Central Laboratory for Evaluation of
Veterinary Biologics, Abbasia, Cairo.

USING OF IMMUNOFLUORESCENT TECHNIQUE FOR TESTING OF LIVE ATTENUATED VACCINES EXPERIMENTALLY CONTAMINATED WITH AVIAN REOVIRUS

(With 2 Tables and One Figure)

By

A.A. BADAWI; S.A. NASSIF; ELHAM A. EL-EBIARY and M.M. TAHA

(Received at 29/9/2005)

استخدام اختبار الإشعاع الفلورسنتى المناعى لاختبار اللقاحات الحية المستضعفة للكشف عن تلوثها بفيروس الريو

أحمد عبد اللطيف بدوى ، سمير عبد المعز ناصف ، إلهام عطا الإبيارى، محمد محمود طه

تم استخدام اختبار الإشعاع الفلورسنتى للكشف عن وجود فيروس الريو كملوث فيروسى فى أربعة أنواع من لقاحات الدواجن المستضعفة هى لقاح الإلتهاب الشعبى، لقاح النيوكاسل، لقاح الماريك، ولقاح جدرى الطيور. أثبت الاختبار حساسيته وتخصصه فى الكشف عن فيروس الريو. وأمكن الاعتماد عليه وحده فى التعرف على الفيروس كملوث فيروسى فى هذه اللقاحات. على العكس فإن التأثير السيتوباثولوجى لفيروس الريو قد تداخل مع التأثير الناتج من وجود فيروسات اللقاحات المختبرة ولذلك فإنه لا يكفى الاعتماد عليه فقط فى التعرف على فيروس الريو فى اللقاحات. هذا التأثير السلبى الناتج عن وجود فيروسات اللقاحات تم التخلص منه عن طريق المعالجة بالحرارة عند درجة ٥٦°م لمدة ٤٥ دقيقة.

SUMMARY

Immunofluorescence (IF) technique was used for detection of reovirus contamination in four types of live attenuated poultry vaccines; IB, ND, MD and fowl pox vaccines. IF technique effectively detected reovirus in all types of vaccines and it proved specificity and sensitivity. In contrast, the cytopathogenic effect (CPE) of reovirus on infected cultures was interfered with that of the vaccine virus and so it could not be taken alone as a criterion for detection of reovirus as a contaminant in live poultry vaccines. Specificity of IF technique slightly affected by the

presence of vaccine virus. This adverse effect was disappeared when the vaccine virus was excluded by heat inactivation at 56°C for 45 minutes.

Key words: *Vaccination, immunofluorescent technique, avian reovirs*

INTRODUCTION

Egg transmission of reovirus was demonstrated by several workers (Deshmukh and Pomeroy, 1969, Menendez et al., 1975 and Van der Heide and Kalbac, 1975). Chicken embryos are considered one of the most important system used for vaccine production either by production of cell cultures or by different routes of egg inoculation. Eggs produced by reovirus-infected flocks is an ideal source for biocontamination of vaccines. Reovirus could be detected by different methods as virus neutralization, agar gel precipitation test, plaque assay and immunofluorecent techniques. The fluorescent antibody test had been used by several workers for detection of reovirus in different tissues and cell cultures (Ni and Kemp, 1995) but it did not used for detection of reovirus in contaminated vaccines. This study was designed to evaluate the efficacy of IF technique in detection of reovirus in some poultry vaccines and also aimed to determine to which extent the specificity and sensitivity of IF test could be affected by the presence of vaccine viruses.

MATERIALS and METHODS

Tissue cultures:

Primary chicken embryo fibroblasts (CEF) cells were obtained from 10-day-old embryos of specific pathogen free eggs. Primary cells were prepared as described by Ni and Kemp (1995) and 5 x 10^5 cells per ml were seeded in MEM containing 10% calf serum and 0.5 gm of sodium bicarbonate /1 litre.

Viruses:

Commercial reovirus vaccine based on the S1133 isolate of L. Van der Heide was used. Reovirus was propagated by inoculation onto confluent monolayers of chicken embryo fibroblast cells.

Titration of reovirus was performed by conventional plaque titration technique using agar overlay as described by Ni and Kemp (1995).

Based on the numbers of plaque forming units, titre was $10^{3.8}$/0.1 ml.

Vaccines:

Four types of poultry-live attenuated vaccines were used in this study, namely; infectious bronchitis (IB), Newcastle disease (ND), Marek's disease (MD) and fowl pox (FP) vaccines were obtained commercially as vaccines ready for use in the field.

Production of antisera:

Twenty SPF chickens 3 to 4 weeks old were inoculated each with approximately 10^6 PFU of purified reovirus preparation as described by Hieronymus *et al.* (1983). Two weeks later, they were reinfected as before. Blood was obtained 2 weeks later and the serum was collected, pooled, heat inactivated at 56°C for 45 minutes and stored in 3 ml aliquots at -20°C. The virus neutralization test was used for titration of antibodies against reovirus. The prepared serum batch had a neutralization index equal to 3.2.

Virus neutralization (VN) test:

The decreasing virus, constant serum method VN test was performed in a microtitre system, using chicken embryo fibroblasts as described by Rau *et al.* (1980). Serial ten fold dilutions of reovirus (10^{-1} through 10^{-8}) were prepared in duplicate. One volume of each virus dilution was added to equal amount of serum. Virus and virus-serum mixtures were added to the wells of CEF cultures, 5 wells were maintained uninfected as negative control wells. Medium was added and cells were incubated then cultures were examined microscopically for titre calculation. Each well with distinct cytopathogenic effect such as syncytial or round cell formation, was considered positive. End points were determined using the method of Reed and Muench (1938). Neutralization indices were expressed as negative reciprocal of the difference between titres of virus and virus-serum mixtures.

Immunofluorescent (IF) technique:

It was used to monitor virus replication in infected CEF cultures as described by Ni and Kemp (1995). Cells in 96-well TC plates were fixed with a mixture of acetone and 95% ethanol (6:4), then it was treated with chicken anti-reovirus serum diluted 1:50 in PBS and incubated for 30 minutes at 37°C in water bath, then washed 3 times with PBS and stained with rabbit anti-chicken FITC conjugated IgG (Sigma) for 30 minutes before washing with PBS and then were mounted by a drop of glycerol diluted 1:2 in PBS and examined in the inverted position with a fluorescent microscope. The scoring criteria for IF were:

* - = non-infected cells detected.
* + = an average of 1-10 infected cells detected per field at 250x magnification.
* ++ = an average of more than 10 infected cells detected per field at 250x magnification.

Experimental Design

Trials (1):

A pool of live attenuated infectious bronchitis (IB) virus vaccine were rehydrated with sterile saline and diluted at the rate of 10 doses/0.1 ml (European Pharmacopoeia, 1997 and British Pharmacopoeia, 2002).

Strain S1133 of reovirus was used for contaminating the diluted IB vaccine. A stock of reovirus preparation with a titre of $10^{3.8}$ $TCID_{50}$/0.1 ml was 10-fold diluted (from 10^{-1} through 10^{-6}) and one volume from each virus dilution was mixed with an equal volume of diluted IBV vaccine in a separate vial. Each vial of contaminated vaccine was then divided into 2 parts, the first part was heat-treated at 56°C for 45 minutes, while the second part was neutralized with specific IBV antiserum (1:1). After either heat treatment or neutralization, the contaminated vaccine was then inoculated onto confluent monolayers of CEF in 96-well tissue culture plate (50 µl/well), each sample was inoculated on each of 5 wells. Plates were incubated for 30 minutes at 37°C for adsorption then medium was added (150 µl/well) and incubated in 5% CO_2 atmosphere at 37°C. Each dilution of reovirus (from 10^{-1} to 10^{-6}) was inoculated on each of 5 wells as positive controls, while another 5 wells were kept uninoculated as negative controls. All cultures were examined daily for abnormalities or CPE. The specific CPE for reovirus was scored as: - = negative, + = moderate, ++ = severe. When CPE appeared in about 50% of infected cultures, immunofluorescent (IF) technique was conducted using rabbit anti-chicken FITC conjugated IgG.

Trial (2):

To check the specificity of reagents used in IF test, three other vaccine types, namely Marek's disease (MD), Newcastle disease (ND), and fowl pox (FP) vaccines were diluted at the rate of 10 doses/0.1 ml and it then deliberately contaminated with reovirus dilutions, one volume of each dilution of reovirus (from 10^{-1} to 10^{-6}) was mixed with an equal volume of each diluted vaccine. Each vial was then divided into 2 parts, one part was inoculated into 5 replicates of CEF cultures, while the second part was treated with reovirus antiserum for 30 minutes at

37°C and then inoculated on CEF cultures and incubated as before. IF test was conducted when CPE was appeared in about 50% of infected cultures. Positive and negative cells were maintained parallel to the test.

RESULTS

In cultures inoculated with reovirus alone (group 1) a specific CPE was observed starting on the second day post infection. Syncytia formation, rounded cells and areas of monolayers had detached. No CPE was recorded in uninoculated cultures. The peak CPE was recorded at 10^{-1} dilution then it was gradually decreased until it completely disappeared in dilution 10^{-5} as shown in Table 1. In cultures inoculated simultaneously with reovirus and IB virus (group 2), the CPE was not clear and non-specific, enlarged, rounded and detached cells were observed from dilution 10^{-1} to dilution 10^{-5}. When reovirus fraction was neutralized with specific antiserum, non-specific CPE still recorded but to lesser extent (group 5).

Immunofluorescence was detected in all groups infected with reovirus either alone or simultaneously with IBV except in group 5 in which antiserum against reovirus was used. The degree of immunofluorescence varied greatly in different groups according to the type of inoculum and treatment used and even in the same group from one dilution to another (Table 1). The peak immunofluorescence was detected in group 1 and group 3, while the least IF activity was recorded in groups 2 and 4.

Discrepancy was recorded between IF and CPE in groups 2, 4 and 5. In group 2, positive immunofluorescent was recorded only in 10^{-1} and 10^{-2} dilutions while CPE was recorded up to the 10^{-5} dilution (Table 1). In group 4 when IB antiserum was used, CPE appeared in the first four dilutions while immunofluorescence could be detected in the 10^{-1} and 10^{-2} dilutions only.

Although there was no IF activities in group 5, a CPE was observed at the first 3 dilutions.

Neither IF activity nor CPE was detected in uninoculated control cultures.

The efficacy of IF test was checked to evaluate its specificity for detection of reovirus only, even in the presence of other avian viruses in the same inoculum.

Results in Table (2) shows a negative IF in groups 2, 4 and 6 in which reovirus antiserum was used to exclude its effect on inoculated cultures. At the same time, when reovirus was not neutralized (groups 1,

3 and 5), a positive IF was obtained indicating the specificity of the test although the degree of immunofluorescence was lesser than that obtained in control group 7.

The presence of vaccine virus adversely affect the results of IF as shown in Table 2. The effect of MDV, NDV and FPV was comparable and all of them reduced IF results when it is compared with the positive control (group 7).

Table 1: Results of immunofluorescence test (IF) and cytopathogenic effect (CPE) in CEF cultures infected with IB vaccine deliberately contaminated with strain S1133 of reovirus

Group No.	Inoculum		Test used	10^x dilutions of reovirus					
	Type	Treatment		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	Reovirus	Untreated	IFA*	+++	++	+	+	-	-
			CPE**	++	++	+	+	-	-
2	IB+reovirus	Untreated	IFA	+	+	-	-	-	-
			CPE	++	++	++	+	+	-
3	IB+reovirus	56°C for 45 min.	IFA	+++	++	+	+	-	-
			CPE	++	++	+	-	-	-
4	IB+reovirus	Antiserum against IBV	IFA	++	+	-	-	-	-
			CPE	++	++	++	+	-	-
5	IB+reovirus	Antiserum against reovirus	IFA	-	-	-	-	-	-
			CPE	+	+	+	-	-	-
6	TC medium (control)	Untreated	IFA	-	-	-	-	-	-
			CPE	-	-	-	-	-	-

* - = Non-infected cells, + = 1-10 infected cells/field, ++ = more than 10 infected cells / field
** - = negative, + = moderate, ++ = severe

Table 2: Results of immunofluorescence test (IF) for detection of reovirus in different types of vaccines deliberately contaminated either with neutralized or non-neutralized reovirus

Group No.	Inoculum		10^x dilutions of reovirus					
	Type	Treatment	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	MDV+reovirus	Untreated	++ *	+	-	-		-
2	MDV+reovirus	Reovirus serum	-	-	-	-	-	-
3	NDV+reovirus	Untreated	+	+	-	-	-	-
4	NDV+reovirus	Reovirus serum	-	-	-	-	-	-
5	FPV+reovirus	Untreated	++	-	-	-	-	-
6	FPV+reovirus	Reovirus serum	-	-	-	-	-	-
7	Reovirus	Untreated	+++	++	+	+	-	-
8	TC medium (control negative)	Untreated	-	-	-	-	-	-

* - = Non-infected cells, + = 1-10 infected cells/field, ++ = more than 10 infected cells / field

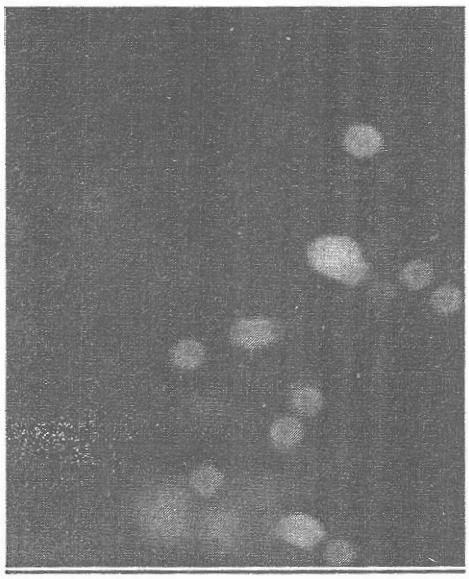

Fig. 1: Reovirus antigen detected by immunofluorescent staining in CEF culture at 48 hours post inoculation. Intracytoplasmic granular are seen in enlarged cells (x250)

DISCUSSION

The present study dealt with detection of avian reovirus contamination in some poultry live vaccines and how could this virus be detected by using immunofluorescence (IF) test and at the same time was aimed to study if there is an adverse effect may be played by the vaccine virus on the efficacy and specificity of IF test.

Results in Table (1) indicated that presence of IBV had an adverse effect on results of IF test. This adverse effect was disappeared when the inoculum was treated either by heat at 56°C for 45 minutes or neutralized by antiserum specific to IB virus. Heat inactivation was more efficient and more potent than neutralization, so it may be used as an alternative tool for detection of reovirus in IB vaccine to exclude the effect of IBV.

IBV is inactivated after 15 minutes at 56°C (Otsuki *et al.*, 1979), but reovirus is heat stable and can resist 56°C for up to 6 hours (Rosenberger and Olson, 1997). Using of antiserum was less efficient and it may be due to the fact that vaccine virus is highly concentrated

(1000 or 2500 doses/vial) and need more quantity of serum to be neutralized.

Cytopathogenic effect (CPE) was augmented when both reovirus and IBV were simultaneously inoculated. This CPE seems to be due to not only reovirus but also IBV may share this effect. IBV strain is a cell culture-adapted and can produce CPE in infected cells (Otsuki *et al.*, 1979). In this test, the recorded picture of CPE is considered false positive because the use of reovirus antiserum did not completely inhibit this CPE. This may explain the discrepancy between results of IF and the obtained CPE in group 2, as the CPE was recorded until the 10^{-5} dilution while IF was detected only in the 10^{-1} and 10^{-2} dilutions which may suggests the specificity of IF test because it detected only the reovirus-infected cells while the CPE was a summation of the dual effect of reovirus and the vaccine virus.

Specificity of reagents used in IF test was checked to be sure that it will detect reovirus only even in the presence of other avian viruses. As shown in Table (2), when reovirus was neutralized with specific antiserum, a negative IF was obtained in the presence of MD, ND and FP viruses. Interestingly, a negative IF was obtained although there was CPE in the inoculated cultures (data not shown). This suggest that the observed CPE was due to the vaccine virus but not to reovirus and also means that IF test is more accurate than the CPE.

It is concluded that IF test is a sensitive and specific test and could be used for detection of reovirus as contaminant in live attenuated poultry vaccines. The presence of vaccine virus may affect the sensitivity but not specificity of IF test.

So, the effect of vaccine virus should be completely excluded either by heat inactivation or neutralization with specific antiserum. Heat inactivation is superior than virus neutralization, but it is limited to heat labile viruses. In case of heat stable viruses, the quantity and quality (titre) of antiserum must be enough to completely neutralize the vaccine virus.

REFERENCES

British Pharmacopoeia (2002): Veterinary, pp. A19-A20.

Deshmukh, D.R. and Pomeroy, B.S. (1969): Avian reoviruses. III. Infectivity and egg transmission. Avian Dis., 13: 427-439.

European Pharmacopoeia (1997): 3rd ed. Strasbourg, France, European Pharmacopoeia Secretariat.

Hieronymus, D.R.K.; Villegas, P. and Kleven, S.H. (1983): Identification and serological differentiation of several reovirus strains isolated from chickens with suspected malabsorption syndrome. Avian Dis., 27: 246-260.

Menedez, N.A.; Calnek, B.W. and Cowen, B.S. (1975): Experimental egg transmission of avian reovirus. Avian Dis., 19: 104-111.

Ni Yawei and Kemp Maurice (1995): A comparative study of avian reovirus pathogenicity: virus spread and replication and induction of lesions. Avian Dis., 39: 554-566.

Otsuki, K.; Noro, K.; Yamamoto, H. and Tsubokura, M. (1979): Studies on avian infectious bronchitis virus (IBV) 2. Propagation of IBV in several cultured cells. Arch. Virol., 60: 115-122.

Rau, W.E.; Van der Heide, L.; Kalbac, M. and Girshick, T. (1980): Onset of progeny immunity against viral arthritis/tenosynovitis after experimental vaccination of parent breeder chickens and cross immunity against six reovirus isolates. Avian Dis., 24: 648-657.

Reed, L.J.; and Munench. H. (1938): A simple method for estimating fifty percent endpoints. Am. J.Hyg. 27: 493-497.

Rosenberger, J.K. and Olson, N.O. (1997): Viral arthritis in: Diseases of Poultry. 10th ed. pp. 711-719.

Van der Heide, I. and Kalbac, M. (1975): Infectious tenosynovitis (viral arthritis): characterization of a Connecticut isolate as a reovirus and evidence of viral egg transmission by reovirus infected broiler breeders. Avian Dis., 19: 683-688.