم الأمصال المقترنة بالهورس راديش بيروكسيدز باختبار الأنزيم المرتبط المناعى الممدص وجد أن الأمصال المقترنة المضاد للنيوكاسل تعطى نتائج إيجابية حتى التخفيفات 1: 500، 1: 400، 1: 500، 1: 400 للأمصال الدجاج والبط والأرناب والماعز على التوالى وأعطت أمصال الالتهاب الشعبى المعدى نتائج إيجابية حتى التخفيفات 1: 500 للأمصال الدجاج والأرناب و1: 400 للأمصال البط والماعز كما تم الحصول على نتائج إيجابية للأمصال الجمبورو حتى تخفيف 1: 500 للأمصال الدجاج والأرناب و1: 400 للأمصال البط والماعز.