PREPARATION OF PEROXIDASE CONJUGATED AND NON-CONJUGATED ANTISHEEP IgM IMMUNOGLOBULIN

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Abstract

Peroxidase conjugated and non-conjugated antisheep immuno-globulin-M (IgM) were prepared from rabbit hyperimmune serum. The optimal dilutions of both products were determined by checkerboard ELISA method which proved their efficacy. Their titres were 1:2000 and 1:100, respectively. Both peroxidase conjugated and non-conjugated antisheep IgM, in comparing with imported standardized product were applied on 28 serum samples collected from sheep infected with Rift Valley Fever virus (RVFV) to detect IgM antibodies against RVFV using both indirect ELISA and IgM capture ELISA. The results revealed that non-conjugated antisheep IgM using IgM capture ELISA was superior to peroxidase conjugated antisheep IgM using indirect ELISA for detection of IgM antibodies in sheep.

INTRODUCTION

Acute infections with different viral diseases and risk of dissemination of these viruses, represent simple, safe serological test for diagnosis of an early infection.

The first immunoglobulins appearing after a primary infection are the immunoglobulins-M (IgM) which disappeared rapidly within few weeks and were replaced by immunoglobulins-G (IgG) which persisted for longer periods. Thus, it was suggested for the first time that detection of specific anti-virus IgM could be of value in recognizing recent virus infection (Schluederberg, 1965).

Transient nature of IgM response appears to hold time for most primary virus infection. Specific anti-virus IgM antibodies, typically appeared between 7 to 10 days after primary infection reaching maximal level within 2 to 3 weeks, then, declined to undetectable level after 3 months (Erdman, 2000).

The most important viral diseases causing severe losses in sheep population are peste des petits ruminants (PPR) (Ikram *et al.*, 1988), Rift valley fever (RVF) (Imam and Darwish, 1977), blue tongue disease (Marhart and Osburn, 1986) and bovine viral diarrhoea (BVD) (Zeidan, 1986).

The present study aimed to produce rabbit anti-sheep IgM peroxidase conjugated and non-conjugated antisheep IgM to be used for diagnosis of different viral infections in infected sheep herds.

MATERIALS AND METHODS

Animals

Rabbits

Five apparently healthy adult male New-Zealand white rabbits of 4-6 months old were used for preparation of hyperimmune serum against sheep IgM immunoglobulin, and two rabbits were kept as control.

Sheep

Sixty ml of sheep sera were collected from two apparently healthy native breed tested with SNT and ELISA proved to be free from viral infections (BVD, sheep pox, RVF, FMD) were used for preparation of antisheep IgM immunoglobulin to be used for immunization of rabbits.

Serum samples

Twenty-eight serum samples were collected daily from two sheep experimentally infected with RVFV from 3rd to 14th days, then, at weekly intervals till 28 days post-infection. Sera were used for comparative evaluation of the locally prepared non-conjugated and peroxidase conjugated anti-sheep IgM with imported one.

RVF ELISA antigens

Cell supernatant Rift Valley Fever antigen (Elian and Botros, 1997) and mice brain sucrose acetone RVF antigen (Taradi, 2003) kindly supplied by RVF Dept., Veterinary Serum and Vaccine Research Institute, Abbasia, Cairo, were used for detection of specific RVFV IgM antibodies in sheep sera.

Rabbit anti-sheep IgM

It was supplied by Holly Ditch Farm, Mile Elm, Calne, Wilshire, Snllopy, and used at a dilution of 1:100.

Rabbit anti-sheep IgM peroxidase conjugate

It was supplied by NAMRU-3, and used at a dilution of 1:3000.

Precipitation and purification of IgM immunoglobulin

They were prepared according to Talwar (1983) as the following steps:

1. Precipitation and purification of sheep IgM from sheep sera

It was conducted as the method described by Talwar (1983).

a. Precipitation of sheep euglobulins (IgM + β-lipoproteins) with 40% of saturated ammonium sulfate by adding gradually 40 ml of saturated ammonium sulfate solution to 60 ml of sheep serum and stirred for two hours at room temperature, then, centrifuged at 3000 rpm for 30 minutes. The precipitate was dissolved in 30 ml distilled water and precipitated again by gradual addition of 20 ml of saturated ammonium sulfate solution, and the cycle was repeated twice. The final precipitate

was dissolved in 15 ml of 0.1 M sodium chloride and dialysed for removal of ammonium sulfate against double distilled water at 4°C for 24 hours.

b. Removal of β-lipoprotein chemical precipitation: lipoproteins were precipitated and removed with dextran sulfate and calcium chloride. For every 10ml volume of serum, 0.2 ml of a 10% solution of dextran sulfate was added and mixed followed by gradual addition of 1.0 M $CaCl_2$, while, mixing maximum precipitation was achieved by addition of 1.0 ml of $CaICl_2$ and was removed by centrifugation. The supernatant was subjected to dialysis for overnight against 0.05 M tris buffer pH 7.5 containing 0.15 M NaCl, and the concentration of IgM immunoglobulins was estimated according to Canon *et al.* (1974), it was 80 mg/ml used for immunization of the rabbit.

Preparation of hyperimmune sera against sheep IgM immunoglobulin

These were prepared in three rabbits according to methods described by Green and Manson (1990) and Iman *et al.* (2003).

Through intramuscular inoculation, each rabbit was injected deeply in the muscle of different legs with 0.5 ml of sheep IgM immunoglobulin mixed with 0.5 ml of complete Freund's adjuvant. Boostering of these rabbits was done weekly for further 3 weeks with emulsion containing incomplete Freund's adjuvant. Ten days after last injection, the rabbits were bled.

The collected blood from each rabbit was allowed to clot and the serum was separated. The anti-sheep IgM immunoglobulin contents were evaluated by agar gel precipitation test against sheep IgM immuno-globulins. Three further bleeds were carried out on successive days.

Conjugation of anti-sheep IgM immunoglobulins with horseradish peroxidase

It was done according to periodate method described by Tijssen and Kurstak (1984), Iman *et al.* (2003).

Estimation of the protein content

It was done according to Canon *et al.* (1974) using Biurt Method.

Agar gel precipitation test

It was done according to Ouchlerlong (1962) to evaluate the prepared antisheep IgM using sheep IgM precipitate as an antigen.

Checkerboard ELISA technique

It was done according to Rose *et al.* (1986).

Indirect ELISA technique

It was done according to Voller *et al.* (1976).

ELISA capture IgM technique

It was done according to Paweska *et al.* (2003).

RESULTS AND DISCUSSION

In this study, the developed antibodies specific to sheep IgM immunoglobulin were tested by agar gel precipitation test. It revealed that the maximal precipitating titre was obtained 10 days post the 4th injection of the rabbit.

Rabbit hyperimmune sera were collected and divided into two parts. The first part was used as anti-sheep IgM non-conjugated and the optimum dilution was determined by checkerboard ELISA technique for determination of the working dilution. The result was represented in Table 1 showing that the optimum dilution was 1:100.

Comparative evaluation of the locally prepared anti-sheep IgM non-conjugated antibodies and the imported one was done on 28 serum samples tested for anti-RVFV IgM antibodies as in Table 2 using known negative and positive serum samples. The results agreed with Elian and Botros (1997) who found that IgM antibodies could be detected through the period from the 7th day up to 21st day post-vaccination with attenuated Rift Valley Fever virus vaccine.

The second part of the rabbit hyperimmune sera was subjected to precipitation with saturated ammonium sulfate till complete removal of the albumin, and the globulin content of the hypersensitized rabbit with sheep IgM immunoglobulin was determined by the method described by Henery and Kawaoi (1974), and it was 36 mg/ml.

Anti-sheep IgM immunoglobulins were labeled by covalent coupling to horseradish peroxidase enzyme as described by Tijssen and Kurstak (1984). The optimum dilution of the conjugate was determined by checkerboard ELISA, the results are represented in Table 3. The optimum dilution was 1:2000.

Comparative evaluation of the locally prepared anti-sheep IgM peroxidase conjugated antibodies was compared with the imported one on 28 serum samples as shown in Table 4.

Table 5 indicated that IgM capture ELISA proved to be highly sensitive when compared with indirect ELISA, and was the best to detect low IgM antibodies in infected sheep. This agreed with the results obtained by Erdman (2000).

The results showed that the locally prepared anti-sheep IgM conjugated and non-conjugated are effective and can be used for diagnosis and economically save both time and money.

Table 1. Optimum dilution of the prepared anti-sheep IgM antibodies using IgM capture ELISA.

Coating with anti-sheep IgM antibodies	Absorbance value (at 492 nm) of reference samples		
	Strongly +ve	Weak +ve	Negative
1:50	0.170	0.150	0.09
1:100	0.291	0.250	0.10
1:200	0.119	0.131	0.02

Table 2. Detection of Rift valley fever IgM antibodies in sheep sera using locally prepared and imported anti-sheep IgM non-conjugated antibodies using IgM capture ELISA.

Serum samples	Absorbance value (at 492 nm) with non-conjugate local and imported anti-sheep IgM			
	Local		Imported	
DPI	Sheep No. 1	Sheep No. 2	Sheep No. 1	Sheep No. 2
3	0.237	0.296	0.232	0.286
4	0.279	0.312	0.309	0.311
5	0.271	0.287	0.307	0.292
6	0.265	0.279	0.257	0.296
7	0.259	0.311	0.292	0.306
8	0.271	0.289	0.282	0.277
9	0.267	0.281	0.274	0.279
10	0.235	0.275	0.258	0.289
11	0.260	0.229	0.299	0.273
12	0.237	0.256	0.285	0.260
13	0.287	0.295	0.292	0.245
14	0.134	0.288	0.286	0.295
21	0.129	0.189	0.120	0.192
28	0.107	0.106	0.102	0.110
Control –ve	0.109		0.103	
Control +ve	0.250		0.265	

DPI: Days Post-Injection

Table 3. Optimum dilution of the prepared peroxidase labeled anti-sheep IgM antibodies using checkerboard ELISA.

Sheep antigen	Absorbance value (at 492 nm) at anti-sheep IgM dilution			
	1:500	1:1000	1:2000	1:4000
Strong +ve	0.988	0.640	0.515	0.332
Weak +ve	0.664	0.205	0.127	0.050
Negative	0.040	0.030	0.020	0.022
PBS	0.014	0.014	0.014	0.015

Table 4. Detection of Rift valley fever IgM antibodies in sheep sera using locally prepared and imported anti-sheep IgM peroxidase conjugated antibodies using indirect ELISA technique.

Serum samples	Absorbance value (at 492 nm) with prepared and imported anti-sheep IgM peroxidase-conjugate			
	Local		Imported	
DPI	Sheep No. 1	Sheep No. 2	Sheep No. 1	Sheep No. 2
3	0.120	0.138	0.126	0.141
4	0.154	0.161	0.157	0.155
5	0.141	0.149	0.153	0.148
6	0.138	0.143	0.139	0.141
7	0.141	0.154	0.148	0.159
8	0.132	0.139	0.131	0.138
9	0.159	0.146	0.153	0.144
10	0.143	0.137	0.140	0.143
11	0.144	0.146	0.148	0.141
12	0.158	0.137	0.158	0.136
13	0.149	0.145	0.153	0.141
14	0.138	0.141	0.146	0.143
21	0.075	0.115	0.076	0.118
28	0.074	0.063	0.069	0.062
Control –ve	0.084		0.086	
Control +ve	0.159		0.161	

DPI: Days Post-Injection

Table 5. Analysis of the results by indirect ELISA and IgM capture ELISA technique on 28 days post-infection sheep sera using different prepared antisheep IgM conjugate and non-conjugated antibodies.

Serological assay		Number of tested sheep sera			
		Local product		Imported product	
		Non-conjugated	Conjugated	Non-conjugated	Conjugated
ELISA +ve	Capture ELISA +ve	25	25	25	25
ELISA +ve	Capture ELISA - ve	-	-	-	-
ELISA -ve	Capture ELISA +ve	1	1	1	1
ELISA -ve	Capture ELISA - ve	2	2	2	2
Total		28	28	28	28

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تحضير أجسام مناعية مقترنة وغير مقترنة بالبيروكسيديز ضد الجلوبيولين المناعى IgM للأغنام

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تم تحضير سيرم مناعى ضد جلوبيولين الأغنام IgM فى الأرانب وتم اقتران جزء منه بأنزيم البيروكسيديز وجزء لم يتم اقترانه. تم حساب التخفيف الأمثل لهما بإجراء اختبار الاليزا الذى أثبت كفاءة المستحضرين، وبإستخدام كل من السيرم المناعى المقترن والغير مقترن بإنزيم البيروكسيديز بإستخدام ٢٨ عينة سيرم أغنام للكشف عن وجود أجسام مناعية IgM ضد فيروس حمى الوادى المتصدع باستخدام كل من اختبار الاليزا capture والاليزا الغير مباشر على التوالى بالمقارنة بالمستحضرات المماثلة المستوردة. وأثبتت النتائج أن الأجسام المناعية الغير مقترنة بالبيروكسيديز أعطت نتائج أفضل من الأجسام المناعية المقترنة بالبيروكسيديز عند إجراء اختبار الاليزا للكشف عن الأجسام المناعية IgM فى الأغنام.