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RAPID DETECTION OF CONGO-CRIMEAN HAEMORRHAGIC FEVER ANTIBODIES

(With 3 Tables and 1 Figure)

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التشخيص السريع لحمى القرم والكونغو النزفية

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اختبر عدد ٣٩٨٥ عينة دم من أغنام وماعز سليمة ظاهريا باستخدام اختبار الاستشعاع المناعي الغير مباشر واختبار الإليزا النقطي. استخدمت في الدراسة خلايا الفيرو المحقونة بفيروس حمى القرم و الكونغو النزفية والتي تم معالجتها بالاشعاع للقضاء على حيوية الفيروس. وأظهرت النتائج وجود أجسام مناعية جي لفيروس حمى القرم والكونغو النزفية في الأغنام فقط بنسبة ٢%. وبالنسبة لتوزيع العينات الإيجابية كان ٨٧ % في الأغنام عمر سنة أو أكثر بينما كان ١٣% في عمر أقل من سنة. لم يحصل على كل من الأجسام المناعية إم في عينات الدم و كذا أنتيجين الفيروس في القراد الذي تم جمعه من الأغنام. وقد بينت الدراسة توافق بين اختبار الإليزا النقطي والاستشعاع المناعي الغير مباشر ليس فقط في العدد الكلي للعينات الإيجابية ولكن أيضا في العينات الفردية، فضلا عن ذلك فإن اختبار الإليزا النقطي كان أكثر حساسية ولكنه احتاج إلى وقت أكثر مقارنة باختبار الاستشعاع المناعي الغير مباشر. ولهذا فإنه يفضل إجراء اختبار الاستشعاع المناعي الغير مباشر لفحص العينات خاصة عندما يطلب إبلاغ نتيجة سريعة في أوقات حرجة.

SUMMARY

Serum sample from 3985 apparently healthy sheep and goats were tested for CCHF by indirect immunofluorescence (IF) and Dot-enzyme linked immunosorbent assay (Dot-ELISA). Infected inactivated Vero cell suspension was used as antigen in the study. CCHF viral antibody prevalence was found among sheep (2%), while not among goats. The percentages of reactive sera were 87% and 13% in age ≥1 year and < 1year subsequently. Neither IgM antibody in sera nor CCHF viral antigen

in tick homogenates could be demonstrated. There was a correlation between Dot-ELISA and IF not only with the respect to the total numbers of positives, and negatives, but also, for each individual serum. Moreover, Dot-ELISA was more sensitive than IF, but needed more time for application. So IF technique was faster especially when rapid result delivery had been highly recommended.

Key Words: *Congo Fever-Crimean Haemorrhagic Fever-Antibodies.*

INTRODUCTION

Crimean-Congo haemorrhagic fever virus is widely distributed throughout the arid regions of Africa, the Middle East, South and Eastern Europe, and Asia (Hoogstraal, 1979), which lie within the geographic range of thirty species of ticks particularly in the genus Hyalomma that act both as reservoir and vector (Camicas et.al. 1990). This virus belongs to the genus Nairovirus, family Bunyaviridae (Casals and Tignor, 1980). It causes dangerous illness including haemorrhagic signs, and mortality outbreaks. Human become infected by tick bites or through close contact with infected animals or man (Hoogstraal, 1979, Yu-Chen et.al. 1985, and Oldfield et.al.1991). The infection is enzootic, but mainly asymptomatic in many species such as cattle, sheep, goats, camels and hares (Hoogstraal, 1979). To prevent the risk of spreading the virus by imported animals, a rapid serodiagnosis should be applied at the gates of the country that have no history of the disease. Because of the ability of the virus to cause laboratory infections and the severity of the human disease, maximum containment laboratory facilities are required for isolation of CCHF virus.

The present investigation focused on detection of antibodies against CCHF virus in sheep and goat sera by indirect immunofluorescence, as well as Dot ELISA to reach a quick and specific method for identification.

MATERIALS and METHODS

Specimens:

A total of 3985 *blood* specimens were obtained by veinpuncture from apparently healthy sheep (3380) and goats (605) from August 1993 to September 1994 at Hadithah quarantine in northern west of Saudi Arabia. After collection and centrifugation, the sera were separated and frozen at -20°C until tested. All samples were tested in duplicate and

under blind conditions by IF and spot ELISA techniques.

Thirty-seven tick samples were carefully picked up by hand from sheep and goats, macerated in a mortar, taken into suspension in half ml of Hanks balanced salt solution containing 10% heat inactivated (30 min, 56°C) fetal calf serum (GIPCO Ltd.) and 100 IU of penicillin and 100 µg of streptomycin per ml. From each tick suspension, a thin spot was prepared (10µ volumes) on slides and fixed by cold acetone then were kept at -20° C.

Antigen:

Antigen was provided from Special Pathogens Branch, Centers for Disease Control, Atlanta, G A. according to Johnson et. al. (1981). Briefly CCHF virus infected Vero cells were mixed with non infected cells at a ratio of 1:10, and exposed to UV light for 20 min. (about 1,200-3,000 W/cm^2) in order to inactivate the virus. So that the antigen could be used outside the maximum containment laboratory and the majority of non-infected cells were in fact a control (blank) for the viral antigen. The mixture, at a concentration of $2x10^6$ cells/ml, was coated in 10 µl volumes onto each of 12-well multitest slides (Flow Lab. UK). The slides were then air-dried and fixed in cold acetone and were kept at -20° C until used. For Dot ELISA, infected inactivated Vero cell suspension was frozen and thawed three times and placed 3 µl volumes onto each center of 96-impression circles on nitrocellulose membrane (Sartorious, Germany) and kept at -20° C.

Antisera:

*Human serum containing antibodies to CCHFV was obtained from Dr. I. Allam. Virologist, Jeddah vet. Lab.

*Sheep serum containing antibodies to CCHFV was kindly supplied by Dr.B.Botros, Namru3.

*Antihuman IgG conjugated with FITC (Wellcome Ltd., Beckenham, England) Sigma.

*Anti sheep IgG & IgM conjugated with FIIC (The Binding site limited, Birmingham, England). The working dilution was 1:20 in PBS.

*Anti goat IgG & IgM conjugated with FIIC (The Binding site limited, Birmingham, England).

*Anti sheep Immunoglobulin-peroxidase-conjugated (Sigma Chemical Co, St. Louis, MO).

Indirect- immunofluorescent Test:

Sera were screened in a 1:16 dilution for the presence of IgG and IgM antibodies against CCHF antigen. Then positive sera were titrated in

two-fold dilutions. The end point titer was considered to be the reciprocal of the highest serum dilution with which 10 or more cells showed fluorescent similar to that of the positive control serum. The staining technique described by Johnson et.al. (1981) was adopted which included 30-min to detect IgG & 90 min to detect IgM primary and secondary reaction steps carried out at 37° C and three vigorous stirred washing steps after each reaction on phosphate buffered saline at pH 7.2 for 5 minutes each. Slides were counterstained with Evans blue dye at a concentration of 1:1000, then air-dried, mounted with cover slips in glycerin phosphate-buffered saline (9:1), pH 9.0, and examined blindly in a Leitz microscope equipped with epi-illumination. A 50 W halogen light source was used, together with a BG 38 red-absorbing filter and KP-500 primary and K 510 secondary filters. The author used 10 X/18 oculars and 40/0.75 power air-objectives designed to maximize fluorescent intensity at the wavelengths delivered by this system.

Dot-enzyme linked immunosorbent assay:

According to Hawkes et.al. (1982), Dot-immuno assay was performed as the following: The marked nitrocellulose membrane containing antigen was taken from-20°C, was kept 5 min. in TBS (50 mM Tris-Hcl, 200 mM NaCl pH 7.4), cut out 3X3 mm squares containing antigen fit into the well microtiter. The squares facing upward were placed into the wells of 96-well tray (A/S Nunc, Reskilde, Denmark), blocked with 150 µl of 3% W/V serum albumin, and 0.5% V/V Tween in TBS for 15 min. Then the blocking solution was aspirated. 150 µl serum samples in a 1:16 dilution (in blocking solution) were added, left for 2 hours at 37°C, washed 30 min in several changes of TBS with continuous shaking and the blocking was repeated. Anti-sheep immunoglobulin proxidase-conjugated was diluted 1:5000 in blocking solution, added to the wells (100µl), incubated 2 hours at 37°C. Then they were washed for 30 min in several changes of TBS. with continuous shaking. 4-chloro-1- naphthol (Merck Inc.) was prepared as 3 mg/ml stock solution in methanol containing 0.01% V/V hydrogen peroxide. The developing solution was added to the wells (100µl), kept for 15 min, washed with distilled water, then allowed to dry and should have been stored in the dark.

RESULTS

As shown in Table (1) of 3985 sheep and goat sera tested by IF for the presence of antibodies to CCHF virus, 57 sheep sera were found positive where displayed bright intra-cytoplasmic fluorescence. The

prevalence of antibody was identified in sheep that originated from Turkey, but non-from Jordan, Syria, Egypt and Lebanon. The percentage of reactive sera were nearly distributed equally (20-30%) all over the months which sera were collected in, except June (9%) hadn't detectable IF antibodies as exhibited in Figure (1).

Attempts to demonstrate IgM antibody in collected sheep and goat sera were unsuccessful. Moreover, neither IgM nor IgG antibodies could be detected in 605 goat sera. There was much higher percentage of positive sera in age ≥1 year (87%), but the lowest in age < 1 year (13%) as shown in table (2).

Table (3) presents the results of 705 random selected sera including the positive ones in IF which were further investigated by Dot-ELISA technique. All IF positive sera were also positive by Dot-ELISA. The antibody levels recorded in Dot-ELISA were 1-2 fold higher than in the corresponding IF test were observed. Furthermore, 6 sheep sera were detected by Dot-ELISA (1/16) which had not been demonstrable by IF.

The observation of CCHF viral antigen in tick homogenate fixed smears was negative using IF and Dot-ELISA.

Table 1: Prevalence of CCHF viral antibody in sheep and goats sera by IF technique.

Origin	Species	Negative	Positive	Positive %
Turkey	Sheep	232	57	25
Jordan	Sheep	1629	–	–
,,	Goat	73	–	–
Syria	Sheep	1455	–	–
,,	Goat	391	–	–
Egypt	Sheep	50	–	–
,,	Goat	101	–	–
Lebanon	Sheep	14	–	–
,,	Goat	40	–	–
Sub total	Sheep	3380	57	2
	Goat	605	-	-
Total		3985	57	1

Table 2: Relationship between age and positive sera to CCHF virus, by IF technique among sheep.

Age of animal	No. of positive sera	Percentage
<1 year	8	13
1 year	55	87
Total	63	100

Table 3: Correlation between reciprocal of antibody titers to CCHF virus in selected sheep sera.

No. of sera	Test	
	Dot-ELIZA	IF
642	Negative *	Negative *
6	16	Negative *
27	32	16
21	64	32
3	128	128
5	256	64
1	1024	256

- *Sera negative at lowest dilution tested, i.e., 1/16 of CCHF viral antibody among sheep.*

DISCUSSION

The detection of antibodies in animal sera can be useful to reveal the presence of otherwise unrecognized CCHF, as well as the prevalence of infection and thus the risk of human exposure to infected tick bites and infected animal body fluids can be avoided (Chumakov, 1971). So this study aimed to the previous purpose, in addition to, evaluation of the test of choice to detect the disease.

The presence of CCHF virus, or antibodies to it, has been demonstrated in the former USSR, Bulgaria, Greece, Turkey, Hungary, Yugoslavia, France, Portugal, Kuwait, Dubai, Sharjah, Iraq, Iran, Afghanistan, Pakistan, India, China, Egypt, Ethiopia, Mauritania, Senegal, Burkina Faso, Benin, Nigeria, the Central Africa Republic, Congo, Kenya, Uganda, Tanzania, Zimbabwe, Namibia, South Africa (Watts et al., 1988), Oman (Scrimgeour et.al.1996). In Saudi Arabia,a number of cases occurred in abattoir workers in Mecca. In 1992 CCHF developed in 2 adults who were exposed to fresh meat purchased in

Mecca as described by Scrimgeour (1995). However, the evidence for France, Portugal, Turkey, India and Egypt is based on limited serological observations (Watts et.al., 1988).

From Table (1) 57 out of 232 sera, originating from Turkey, were positive (25%); this coincides with the findings of Casals (1978), who reported that 26 of 1100 human sera from Turkey were positive (2%). This strengthens the presence of CCHF in Turkey.

Possible explanation for the low incidence of human infection includes the fact that viraemia in animals is short-lived and of low intensity compared to that in other zoonotic diseases as Rift Valley fever, which is more readily acquired from contact with infected tissues, and that humans are not the preferred hosts of Hyalomma ticks.

On the contrary, negative results have been observed in sheep and goat sera which had come from Egypt, Lebanon, Syria and Jordan. However, a more plausible reason towards this instant is the animals in these countries may be held indoors where less ticks exposure than animal feeding in pastures and the governmental and biomedical security are intense in controlled agroeconomical projects.

As regards the incidence of reactive sera all over the year months, Karinskaya et. al. (1970) demonstrated a high rate of antibody reactions among domestic animals in September 1968 in Rostov Oblast [at the end of the period of adult activity of the ticks], antibody rates were low in January- February 1969, and there were no antibodies in the same animals in May 1969. But Vasilenko et. al. (1977) found in Bulgaria seasonal dynamics of CCHF, appearing in April, reaching peak numbers in June, and disappearing by October, [followed by about a month the first appearance, peak numbers, and declining numbers of adult ticks]. In South Africa, Rechav (1986) recorded human disease in most months of the year, but there has been slight preponderance of cases in February- March and October- November, [when adult Hyalomma ticks tend to manifest peak questing activity]. This study showed nearly equal distribution of antibody all over the months which Turkish sheep sera were collected in, except June (Figure 1). This was attributed to favorable ecological and climatic factors beneficial for survival of large numbers of Hyalomma ticks and of the hosts which provide the opportunity for a serious CCHF cases to develop; these factors differ from country to other.

In this study both IF and Dot- ELISA showed negative results for detection of CCHF antigen in tick homogenates. The tick sample size was obviously too small owing to the effective acaricide treatment of

animal before shipment. However, Chumakov et al., (1973) obtained high positive results of the tick preparations in Kazakhistan and Tadzhikistan where the virus was established to be enzootic.

In sheep less than 1 year of age, the presence of CCHF antibodies was significantly lower than older ones as shown in Table (2). It seems likely that either Hyalomma ticks are less attracted by young age sheep or due to the maternal care.

Attempts to demonstrate IgM antibody in collected sheep sera were unsuccessful. This indicates neither exposure to the virus nor this type of antibody appeared. IgG antibody could be yielded by both FA and Dot-ELISA. These findings were in close agreement with the results of Shepherd et al., (1989) and Burt et al., (1994) that stated that the titer of IgM of CCHF in human declined gradually thereafter and were low or negative by the fourth months, while the titer of IgG increased markedly between 2 and 4 months after onset of illness and remained readily demonstrable up to 3 years after infection.

Several authors have discussed many problems encountered in performing tests for antibody to CCHF virus (Donets et al., 1982). Neutralization tests are complicated by the fact that the sera of many species contain non-specific inhibitors of virus infectivity, which have to be removed by acetone- ether extraction (Casals and Tignor, 1974). The use of mice in neutralization tests is inconvenient for large scale surveys, while plaque reduction tests are complicated by the need for prolonged incubation of cell cultures under overlay medium (Buckley, 1974), and by difficulty in preparing good plaque-production stocks of virus (Tignor et al., 1980). Complement fixation and agar gel immunodifusion techniques lacked sensitivity and reproducibility (Casals, 1978), while HAI is difficult to perform with CCHF virus (Donets et al., 1982). The immunofluorescence and Dot-ELISA techniques appeared to be most suitable for use in surveys and hence were investigated here.

IF technique has several advantages for serological diagnosis. It could be performed easily with the use of prepared antigen slides stored in the frozen state. The present study found that the IF test is the most convenient, inexpensive, specific and efficient tool to screen sera for antibody. Often on arrival of animals once a positive result was obtained would allow a tentative diagnosis to be reported in little more than an hour. The usefulness of IF technique for potentially dangerous viruses, such as CCHF, is enhanced by the fact that inactivated antigen can be used as effectively as live antigen. So it is of great help for diagnostic laboratories which have no a maximum containment facilities (Zgurskaia

and Chumakov, 1977).

Dot-ELISA that had been employed, allowed both a considerable simplification of the procedure and the simultaneous screening of a large number of samples. The methods had the merit of being more sensitive than comparable existing procedures. It had two advantages over than enzyme immuno assay, which employed, for example, microtiter plates as supports. First the amount of the antigen needed is greatly reduced because of the small spot size. A second fundamental advantage is that the use of nitrocellulose permitted tight bound with the antigen, moreover, an insoluble fixed coloured precipitate reaction to be viewed against a white background. In this way the discriminatory power is greater in detecting positive reactions.

Table (3) shows close agreement between the results of Dot-ELISA and IF tests with the sera of sheep, not only with the respect to the total numbers of positives, and negatives, but also, for each individual serum. Moreover, Dot-ELISA demonstrated antibody 1-2 fold dilution higher than did IF and found out more 6 sera having low antibodies which could not be detected by IF.

It was postulated that using Dot-ELISA stands on the way of CCHF diagnosis with somewhat higher sensitivity than IF technique without requiring special equipment. However Dot-ELISA needs several washing steps and long incubation periods, which render it cumbersome and time- consuming. Thus it is unsuitable for rapid diagnosis especially in critical positions where fast results for higher titers are ordered.

From this work it could be concluded that IF technique is convenient for rapid detection of CCHF antibodies in sera of animals especially at a country gate. In spite of the sensitivity of using Dot-ELISA is more than IF, it needs further study to render it much faster in diagnosis.

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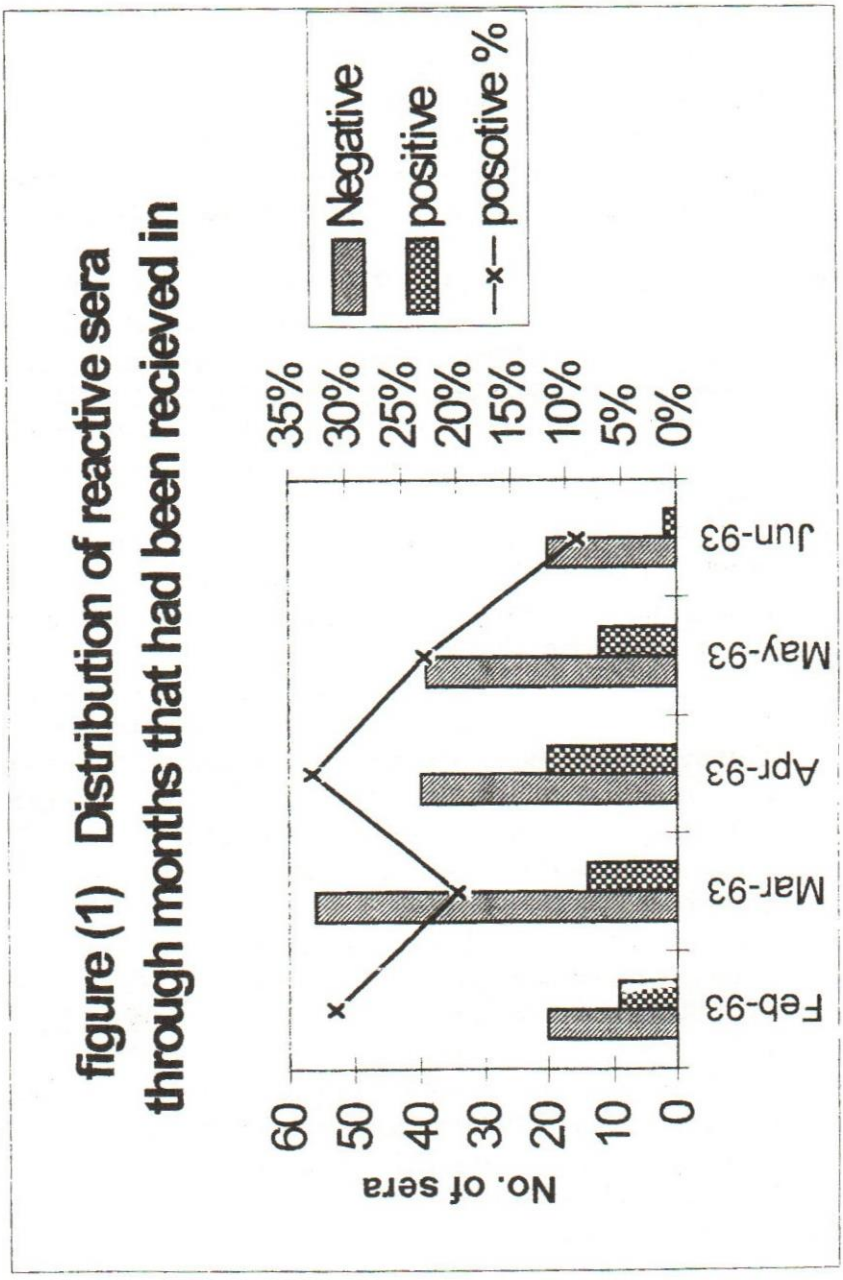

figure (1) Distribution of reactive sera through months that had been recieved in