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PRODUCTION OF SPECIFIC IMMUNOFLUORESCENT HYPERIMMUNE SERUM FOR DIAGNOSIS OF BOVINE DIARRHOEA VIRUS

ZEIDAN, S.M., M.M. TAHA, AND IMAN K.A. KASSEM

Serum and Vaccine Production Research Institute, Agricultural Reasrch Centre, Ministry of Agriculture, Dokki, Giza.

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Abstract

A preliminary study was conducted in which polycolonal antiserum against bovine viral diarrhoea virus (BVDV), was prepared in Newzeland white rabbit. The antibodies were titrated by both serum neutralization test (SNT) and enzyme linked immunosorbent assay (ELISA) techniques.

The immunoglobuline was precipitated from the serum by concentrated ammonium sulphate solution and was conjugated with fluorescine isothiocyanate (FITC). Both non-reacted FITC and heavily FITC conjugated immunoglobuline were removed. The optimal dilution of the conjugate and its specificity were determined by using BVDV infected tissue cultures.

Seventy-five bovine tissue specimens collected from Cairo abattoir were used for BVDV isolation, as well as for direct fluorescence antibody (FA) techniques and their results were compared.

INTRODUCTION

Bovine viral diarrhoea (BVD) is a multisystemic viral disease of cattle with widely disparate clinical manifestations (Perdrizet *et al.*, 1987). The diseases induced by BVD virus range from an acute, inapparent BVD to highly fatal mucosal disease. Convalescent cattle produce high antibody titre to the virus, yet, immunosuppression, immunotolerent and persistent infection occur (Steven, 1990).

Both BVD virus and its antibodies frequently contaminate foetal calf serum cells are used in diagnostic and research laboratories (Potts *et al.*, 1989). Laboratory contamination of cells or media with BVD virus or its antibodies may lead to false positive or false negative results. Non-cytopathic BVD virus may infect cell cultures resulting in viral interference with cytopathic BVD virus used in serum neutralization test and vaccine production (Gillespie *et al.* , 1962).

A presumptive diagnosis of BDV can often be made on clinical examination and necropsy finding. In most outbreaks, this must be relied upon, since definitive diagnosis

(other than immunofluorescence) usually requires 2 to 3 weeks (Vantsis *et al.*, 1980). Thus, the most rapid diagnostic techique available is the detection of the viral antigen in tissue by fluorescent antibody staining. Moreover, this techinque is also used to detect non-cytopathic BVD virus in cell culture (Ohmann and Dalsgaard, 1980).

The present studies aimed to prepare a specific BVD hyperimmune serum conjugated with fluorescine isothiocyanate to be used in the fluorescent antibody technique as a local cheap preparation instead of expensive imported one.

MATERIALS AND METHODS

MATERIALS

1. Animals

A-Rabbits: Six apparently healthy adult rabbits of 6-8 months old were used for preparation of BVD hyperimmune serum. The average weight was 4 Kilograms. They were kept under observation and hygienic measures and receiving balanced ration containing about 17% proteins.

B-Mice: Fifteen albino Swiss mice were used for the preparation of liver powder according to the method described by Narin and Marrack (1964). Mice liver powder was used to remove non-specific staining caused by globulin which is too heavily conjugated with FITC (Goldstein *et al.*, 1961).

2. Virus: BVD Iman strain (Baz, 1975) isolated from calves that were suffering from pneumoentritis at Tahrir Province Egypt was used for inoculation of laboratory animals and SNT.

3. Tissue culture: Madin Darby Bovine Kidney (MDBK), cell line (Marcus and Moll, 1968) proved to be free from non-cytopathic BVD virus, were used for serum neutralization test and viral isolation.

4-Tissue samples: 75 random samples were collected from different organs of apparently healthy calves slaughtered at Cairo abattoir. The samples used were 15 livers, 10 hearts, 15 kidneys, 10 intestines, 10 spleens and 15 mesenteric lymph nodes. The samples were used in trails for viral isolation, as well as antigenic detection as a field application of the locally prepared FITC conjugated anti-BVD hyperimmune serum.

METHODS

1. Preparation of polycolonal BVD hyperimmune sera

Polycolonal BVD hyperimmune sera were prepared through inoculation of New-zeland white rabbit according to the method described by **Green and Manson (1990)**. The antigen for first immunization was prepared with complete Fruend adjuvant. Subsequent injections were done with incomplete adjuvants.

For primary immunization of rabbit, 0.5 ml. was deeply injected into each thigh muscle and also, 0.5 ml. into each of two sites through the skin of shoulders.

The injections were repeated weekly for further three weeks, but the emulsion was prepared with incomplete adjuvants. Seven days after each injection, the rabbits were bled from the marginal ear vein and the collected blood was allowed to clot and the serum was separated. The antiserum was tested by both SNT and ELISA tests. If the antibody has a high titre, three further bleedings were collected on successive days. If it was unsatisfactory, 1ml was injected and blood was tested ten days later.

2. Serum neutralization test

Two fold serial dilution of serum (1:2 to 1:1024) inactivated at 56°C for 30 minutes, were mixed with equal volume of the BVD Iman strain diluted to give 100 $TCID_{50}$/0.1ml. The mixture was incubated for 60 minutes at 73°C. 0.2 ml of serum-virus mixture was inoculated into each of two MDBK cell culture tubes. The cultures were checked for cytopathic changes and final readings were made three to five days after inoculation when the virus control had undergone complete cytopathic changes. The highest dilution of serum which inhibited cytopathic activity, was taken as the end point titre. Appropriate negative and positive controls and virus titration were included in each test. The test was conducted for testing the prepared antiserum.

3. Indirect ELISA techique

Five fold dilution of rabbit sera samples in phosphate buffer saline containing 0.05% Tween-80 (PBS-T) starting from 1:10, were incubated in BVDV ELISA plates for 1 hour at 37°C. After washing, 100 ML of horse reddish peroxidase HRP conjugated anti-rabbit monoclonal antibody MAB diluted 1:1000 in PBS-T, were added per well and inculbated for one hour at 37°C. The plates were washed twice in PBS-T and 200ML of the substrate were added per well. After 10 minutes at room temperature, 50ML H_2SO_4 were added to stop reaction. The absorbent value at 450 nm was measured.

4. Precipitation of gammaglobulin

Precipitin of globulin was conducted according to the method described by Peter (1969) using saturated ammonium sulfate. Finally, remaining sulfate was removed by dialysis against 0.15M. Nacl. The globulin concentration was determined and adjusted to be 20mg/ml using phoshate buffer solution.

5. Conjugation of immunoglobulin with fluorescine isothiocyanate (FITC)

Globulin solution was adjusted to be 5% protein in 0.15 M. Nacl and mixed with FITC solution at a ratio of 1:0.8. The pH was adjusted to 9.5 by addition of 0.04 M. Nacl. Conjugation was completed after 30 minutes stirring at room temperature (Spendlove, 1966).

Non-reacted FITC was removed by dialysis against phoshate-buffer saline. This might require several days and should be continued until the dialysate became free of fluorescent material. The non-specific stainings caused by globulins which were heavily conjugated with FITC were removed by using mice powder according to the method described by Goldstein *et al.* (1961. Conjugates might be frozen at-20°C or below.

6. Direct fluorescent antibody techinique (FAT)

The locally prepared anti-BVD-conjugated sera was tested and evaluated through application of direct FAT according to the method described by Ohmann and Dalsgaard (1980). The test was conducted in experimentally inoculated tissues by using five-fold dilution of the prepared conjugate.

7. Viral isolation and antigenic detection

Attempts were made to isolate BVD virus from tissue samples and/or to detect its antigen by direct FAT. A 10% suspension of tissues was made in Hank's balanced salt solution (HBSS), centrifuged to remove debris, and the supernatant was used for virus isolation. Each sample was inoculated into 2 Lighton's tubes containing confluent layer of MDBK cell line and coverslips. Infected cells were incubated at 37°C and examined daily for cytopathic effect. Coverslips were harvested on the seventh day post-inoculation and fixed in acetone for 15 minutes and stained directly with different dilutions of the prepared anti-BVD conjugated hyperimmune sera. Controls infected and non-infected MDBK cells were included.

EXPERIMENTS AND RESULTS

Experiment one: Preparation of hyperimmune serum

Preparation of polycolonal antibodies against BVD are conducted by using 6 rabbits through inoculation of each one with 2mL of BVD virus with a titre of 10^7 $TCID_{50}$/0.1mL mixed with equal amount of complete Frued adjuvant. Viral inoculation was repeated weekly mixing with incomplete Fruend adjuvant for three successive weeks.

Sera samples were collected from inoculated and control rabbits seven days post-each inoculation. Specific developed antibodies were quantitated by both ELISA technique and SNT test.

Results

Specific developed anti-BVD antibodies were evaluated both SNT test and ELISA technique and the obained resuls are represented in Table 1.

Experiment two: Precipitation of immunoglobulin

The immunoglobulin content of the locally prepared hyperimmune sera was precipitated by using saturated ammonium sulfate solution. After removing the remaining sulfate from the precipitant by dialyzing, both protein and albumin contents were quantitated. Precipitation process was repeated till complete removal of albumin content.

Results

After accurate precipitation of gamma globulin and complete removal of albumin and sulfate, the concentration of the protein was 1.5 gm/100ml, while, albumin declared undetected.

Experiment three: Conjugation with FITC

After complete conjugation and removal of both unreacted FITC and the heavily FITC conjugated immunoglobulin molecules, the product was tested against BVDV by experimentally inoculateding tissue culture MDBK cells, as well as, control non-infected cells.

The optimal conjugate dilution was determined by titration using cell cultures infected with BVD virus.

Results

Locally prepared anti-BVD conjugated sera gave strong positive fluorescent reaction with experimentally infected tissue culture cells as seen in picture1, while, control non-infected cells are seen dark.

Immunofluoresent conjugate titration assay indicated that the optimal conjugate dilution is 1:25 as represented in Table 2.

Experiment four: Field application

Trails of viral isolation and antigenic detection were conducted on 75 random bovine tissue specimens by using locally prepared anti-BVD conjugate. Isolation was conducted by using Lighton's tubes containing MDBK cells. Inoculated cells were incubated at 37°C for 7 days with daily examination for cytopathic effect and finally, fixed in acetone and stained directly with anti-BVD conjugate and examined for fluorescent cells.

Results

Results of trails of BVDV isolation and/or its antigenic detection, on 75 random bovine tissue specimens cellected from Cairo abattoir by using of locally prepared conjugated anti-serum, are represented in Table 3.

DISCUSSION

The criteria for diagnosing and evaluating the role of BVD in a herd include recognizing the clinical signs of infection, examining gross and microscopic lesions, identifying the virus and evaluating the results of clinical, pathological and virological examination. Interpreting the reults of these examinations is difficult because of a variety of factors in the bovine host, some are in the viral agent and, in the interaction between the host and the virus **(George Ruth 1985)**. Moreover, the serological tests have a limited usefulness essentially in diagnosis of transient infection **(Littlejohns and Walker, 1985)**. On other hand, the immunofluorescence technique has proven a valuable tool in the investigation of viral replication in vivo and in vitro. Therefore, our studies aimed to prepare a specific BVD hyper-immune serum conjugated with fluorescine isothiocyanate to be used for direct fluorescent antibody techique.

In this experiment, hyperimmune serum against BVD virus was prepared in New-

zeland white rabbits according to Green and Manson (1990). Serum with a titre 512 or more was used to conjugate with FITC serological tests. Table1, indicated that the specific neutralizinig antibodies began to appear 7 days post-1st inoculation (containing complete Freund adjuvant), with a 0.75 Log_{10} mean serum neutralizing antibody titre (Log_{10} MSNAT). The titre was increased gradually and reached its peak after the 4th inoculation (5 weeks post-1st inoculation) with a titre of 2.85 Log_{10} MSNAT. These results were in harmony with those obtained by Ward *et al.* (1984) but, they had used serum with a titre of 640 for labeling.

Concering Immunofluoresent conjugate titration, assays indicated that the optimal conjugate dilution was 1:25, and it gave a positive result with the BVD inoculated MDBK cells (7 days post-inoculation). These results coincide with previous report by Synder *et al.* (1979), who indicated the optimal conjugate dilutions of 1:30 and 1:15 for virus isolation and tissue section techniques, respecively.

In the present studies, direct fluorescent antibody technique (FAT) conducted on 75 specimens collected from Cairo abattoir indicated that, 16 out of them confirmed BVD virus infection. These results generally agreed with those obtained by viral isolation with the exception of two specimens, which were positive to FAT in spite of no viral isolation. It seems very possible that, the isolation was negative and is mainly due to non-viable virus, or it may contain non-cytopathic virus. Therefore, FAT is more sensitive than viral isolation. The present results coincide with those obtained by Clark *et al.* (1985), who concluded that the FAT can check for non-cytopathogenic strains of bovine virus diarrhoea virus.

From all above mentioned results, it would be clear that the immunofluorescene technique is a valuable tool in the investigation of viral replication in vivo and in vitro, and it can easily detect the non-cytopathic BVDV infection. Also, it would clear the situation of BVDV infection in cattle in Egypt, and dictates the development of control measures through application of highly effective vaccine with detection of immunetolerent and persistently infected animals.

Table 1. Mean serum neutralizing and ELISA titers in experimentally immunized rabbits.

Tests used		Weeks post immunization						
		0	1	2	3	4	5	6
Log_{10} SNAT	M	0.0	0.75	1.2	1.725	2.25	2.85	2.625
	SD	0.0	0.17	0.24	0.15	0.173	0.173	0.15
ELISA titre	M	67.3	117.5	256.5	609	1469	2854	2558
	SD	+7	+5	+21	+13	+58	+108	+94

SNAT: Serum neutralizing antibody titers. Sd: Standard deviation.

ELISA: Enzyme linked immunosorbent assay. M: Mean

Table 2. Titration of FITC conjugate anti-BVD immune serum.

Conjugate dilution	Reaction with	
	BVD infected cells*	Control cells*
1 : 5	+ve	-ve
1 : 10	+ve	-ve
1 : 15	+ve	-ve
1 : 20	+ve	-ve
1 : 25	+ve	-ve
1 : 30	-ve	-ve

FITC: fluorescent isothiocyanate.

*= **MDBK:** Madin Darby bovine kidney.

BVDV: bovine virus diarrhoea virus.

Table 3. Results of viral isolation and antigenic detection on bovine specimens from Cairo abattoir.

Specimens & numbers		Viral isolation			Antigenic detection		
		+ve	-ve	%ve	+ve	-ve	%ve
Liver	15	2	3	13.3	2	13	13.3
Spleen	10	2	8	20	3	7	30
Heart	10	1	9	10	1	9	10
Mes. Ln	15	4	11	26.6	5	10	33.3
Kidney	15	3	12	20	3	12	20
Intestine	10	2	8	20	2	8	20
Commutative		14/75	61/75		16/75	59/75	
Percentage		18.7	81.3		21.3	78.7	

Mes. Ln.: mesenteric lymph node.

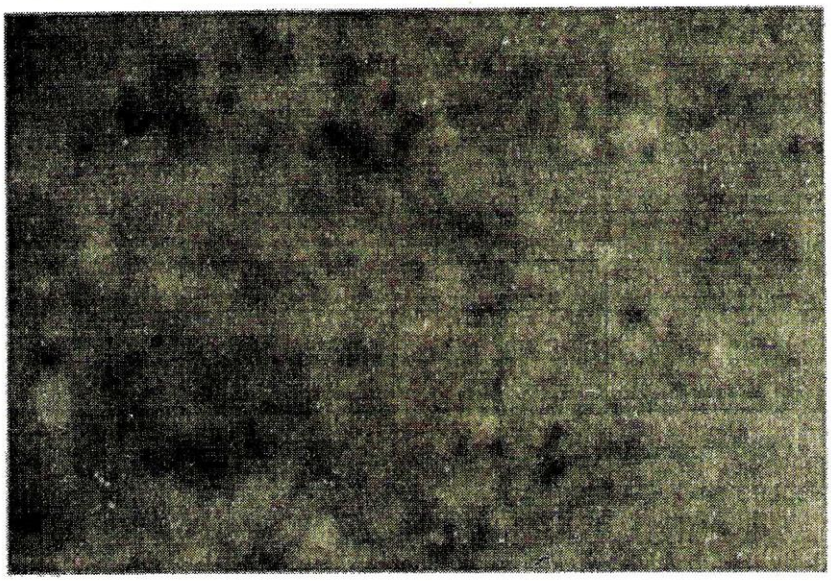

Fig.1. Positive immune-fluorescence reaction on BVDV infected MDBK tissue culture cells

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إنتاج سيرم نوعى عالى المناعة مشع لتشخيص مرض الإسهال الفيروسى البقرى

سيد محمد زيدان، محمد محمود طه سلام
إيمان كامل أحمد قاسم

معهد بحوث وإنتاج الأمصال واللقاحات البيطرية - العباسية - مركز البحوث الزراعية - وزارة الزراعة - الجيزة.

تم تحضير مصل عالى المناعة ضد فيروس الإسهال الفيروسى البقرى فى الأرانب النيوزيلندية البيضاء وتم معايرة الأجسام المناعية بكل من اختبار المصل التعادلى واختبار الاليزا.

تم ترسيب الجلوبيولين المناعى من المصل باستخدام محلول مركز من كبريتات الأمونيوم ثم تم إقرانه بمادة الفلوريسين ايزوثيوسيانيت، تم إزالة كل من الفلوريسين الذى لم يتفاعل وكذلك الجلوبيولين المقترن بالفلوريسين بغزازة، تم قياس نوعية وعيارية الجلوبيولين المناعى المقترن بالفلوريسين والمضاد لمرض الإسهال الفيروسى البقرى باستخدام خلايا نسيج زرعى محقونة بفيروس الإسهال الفيروسى البقرى.

جمعت خمسة وسبعون عينة عشوائية من أعضاء مختلفة من البقر من مجزر القاهرة واستخدمت تلك العينات لمحاولات عزل الفيروس وكذلك للكشف عن وجود أنتيجيناتة باستخدام مادة الجلوبيولين المناعى المقترن بالفلوريسين والمضاد لفيروس الإسهال الفيروسى الذى تم إعداده وذلك باختبار الفلوريسين المباشر ثم تم مقارنة النتائج.