Department of Virology,
Animal Health Research Institute,
Agricultural Research Center, Egypt.

PRODUCTION OF EGG YOLK - BLUETONGE SPECIFIC ANTIBODIES (IGY) FOR THE DIAGNOSTIC PURPOSES OF BLUETONGUE VIRUS INFECTION

(With One Table, 3 Figures and 2 Photos)

By

A.R.A. HABASHI; I.A. KARIM and H.B. HASSAN*

*Faculty of Veterinary Medicine, Tanta University Kafr-El-Sheikh Branch

(Received at 18/8/2005)

انتاج الأجسام المناعية المضادة لفيروس مرض اللسان الازرق فى مح البيض وإستخدامها فى تشخيص المرض

أحمد رفعت احمد حبشى ، اكرام عبد الكريم اسماعيل ، حسن بدر حسن

يمثل هذا البحث دراسة تجريبية لتقييم إنتاج الأجسام المناعية المضاده لفيروس اللسان الأزرق فى الدجاج المحصن والمحقون باللقاح المتعدد ضد فيروس اللسان الأزرق وكفاءة استخدامها فى أغراض تشخيص المرض: تم الكشف على تكون الأجسام المناعية المضادة لفيروس السان الأزرق في سيرم الدجاج المحصن بأستخدام اختبار الأجار الترسيبى. تم تجميع عينات سيرم وبيض خلال فتره التجربة (٢١ اسبوع) وكذلك فصل الجلوبيولين المناعى (IgY) من مح البيض بترسيبه بسلفات الأمونيوم. بإستخدام اختبار الأليزا ثم مقارنة بين الأجسام المناعية فى السيرم و تلك المستخرجة من مح البيض حيث وجد ان مستوى الأجسام المناعية بهما متساوياً ويستمر مستوى الأجسام المناعية عالياً فى مح البيض لفترة أطول بالمقارنة بمستواه في الدم مما يتيح لنا انتاج كمية كبيرة من الأجسام المناعية المضاده للفيروس من كل دجاجة محصنة ضده معتمدا علي حقن جرعات منشطة من الفيروس واستمرارية انتاج البيض. تم ايضا إثبات كفاءة استخدام هذه الأجسام المناعية المستخرجه من مح البيض فى الكشف عن انتيجين فيروس اللسان ألأزرق وتشخيص المرض بإستخدمها فى اختبار الفلوسنت المناعى المشع للتعرف على ١٣ معزلا تمثل ٥ عترات من الفيروس.

SUMMARY

An experimental study was carried out to evaluate a new simple method for massive production of bluetongue virus (BTV) specific antibodies by specific IgY extraction from the egg yolk of immunized hens with BTV polyvalent vaccine. After detection of the development of the serum precipitating antibodies, serum samples and egg batches were collected from the immunized hens at regular intervals then

titrated by the indirect ELISA. The serum antibody titres were compared with the extracted egg yolk IgY antibody titres during the period of the study which elapsed 21 weeks and during this period 3 doses were injected. The results revealed that the egg BTV specific antibody titres were nearly similar to the serum antibody titres providing a higher yield of specific antibodies can be collected from the immunized hens depending on the availability egg production and occasional posturing. The diagnostic efficiency of the prepared egg yolk antibodies was also proved by successful detection of 13 BTV isolates using it in the procedures of indirect fluorescence antibody technique.

Key words: *Virology, blue tongue, antibodies.*

INTRODUCTION

The use of chicken as alternative labanimal for polyclonal antibodies production has described by (Polson *et al.,* 1980). Hens could be the antibody factories of the future and the production of specific polycolonal antibodies in the chicken egg yolk may be used for diagnosis of various diseases of poultry, livestock and human (Anonymous 1994). The egg yolk of chicken is recognized as a rich source of specific antibodies (Arshad *et al.,* 1996). These include the advantages of producing more specific antibodies against mammalian antigens in birds compared to mammals because of the phylogenic distance between birds and mammals, low cost of production, convenience and what is becoming important, compatibility with regulation for modern animal production and animal welfare. Relatively small amount of highly conserved mammalian protein are sufficient to induce a sustained immune response. Chicken egg- yolk antibodies are also phylogenetically distinct from mammalian antibodies. Therefore they can be used in a multiple antibody assay with mammalian antibodies with minimal cross-reactivity (Sim and Nakai 1994).

There is a prerequisite for full understanding and evaluation the efficiency of this system for production specific antibodies against important diseases and their use for diagnostic purposes. Bluetongue (BT) is an infectious non contagious insect-born viral disease of sheep, caused by an orbivirus. Severe disease usually occurs in certain breeds of sheep, particularly the fine-wool and mutton breeds. Cattle, goats and wild ruminants can be also infected, but usually with mild or no clinical signs (Howell, 1963; Erasmus, 1975; Mellor, 1996). The Disease is worldwide distributed and endemic in temperate climate areas (Gibbs *et al.* 1989). The importance of bluetongue virus became very clear especially after occurrence recent out breaks in many

countries around the world since a long period of silent circulation of the virus without showing clinical disease conditions (OIE disease information 1999- 2001).

The aims of present study are:

1. Evaluation of a new simple method for massive production of specific antibodies against bluetongue virus by extraction of IgY from the egg yolk of hens immunized with BTV polyvalent vaccine.
2. Determination of the diagnostic efficiency of the experimentally prepared egg yolk antibodies.

MATERIALS and METHODS

According to Hudson and Hay (1991), Immunization of laying hens with BTV was done by 1ml oil emultion of an equal volume of both the tissue culture bluetongue polyvalent vaccine was obtained from Serum and Vaccine production Institute, Abassia, Cairo, Egypt and Ferund's complete adjuvant "Sigma" injected subcutaneously at both sides of the chest and the inoculation was repeated after 3 weeks and 3 months from the frist dose.

24 serum samples, and 17 egg batches were collected from the immunized hens at regular intervals from the zero day during the period of this study (21weeks) depending on the availability of laying eggs. Six normal chicken control sera (with no record of BTV antigen inoculation) were used as control negative.

According to the method described previously by Pearson and Jochim (1979), development of specific antibodies was detected by AGPT in the serum samples collected 2 weeks after the 2^{nd} and 3^{rd} imunization of the hens. 0.9% agarose was prepared in isotonic saline solution (0.85% NaCI in distilled water). A test pattern with a central well and 6 peripheral wells was used, with each well measuring 4 mm in diameter and separated by a distance of 2.4 mm between each well and positive-control serum was placed in 3 peripheral wells alternately with 3 undiluted test sera; and the central well was filled with the Bluetongue AGID reference antigen "Pirbright England" Each well contained approximately 0.03 ml. Three samples were tested with each pattern. Test plates were held at room temperature in a humidified chamber for 24 hours. Serum was recorded negative when the positive-control precipitin line went into the test serum well and was recorded as positive when the precipitin lines formed lines of identity with the

positive-control serum or when they caused the positive control precipitin line to bend toward the antigen well (weak positives).

Extraction of egg yolk IgY:

Ammonium sulfate precipitation method (Hadsun and hay 1991) was used for extraction of the egg yolk IgY as following:

After careful separation of the egg yolk from the egg white, the yolk was rolled on a filter paper in order to free it completely from albumen material. The membrane of the egg yolk was perforated, so that the content (approximately 10 - 15 ml) may drip into a 100 ml measuring cylinder. Distilled water was added to make a dilution of 1:5. The mixture was stirred for homogenizing and then frozen at -20°C for a minimum of 72 hours. The frozen suspension was thawed slowly at 4 C and centrifuged at 2800 x g for 20 minutes. For each ml of supernatant 0.5ml of ammonium sulfate saturated salt solution was added (2:1). After stirring for two hours at room temperature the mixture was centrifuged at 2800 x g for 20 minutes and then the supernatant was discarded and the precipitate was dissolved in DDW to half of its original volume. 0.5 ml of ammonium sulfate solution was added for each 1ml of the solution. The solution was stirred at room temperature for 2h. , and then centrifuged at 2800 x g for 15 mm. The supernatant was discarded; the precipitate was dissolved in 2.5 ml of PBS per egg yolk and dialysed against PBS at 4°C for 48 hours. After filtration (0.45 µm) the immunoglobulin suspension was stored at -20 °C.

Indirect ELISA:

According to Voller *et al.*, (1976), Indirect ELISA was used for monitoring of BTV antibodies in the serum and the extracted egg yolk IgY of the immunized hens:

- Cheak Board titration of BTV reference antigen:

BTV reference AGID stock antigen "Pirbright England" was titrated against mouse BTV reference positive serum "Pirbright England and normal negative mouse serum for detection of the proper dilution could be used in the plates coating for the indirect ELISA procedures was carried out as following:

50 µl carbonate buffer was placed into each well of a microtitre plate. 50 µl of antigen (Bluetongue AGID antigen) at a starting dilution 1:50 was placed into all wells of row A. After mixing, the antigen was serially diluted by 12 channel multichannel pipette in 50µl amounts from row A to G from where 50 µl are discarded. The plate was incubated on the shaker at 37°C incubator for one hour then at 4 °C over night. The antigen was removed by flicking the plate, and subsequently washed with PBS-T 3 times. 50 µl PBS-T with 6% FCS

(Blocking buffer) was placed into each well of the plate then 50µl of reference positive BTV mouse serum previously dilluted at 1:5 in the same blocking buffer and placed into wells of column 1 and 50µl of negative control serum (normal mouse serum previously dilluted 1:5) placed in the wells of column 7, reaching a final starting dilutions at 1:10 for both positive and negative serum. After mixing the serum was serially diluted by 8 channel multichannel pipette in 50 µl amounts from column 1 to 6 from where 50 µl are discarded and from 7 to 11 after changing the tips. The plate was then incubated on a shaker at 37°C for 1h. The plate was then washed and soaking with PBS-T 3 times. Anti-mouse Ig-HRPO-conjugate "Sigma" at working dilution in PBS-T (1:1000) was added in 50µl amounts to all wells. The plate was incubated in a moist chamber at 37°C for 1h, and supsequtly washed with PBS-T 3 times. 50 µl of freshly prepared substrate indicator system (OPD) was added to all wells then incubated for 10 min at room temperature where the color reaction was stopped by addition of 50µl H_2SO_4 stopping solution and finally the plate was read on ELISA reader (MCC Multiscan) at filter 492.

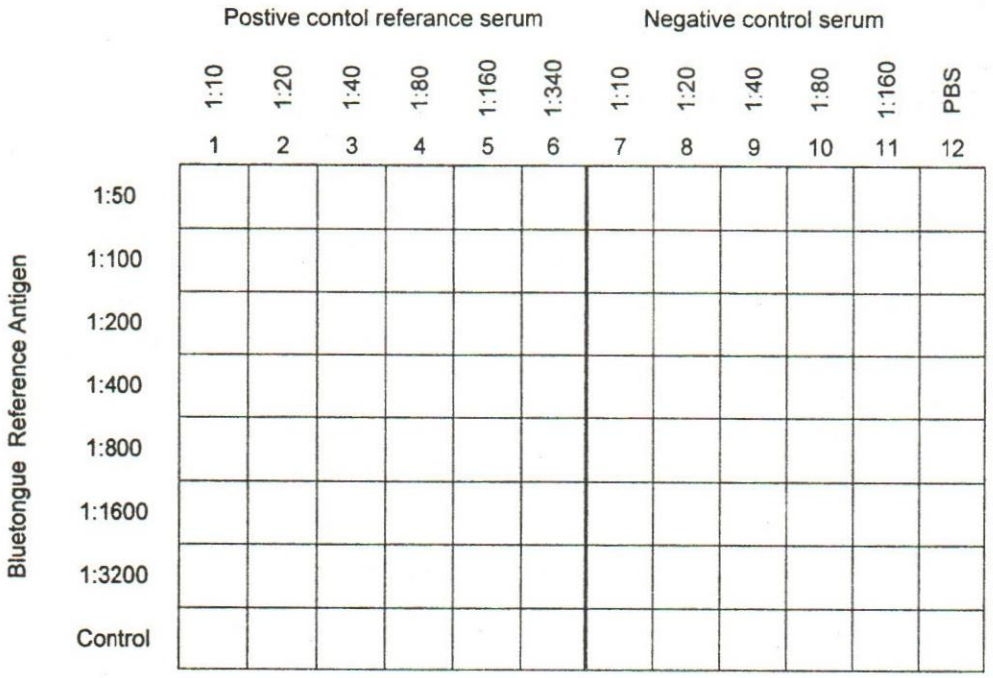


Fig 1: Plate layout in cheak board titration of reference BT antigen.

-Test procedure:

50 µl of BTV antigen at a predetermined dilution in carbonate-bicarbonate buffer was placed into all wells of the plate. The plate was placed on a shaker at 37 °C incubator for 1 h. and then at 4 °C over night. The antigen was removed by flicking the plate, and washed with PBS-T 3 times. Test and negative control sera were double fold diluted in PBS-T+6% FCS (Blocking buffer) in rows from A-H, 50 µl volume and the plate was incubated 1 h at 37 °C then washed with PBS-T 3 times. Anti-chicken Ig-HRPO-conjugate at working dilution (1:5000) in Blocking buffer was added in 50u1 amounts to all wells and the plate was incubated for 1h. at 37 °C then washed with PBS-T 5 times. 50 µl of freshly prepared OPD substrate indicator system was added to all wells and incubated for 10 min at room temperature then the color reaction was stopped by addition of 50 µl of H_2SO_4 stopping solution. The antibody titres of the serum samples, and egg batches were collected from the immunized hens during the study were estimated according to the OD of the negative control and the cut of was determined as following:

Statistical Analysis for determining cut of value:

Limit for negative serum values was calculated for comparison of OD values of negative serum and weak-positive serum. One of the difficulties in identifying weak-positive reactions aroused from the fact that even the negative control sera exhibiting some degree of reaction. Moreover, this background reaction is not uniform, but differed from one control serum to another in a random fashion. The problem was then to establish a borderline which could separate weak-positive sera from true negative sera (Hubschel *et al.* 1980). This was done as following:

- Six normal chicken control sera (with no record of BTV antigen inoculation) were used as controls. The OD of those sera were: 0.218, 0.162, 0.183, 0.193, 0.221, and 0.226. by indirect ELISA
- The arithmetic mean (M) and the SD were calculated as M = 0.20 and SD = 0.0254, respectively. Then the cut off value (COV) was estimated as (Chris *et al.*, 1993).

Cut off value (COV) = M + 3SD

According to this rules the samples with an OD of 0.277 or higher was classified as positive; and any samples with an OD of less than 0.277 was considered negative. By this way we can easily estimate and compare the ELISA titre of different samples

Tissue Culture-Indirect Fluorescent Antibody Test:

According to Foster *et al.*, (1972), this test was done for detection of (13) BTV local isolates constitute 5 serotypes (4, 10, 12, 13, and16) were isolated and identified during September–November 2000 (Habashi 2002).

-Test procedures:

Vero cells were cultivated in Leighton tubes with cover slips (3 tubes for each sample) were inoculated with 0.2 ml of each isolate. Negative controls were also included. Adsorption of virus was allowed for 90 min at 37°C. Cells were washed and maintenance medium was added (MEM without serum). Forty-eight hours later cells were fixed on the coverslips with cold acetone at 4°C for 15 min. The indirect immunofluorescent test was then performed at room temperature:

Initially the cells were covered with the pre-diluted egg yolk antibodies diluted (1/10), (1/20) or (1/40) in PBS at pH 7.2 and incubated at 37°C for 1h in a moist champer then washed three with PBS for 5min. Antichicken fluorescence conjugate (1:40) was added and incubated for1 h and subsequently washed 3 times with PBS, for 5 min and Then the cover slips were dried and examined under fluorescent microscope (Under dark filed with X10, X25, and X40) for the presence of the intracytoplasmic fluorescent inclusions characteristic of BTV after adding a drop of mounting glycerol buffer.

RESULTS

Detection of serum bluetongue specific antibody development in immunized Hens:

The developments of bluetongue specific antibodies were detected in the serum samples that collected from the polyvalent BTV vaccine immunized hens, 2weeks after the 2nd and 3rd immunization by AGPT. The test was done against reference antigen with complete line of identity with the positive control reference BTV serum. While serum samples collected at 0 day were recorded negative.

Indirect ELISA for comparison of BTV antibody titres in the serum and the extracted egg yolk IgY of the immunized hens:

The pattern of specific BTV antibody production was studied on both of the serum samples collected from the immunized hens and the extracted immunoglobulin from the egg yolk (IgY) by using indirect ELISA.

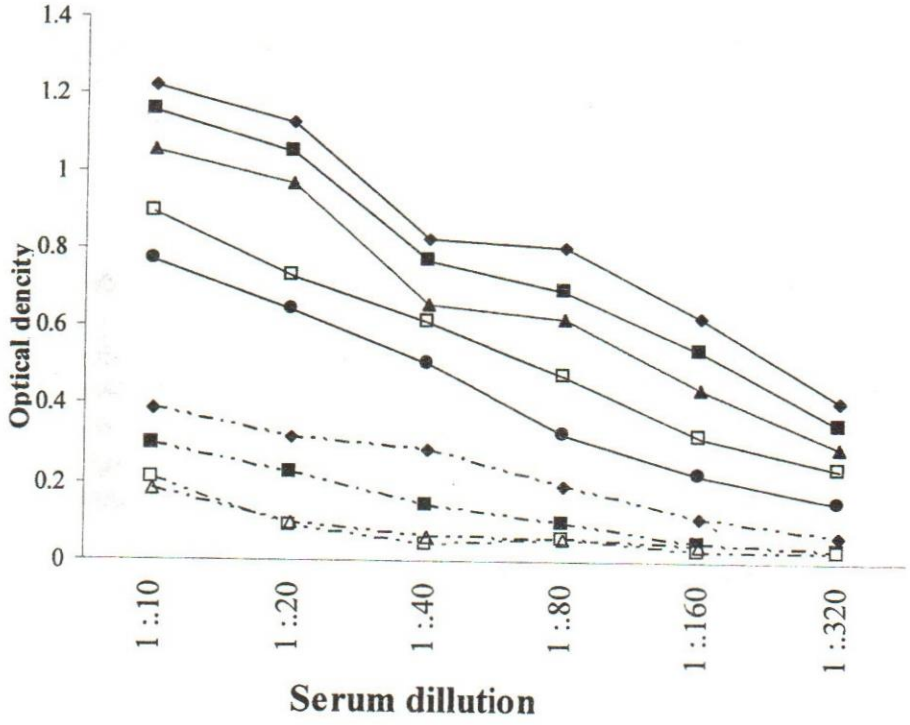
- Cheak Board titration of BTV reference antigen:

The results of titration standard reference BTV antigen, against reference positive antisera and negative serum to determine the optimal dilution that could be used for coating the ELISA plates during the test procedures as follow in the further steps.

As presented in Fig. (2), from which it is clear that: Decreasing the antigen concentration used for coating of the plates led to reducing the absorbance reading for the same serum. This shift was even more

pronounced at lower concentration, whereby the optimum antigen concentration could be judged by the comparison between both negative and positive serum values. On the basis of this result, 1:200 dilution of the purified antigen was considered optimal with lowest background of the negative serum and was used for coating the polystyrene micro-titration plates in all further steps of the experiment.

Fig. (2): Cheak board titration of BTV reference antigen (The influence of antigen concentration on the BT virus ELISA).



-ELISA antibody titres in serum and egg yolk extracted IgY of the BTV immunized hens:

Results were shown in (Table 1, Fig. 3).

Specific BTV antibodies in the serum sample collected from the 2 immunized hens were detected in the serum samples collected at the 3rd week post inoculation (PI) at low end point titre (20), and much more antibodies were detected after the 2nd inoculation reaching the first peak value at week 5 and 6 in both hens respectively with end point titre (80), followed by gradual decreasing after 8 weeks (PI). The highest antibodies levels were detected after the 3rd dose of immunization getting the second beak at week 14 (PI) with end point titre of (640 and 320) in both hens respectively. High level and long sanding antibodies platue were recognized after the 3rd dose of immunization to the last week (week 21) of investigation when the experiment was terminated.

Compared with the serum antibodies levels the pattern of antibodies extracted from the egg yolk were nearly the similar (homologous) to that of serum but during the antibodies decreasing period the egg yolk antibodies titre were higher and longer standing. Therefore the production of BT antibodies through extraction of these specific antibodies from the egg yolk of immunized hens has the advantages of giving very large amount of antibodies from a single immunized hen depending on regular laying of the hens and occasional immunization.

Testing the diagnostic efficiency of the prepared egg yolk BTV specific IgY by Fluorescent antibody technique.

An indirect Fluorescent antibody technique was used for the detection the BTV antigen in infected cells using the prepared egg yolk bluetongue specific IgY at different dilution (1:10, 1:20, and 1:40) for each BTV isolates.

The results revealed that all the 13 BTV isolates were detected and specific fluorescence was observed in the cytoplasm of the infected cells and the most consistent results with brightest specific fluorescence throughout the experiment at dilution 1/20 in PBS, while no specific reaction was observed in both control non infected cells and infected cells with normal negative BTV egg yolk IgY (Photos 1&2).

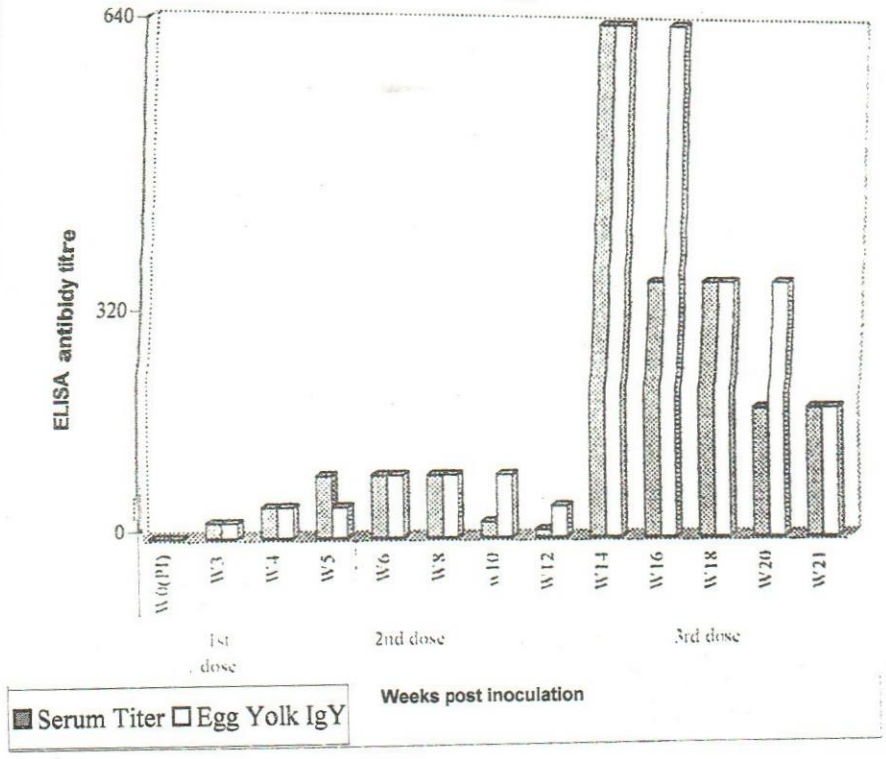
Table 1 : ELISA antibody titres in serum and egg yolk extracted IgY of the BTV immunized hens

Hen number	Antibody source	Pre-inoculation	1st dose	2nd dose(PI)						3rd dose(PI)				
			W3	W4	W5	W6	W8	W10	W12	W14	W16	W18	W20	W21
1	Serum Titer	>10	20	40	80	80	80	20	10	640	320	320	160	160
	Egg Yolk IgY	>10	20	40	40	80	80	80	40	640	640	320	320	160
2	Serum Titer	>10	20	40	40	80	_	40	_	320	320	320	160	160
	Egg Yolk IgY	_	_	_	_	_	_	_	_	_	320	320	320	160

(--) *samples not avilable*

(W) *Weeks post inoculation*

Fig.(3) ELISA BTV specific antiboby titres in serum and egg yolk extracted IgY of one immunized hen.



DISSECTION

The development of bluetongue specific antibodies in immunized hens' is of great interest as it used for the first time in the diagnosis of BTV infection. Polyclonal antisera are produced by blood collection and serum separation from immunized mammals. An alternative to this conventional method the production of antibodies in chicken eggs which can easily exceed 50-100 times the amount which could be gained by exsanguinations of immunized Rabbit during life time of the immunized Hen (Hadsun and hay 1991, Salchow and Schmoldt 1999). The egg yolk of immunized chicken is a rich source of specific polyclonal antibodies (Gassmann *et al.* 1990). Selective transport of all the specific IgY is transferred through the ovarian follicle to the egg yolk representing maternal immunity (Locken and Ruch 1983). Keeping in mind the modern diagnostic tools for disease control, a convenient and low cost effect, this experimental part was conducted in the present study.

After early detection of the development of specific BTV antibodies in the serum of immunized hens by AGPT, both serum

samples and egg yolk were collected at regular intervals during the period of investigation. The IgY from the egg yolk was extracted by ammonium sulphate precipitation method followed by titration of BTV specific antibodies by the indirect ELISA technique in both the collected serum samples and the IgY extracted from the egg yolk.

As shown in (Table 1, Fig. 3); The results were very significant where considerable antibody titre was obtained in the serum samples 2 weeks after the 2nd immunization and the high yield was obtained after the 3rd immunization.

The pattern of antibodies titres extracted from the egg yolks was nearly similar to that of the serum but during the antibody declining period, the egg yolk antibody titres was longer standing and gradually came closer to the antibody titre of sera, which provides an evidence of high yield of large amount of BTV specific antibodies from a single immunized hen. A number of immunologists had already described the application of egg yolk antibodies in the detection and characterizations of large number of structures, viruses, receptors, etc.... The fact that yolk antibodies are exclusively IgG-isotype antibodies and the fact that mammalian structures are highly immunogenic and phylogenetically distinct from the avian structures are both clear advantages of the egg yolk antibodies (Shafiq *et al.* 1997).

Another significant point was conducted in the present study is the quality and the diagnostic efficiency of prepared antibodies for detection of BTV through the florescent antibody test which could be considered as one of the recommended test in BTV identification. The results of indirect florescent antibody test using the prepared BTV egg yolk specific antibodies indicated its capability for detection of the 13 isolates constituting 5 different BTV serotypes proving its efficiency to be used in diagnostic purposes (photo 1, and 2).

Finally in conclusion, the massive production of specific antibodies against BTV in immunized hens is a simple and easy method. As well as the prepared antibodies can be used for diagnostic purposes such as detection of BT viral antigen using IF technique. preparation of egg yolk antibodies offers the advantages of greater ease of injection and greater stability of storage. Further more, there would be no good reason why the laying hens could not be used as a general source of specific diagnostic antibodies as long as the immune response which occurs dose not interfere with egg production depending on intervals of egg collection, occasional booster injection provided a harvest of specific antibodies only limited by aging of laying hens.

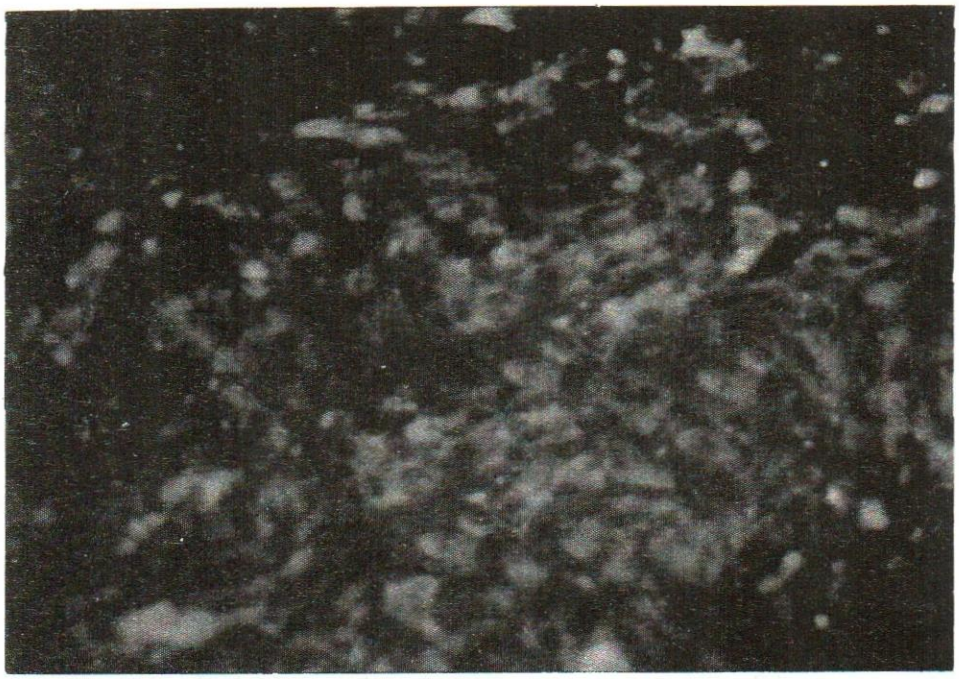


Photo 1: Immunofluorescence of VERO cells 48 h after infection with cytopathic BT isolate using the prepared egg yolk antibodies (x 40).

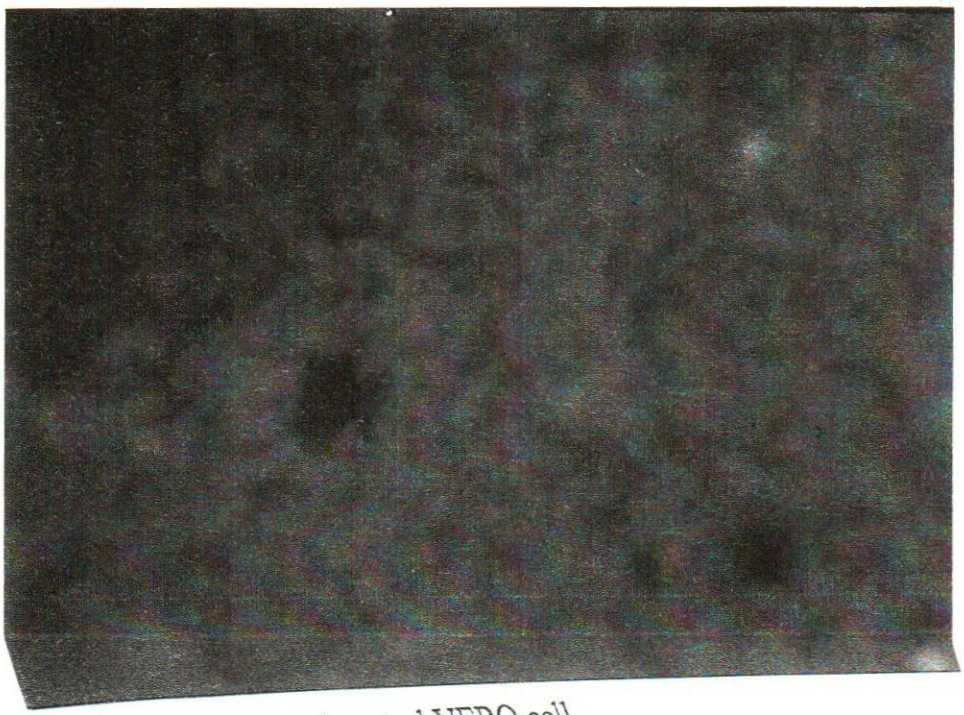


Photo 2: Noninfected control VERO cell.

REFERENCES

Anonymous, (1994): Hens could be the antibody factories of the future, *World Poult.*10 (1/2): 67-68.

Arshad, M.; Afzal, H. and Sindhu, S.T.A.K. (1996): Egg yolk as a therapeutic and diagnostic tool. Agro, Vet. New, 8 (5): 10.

Chris, S. Hayhow and Saif, Y.M. (1993): Development of an Antigen cupter Enzyme-Linked Immunosorbent assay for detection of Enterovirus in Commercial Turkeys, Avian diseases. 37: 375-379.

Erasmus, B.J. (1975): Bluetongue in sheep and goats, Aust, Vet. J., 51(4) 165-70.

Foster, N.M.; Luedke, A.J. and Metcalf, H.E. (1972): Bluetongue in sheep and cattle: efficacy of embryonated chicken eggs in viral isolations, AM. J. Res., 33: 77-81.

Gassmann, M.; Weiser, T.; Thommes, P. and Huscher, U. (1990): The egg yolk as a rich source of polyclonal antibodies. Schweizer Archive fur Tierheilkunde. 132: 289-294.

Gibbs, E.P.J.; Geriner, E.C.; Homan, E.J.; Yuill, T.M.; Mo, C.L. and Walton, T.E. (1989): Arbovirus research in Australia, Proceedings of the 5Th Symposium. Eds M.F. Uren, J. Blok, L.H. Manderson. Brisbane, CSIRO Division of Tropical Animal Science and Queensland Institute for Medical Research. P 29

Habashi, A.R.A. (2002): Studies on Bluetongue virus in sheep and goats, PhD Tanta university – Kaffr-El-sheikh branch, Microbiology Dep.

Howell, P.G. (1963): Bluetongue, In Emerging Diseases of Animals, No, 61. FAO/UN (Rome, Italy), pp. 111-53.

Hubschle, O.J.B. (1980): Bluetongue virus hemagglutination and its inhibition by specific sera, Arch, Virol, 64: 133-40.

Hudson, M.A. and Hay, C.G. (1991): Egg Yolk Diagnostic antibodies, Practical Immunology, Blackwell Scientific Publications, Oxford.

Locken, M.R. and Roth, R.E. (1983): Analysis of maternal IgG subpopulations which are transported into the chicken oocyte, Immunology, 49: 21-28..

Mellor, P.S. (1996): Culicoid Vectors, climate change and disease risk. Vet. Bull. 66, 301-306.

OIE disease information (1999- 2001): follow up emregency reports. Volum 12, 13 and 14.

Pearson, J.F. and Jochim, M.M. (1979): Protocol for immunodiffution test for bluetongue, Procc. Annu. Meet. Am. Assoc. Vet. Lab. Diagnosticans, 22, 463-471.

Polson, A.; Wechmar, B. and Fazakeslay, G. (1980): Antibodies to proteins from immunized hens, Immunol. Commun., 9: 445-514.

Salchow, F. and Schmoldt, R. (1999): Egg yolk as a source of diagnostic antibodies. GTZ-BgVV Service Laboratory Manual. Superaregonal Project Health Protection through Adapted Veterinary Laboratory Diagnosis, Germany.

Shafiq, M.K.; Afzal, H.; Khanum, N. and Arshad, M. (1997): Isolation of egg yolk immunoglobulens (IgY) by chloroform polyethelene glycol technique and assaying of antibodies againest avian infectious bronchitis, Vet. Med. J., Giza. 45:273-278.

Sim, J.S. and Nakai, S. (1994): Egg Use and Processing, New Development. CAB International, Willingford, UK.

Voller, A.; Bidwell, D.E. and Bartlett, A. (1976): Enzyme inimunoassays in diagnostic medicine, Bull WHO 53: 55-65.