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THE THIRD RIFT VALLEY FEVER (RVF) OUTBREAK IN KINGDOM OF SAUDI ARABIA SINCE THE FIRST RECORDING OF THE DISEASE OUTSIDE AFRICA CONTINENT IN 2000

(With 3 Tables and One Figure)

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الوباء الثالث لمرض حمى الوادى المتصدع فى المملكة العربية السعودية منذ تسجيل المرض لأول مرة خارج قارة أفريقيا عام ٢٠٠٠

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تم دراسة وباء ظهر فى المملكة العربية السعودية فى شهر فبراير عام ٢٠٠٧ وتميز بنفوق عدد كبير وحدوث بعض الإجهاضات فى الأغنام . محاولة التعرف علي سبب هذا الوباء تمت بإستخدام بعض الطرق التقليدية للتشخيص مثل اختبار الإليزا والعزل الفيروسى بالإضافة إلى استخدام التفاعل التبلمرى المتسلسل كأحد التقنيات الحديثة للتشخيص. هذه الاختبارات الثلاثة تم إجراءها مرتين متتاليتين يفصل بينهما أسبوع على عدد ١٠٠ رأس من الغنم ٥٠ منها كانت تعانى من أعراض إكلينيكية و ٥٠ كانت تبدو سليمة ظاهريا. كل الحيوانات المختبرة أعطت نتائج سلبية مع إختبار الإليزا الأول بينما فى العزل الفيروسى الأول تم عزل فيروس حمى الوادى المتصدع من ٦١ حيوان كما تم التعرف على الحامض النووى لهذا الفيروس فى ٦٨حيوان بعد إجراء التفاعل التبلمرى المتسلسل الأول. تم التعرف على الأجسام المضادة لفيروس حمى الوادى المتصدع (IgM) فى ٥٨ حيوان بعد إجراء إختبار الإليزا الثانى. بينما نتائج العزل الفيروسى والتفاعل التبلمرى المتسلسل الثانى لم تتغيرعن نتائج الاختبار الأول. نتائج هذا العمل أوضحت أن التفاعل التبلمرى المتنسلسل أشد حساسية من العزل الفيروسى ومن اختبار الإليزا كما أن العزل الفيروسى أشد حساسية من إختبار الإليزا بالإضافة إلى أن التفاعل التبلمرى المتسلسل والعزل الفيروسى تمكنا من التعرف على الفيروس فى مرحلة مبكرة من الإصابة بينما فشل إختبار الإليزا فى التعرف على الأجسام المضادة (IgM) فى هذه المرحلة. كما تميز التفاعل التبلمرى المتسلسل بسرعة الحصول على النتائج. هذه هى المرة الثالثة التى يتم فيها تسجيل وباء لمرض حمى الوادى المتصدع فى جيزان (المنطقة الجنوبية من المملكة العربية السعودية) منذ تسجيل المرض لأول مرة فى المملكة العربية السعودية عام ٢٠٠٠ إلا أنها المرة الأولى التى يحدث فيها الوباء بعيدا عن موسم الأمطار (أغسطس – نوفمبر) وفى غياب البعوض. بعض الحيوانات التى كانت تبدو سليمة ظاهريا أعطت نتائج إيجابية مع إختبار الإليزا الثانى (٨) والعزل الفيروسى الأول والثانى (١١) والتفاعل التبلمرى المتسلسل الأول والثانى (١٨) مما يبين الدور الذى يمكن أن تلعبه هذه الحيوانات فى استمرار وجود الفيروس وانتشاره. من النتائج التى تم الحصول عليها يمكن أن نستنتج أن التفاعل التبلمرى المتسلسل هو أفضل اختبار متاح فى الوقت الحالى يمكن استخدامه فى التشخيص المبكر للإصابة بفيروس حمى

الوادى المتصدع كما يمكنه التعرف عل الحيوانات المصابة والتى تبدو سليمة ظاهريا. كما أن استمرار حدوث هذا الوباء وفى الظروف التى تم بها حدوث الوباء الأخير تثبت أن مرض حمى الوادى المتصدع أصبح متوطن فى المملكة العربية السعودية وبذلك تكون هى أول بلد يستوطن فيها هذا المرض خارج قارة أفريقيا.

SUMMARY

An outbreak appeared in kingdom of Saudi Arabia in February, 2007 and characterized by high mortalities and some abortions among sheep was studied. Trials for identify the cause of this outbreak were done by performing some traditional diagnostic tools like enzyme linked immunosorbent assay (ELISA) and virus isolation (VI), in addition to using of Reverse Transcription-Polymerase Chain Reaction (RT-PCR) as a recent technique. These assays were performed two successive times one week apart to 100 sheep, 50 of them were suffered from clinical signs and 50 were apparently healthy. All examined animals were negative for first ELISA while Rift Valley Fever virus (RVFV) was isolated from 61 animal in first virus isolation and RVF viral nucleic acid was detected in 68 animals after applying first RT-PCR. IgM antibodies to RVFV were identified in 58 animals with second ELISA while results of virus isolation and RT-PCR remained without change. Results of this work showed that RT-PCR was more sensitive than ELISA and virus isolation while virus isolation was more sensitive than ELISA. RT-PCR and virus isolation could detect early RVFV infection while ELISA failed to detect early infection. This is the third recording of RVF outbreak in Jizan (Southern region of Kingdom of Saudi Arabia) since 2000 when the disease reported for the first time in Kingdom of Saudi Arabia but it is the first to record the outbreak away from rainy season (August-November) and mosquitoes. Some apparently healthy sheep gave positive results with 2^{nd} ELISA (8), with 1^{st} and 2^{nd} virus isolation (11) and with 1^{st} and 2^{nd} RT-PCR (18).From the results of this work I can conclude that RT-PCR is the best available test for diagnosis of early infection of RVF and can be used also in detection of apparently healthy infected animals. Repeated occurrence of RVF outbreaks with the circumstances observed in the last outbreak may proved that the disease became endemic in Kingdom of Saudi Arabia so, it will be the first endemic area outside Africa continent.

Key words: *Rift Valley Fever (RVF), RVFV, Enzyme Linked Immunosorbent Assay (ELISA), Virus Isolation (VI), Reverse Transcription Polymerase Chain Reaction (RT-PCR)*

INTRODUCTION

Rift Valley Fever (RVF) is an arthropod-borne viral disease infects animals and humans and transmitted by mosquitoes. In animals Rift Valley Fever virus (RVFV) induces almost 100% mortality among young animals and a high rate of abortion in pregnant females .The disease is endemic in Africa (Turell *et al.,* 1996; Gerdes, 2002; Clements *et al.,* 2007a) and cause great economic losses (Yossef, 2001; Woods *et al.,* 2002)

The initial description of the disease dates back to 1930, when animals and human outbreaks appeared on a farm in Lake Naivasha, in the Great Rift Valley of Kenya. Until 2000, the disease was only described in Africa, and then outbreaks were also declared in the Kingdom of Saudi Arabia (2000-2001 and 2004) and in Yemen (2000-2001) (Jup *et al.,* 2002; Al-Afaleg *et al.,* 2003; Abdo-Salem *et al.,* 2006). Virus transmission appears to occur whenever sheep and cattle are present with abundant mosquito populations (Jup *et al.,* 2000; Miller *et al.,* 2002; Ikegami and Makino, 2004).

Every year some 10 million to 15 million small ruminants may be slaughtered during haj. Most of these animals are imported from countries in East Africa and the horn of Africa, where RVF is known to be enzootic (Shawky, 2000; Davies, 2006). This was an explanation of cause of the first RVF outbreak in Kingdom of Saudi Arabia in 2000.

The viral agent is an arbovirus negative-strand RNA virus, which belongs to the *Phlebovirus* genus in the *Bunyaviridae* family (Flick and Bouloy, 2005; Ikegami *et al.,* 2005;). This virus is responsible for large explosive outbreaks (Le May *et al.,* 2005; Won *et al.,* 2006; Bird *et al.,* 2007a; Clements *et al.,* 2007b).

Diagnosis of RVF is based on serology and virus isolation. The disadvantage of these former assays include poor sensitivity, high coast, the lengthy duration, cross reactivity with other Phleboviruses (Espach *et al.,* 2002), as well as risk associated with using infectious virus as antigen because serodiagnosis of RVF currently relies on the use of live whole virus as antigens (Garcia *et al.,* 2001; Paweska *et al.,* 2003a; Fafetine *et al.,* 2007).

The deadly RVF outbreak in the Arabian Peninsula dramatically illustrated the need for rapid diagnostic methods (Garcia *et al.,* 2001; Anyamba *et al.,* 2006), but rapid diagnosis are not available for several pathogens in the genus *Phlebovirus* of the *Bunyaviridae* family (Weidmann *et al.,* 2007). Therefore, there is a critical need for a highly sensitive and specific molecular diagnostic assay capable of detecting the natural genetic spectrum of RVFV (Bird *et al.,* 2007b; Xu *et al.,* 2007).

Complete genome of RVFV has been published (Albarino *et al.*, 2007; Bird *et al.*, 2007a; Bird *et al.*, 2007c) and thus make tests based on molecular biology are available. Therefore, this work was planned to identify the cause of this outbreak as fast as possible and at the same time evaluation and comparison between different methods used in diagnosis of RVF for selection of the most suitable assay was another aim of the study.

MATERIALS and METHODS

History of the outbreak

In February 2007, a high incidence of abortion and mortalities was observed among sheep in Jizan (Southern region of KSA) which has a previous history of two RVF outbreaks in 2000 and 2004). Areas and farms with suspected clinical signs of RVF were visited for collection of epidemiological data, examination of clinically affected animals and for collection of samples for laboratory investigations.

Animals

100 sheep, 50 clinically affected and 50 apparently healthy sheep, from Jizan district were used in the study.

Serum

5 ml blood from each examined clinically affected or apparently healthy sheep was collected two times one week apart to obtain serum for c-ELISA and virus isolation.

Blood

5 ml blood from each examined clinically affected or apparently healthy sheep was collected two times one week apart for reverse transcription polymerase chain reaction (RT-PCR).

Laboratory diagnosis:

(a) Capture enzyme linked immune osorbent assay (c-ELISA) for detection of anti-RVF IgM in sera

1- Coat plate with 100µl rabbit anti-sheep IgM diluted 1:500 in phosphate-buffered saline (PBS) and incubate plate covered with lids at 4º C overnight
2- Wash plates 3 times using 300µl wash buffer per well.
3- Add 200 µl / well blocking buffer and incubate for 1h in moist chamber at 37ºC.
4- Wash plates 3 times.
5- Add 100µl of test and control sera diluted 1: 400 in diluent buffer into wells as shown in plate layout and incubate for 1h at 37ºC.
6- Wash plates 3 times.

7- Add 100µl of RVF Ag and control Ag diluted 1:200 in diluent buffer to rows A-D 1-12 and rows E-H 1-12 respectively and incubate for 1h in moist chamber at 37ºC.
8- Wash plates 6 times.
9- Add 100µl /well of mouse anti-RVFV serum diluted 1:1000 in diluent buffer and incubate for 1h in moist chamber at 37ºC.
10- Wash plates 6 times.
11- Add 100 µ`l / well goat antimouse IgG horseradish peroxidase (HRPO) conjugate diluted 1:5000 in diluent buffer and incubate for 1h in moist chamber at 37 ºC.
12- Wash plates 6 times.
13- Add 100 µl of ABTS substrate/well.Leave plates for 30min at 22º-25ºC in dark.
14 -Add 100µl of 1% SDS stop solution and read optical density at 405 nm.
15 -Results evaluation and diagnostic interpretation:-

a. The results of the tested microplates are accepted if at least three of the net optical density (OD) values recorded for high positive control (C++) fall within the ring 0.8 – 1.85, if the results of two or more of the four replicates of C++ fall outside this limit the plate must be rejected.
b. If the result of the plate is accepted, the net OD of each serum sample is calculated as follow: Net OD = OD determined with RVFV Ag - OD determined with control Ag.
c. Calculation of percentage positivity (PP) of C+ C- and test sera :

-Percentage positivity (PP) = $\frac{\text{Net OD of (serum or C+ or C-)}}{\text{Net mean OD of C++}} \times 100$

The sample considered positive if PP value ≥ 8

Table 1: ELISA plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	C++	C++	1	5	9	13	17	21	25	29	33	37
B	C++	C++	2	6	10	14	18	22	26	30	34	38
C	C+	C+	3	7	11	15	19	23	27	31	35	39
D	C-	C-	4	8	12	16	20	24	28	32	36	40
E	C++	C++	1	5	9	13	17	21	25	29	33	37
F	C++	C++	2	6	10	14	18	22	26	30	34	38
G	C+	C+	3	7	11	15	19	23	27	31	35	39
H	C-	C-	4	8	12	16	20	24	28	32	36	40

C++ High positive control serum 1-40 Test sera
C+ Low positive control serum Rows A-D 1-12 RVFV Ag
C- Negative control serum Rows E-H 1-12 Control Ag

- c ELISA was performed according to Paweska *et al.,* (2003b) and manufacturer's (Biological Diagnostic Supplies Limited, BDSL. Flow laboratories and Special Pathogen Unit, Scotland, UK) directions.

(b) Virus isolation (VI) and identification:

VI from the samples was attempted with both Vero E6 cells and suckling mice. Confluent monolayer of Vero cells in $25C^3$ flask were infected with 0.5 ml diluted serum samples (100µl of serum and 400µl of Leibovitz15 medium with 5% fetal calf serum and antibiotics) and 4 ml of maintenance medium. When mice were used, VI was attempted by intracerebrally inoculating 30µl of serum samples into each mouse. Cytopathic effects and mortality were examined. Identification and immunofluorescent test using pools of hyperimmune ascitic fluid were performed three days postinfection by methods described by Digoutte et al., (1992).

(c) Reverse transcription-polymerase chain reaction (RT-PCR):

1- RNA extraction and preparation
   - Sera was cleared by centrifugation at 10000 xg for 10 min.
   - Viral RNA was prepared from 140ul of serum by using Qiamp viral RNA kit (Qiagen. Hilden, Germany).
   - RNA was eluted in 50 µl.

2- Oligonucleotide design
   - Oligonucleotide primers were selected according to genetic map of RVFV published by Albarino *et al.,* (2007); Bird *et al.,* (2007a) and Bird *et al.,* (2007b)
   - Sequences and characterization of used primers are shown in table 2.

Table 2: Oligonucleotide primer sequences used for PCR for the detection of RVF RNA genome.

PRIMER	NUCLEOTIDE SEQUENCES 5` - 3`	POSITION
Nsca	CCTTAACCTCTAATCAAC	824-841
NSng	TATCATGGATTACTTTCC	31-48

3- RT-PCR
   - The one step RT-PCR system combining superscript reverse transcriptase with platinum Taq-polymerase (Life Technologies, Karlsruhe, Germany) was used.
   - The reaction mixture was prepared as follow: 10 µl of reaction mix provided with the kit and including basic level of MgSO4 + 40 ng of bovine serum albumin (Sigma, Munich, Germany) per µl + 2µl extracted RNA.
   - The cycling profiles for amplification of RVFV nucleic acid involved the following steps: reverse transcription at 50ºC for 20 min; initial denaturation at 95ºC for 5 min; 10 precycles with 95ºC for 5s, 60ºC for 5s with a temperature decrease of

1ºC for cycle, and 72Cfor 25s; and 40 cycles with 95ºCfor5s, 56ºC for 10s and 72ºC for 30s.

4- Identification of amplified nucleic acid

PCR products were electrophoresed at 100V for 30 min in Tris acetate EDTA buffer (TAE) on 1.5% agarose gel and stained with ethidium bromide (0.6 ug/ml). DNA molecular weight marker type 100 base pair (bp) ladder (Gibco B RL) was applied to identify the size of the PCR products.

- RT-PCR was performed according to Sall *et al.*, (2001); Drosten *et al.*, (2002).

RESULTS

1- Clinical and epidemiological studies

-Clinical examination of sheep showed mucopurulent nasal and ocular discharge, high fever, bloody diarrhea and abortion.

-The abortion rate in pregnant ewes was approximately 55-60% (collected data).

-A mortality rate of about 40-50% in young lambs and 25-3o% in adult sheep was recorded (collected data).

-Some infected sheep (18) were apparently healthy.

-At the time when the outbreak occurred (from February to March, 2007), there was no rain or mosquitoes.

2- Laboratory investigations

(a) c.ELISA

1 - First examination: All examined samples were negative for IgM.

2 - Second examination: 58 examined samples were positive for IgM.

(b) VI

1 - First examination: RVF virus was isolated from 61 sample.

2 - Second examination: RVF virus was isolated from 61 sample.

(c) RT-PCR

1 – First examination: RVF viral nucleic acid was detected in 68 sample.

2 – Second examination: RVF viral nucleic acid was detected in 68 sample.

Results of laboratory investigations are summarized in Table (3), and results of RT-PCR are shown in Figure (1). Positive result of PCR detected by presence of white band which represent the RVF viral nucleic acid.

Table 3: Results of laboratory investigations.

c.ELISA				VI				RT-PCR			
1st		2nd		1st		2nd		1st		2nd	
+	-	+	-	+	-	+	-	+	-	+	-

0	0	58	42	61	39	61	39	68	32	68	32

Fig. 1: Agarose gel electrophoresis analysis of PCR products in examined sheep. Lane1 (100bp DNA ladder) Lanes 2, 3, 4, 5, 6, 8 (negative results). Lane7 (positive result)

DISCUSSION

RVF is considered to be one of the most important viral zoonoses in Africa. In this study I reported an outbreak of RVF in Jizan (Southern region of Kingdom of Saudi Arabia) In 2000, the RVFV spread to the Arabian Peninsula and caused two simultaneous outbreaks in Saudi Arabia (Jizan region) and Yemen (Galgalo *et al.,* 2000; Balkhy and Memish, 2003; Flick and Bouloy, 2005).Another outbreak was recorded in the same region (Jizan) in Saudi Arabia in 2004 (Abdo-Salem *et al.,* 2006; Elfadil *et al.,* 2006a; Elfadil *et al.,* 2006b). RVFV usually associated with periodic outbreaks (Evans *et al.,* 2007; LaBeaud *et al.,* 2007; Nguku *et al.,* 2007).

Unlike the first and second RVF outbreaks recorded in Kingdom of Saudi Arabia, time of occurrence of the present outbreak was away from rainy season and in absence of mosquitoes. Gerdes (2004) concluded that localized heavy rainfall is seldom sufficient to create

conditions for an outbreak of RVF .The same author reported that after virus amplification in vertebrates, mosquitoes act as secondary vectors to sustain the epidemic. RVFV can be greatly amplified during periods of epizootic (Davies, 2006) and prior to outbreak (Clements *et al.,* 2007b; Evans *et al.*, 2007; Paweska *et al.,* 2008).

Although, the present RVF outbreak was the third outbreak observed in the same area (Jizan), abortion and mortality rates were high. These results were in agreement with observation of Fenner *et al.,* (1993) and results of Abd El-Rahim *et al.,* (1999).

Results of this study clearly showed that large number of infected animals (18) were apparently healthy, and this is very important from epidemiological point of view. I think that these animals were the source of infection and the cause of this outbreak. This observation could be explained by the results of Evans *et al.,* (2007) and Paweska *et al.,* (2008) who reported that wild ruminants are act as reservoirs for RVFV during inter epidemic periods and as amplifiers during epidemics and therefore, they play a role in RVFV maintenance between outbreaks and virus amplification prior to a noticeable outbreak. I can add that this role is not played by wild ruminant only but also sheep which are apparently healthy and carry RVFV could play this role. Both sub-clinical and clinically affected animals represent a hazard as a source of infection (Davies, 2006).

Currently, beside recognizing clinical and biological features, diagnosis of RVF infection is based on the detection of specific immunoglobulin M or virus isolation in mammalian or mosquitoes cells or in the brains of suckling mice (Digoutte *et al.,* 1992; Abd El-Rahim *et al.,* 1999). Recently, Sall *et al.,* (2001) described a single tube RT-PCR that allows for the detection of RVFV in clinical samples (Garcia *et al.*, 2001). So, I used all these techniques (except using of mosquitoes cells for virus isolation) in my study for detection of the cause of the outbreak and for selection of the most available sensitive test for diagnosis of early infection of RVF.

ELISA used in this study failed to detect IgM in the first few days of outbreak. In experimentally infected sheep, IgM antibodies were detected from 3 to 4 days post infection (Van Vuren *et al.,* 2007). While IgM were detected after about one week in this work. The same result was recorded by Paweska *et al.,* (2003b); Paweska *et al.,* (2005a); Paweska *et al.,* (2005b); Paweska *et al.,* (2007) and Soumare *et al.,* (2007).

The occurrence of the first confirmed outbreaks of RVF in human and animals outside African region (in the Kingdom of Saudi Arabia and Yemen) is of global medical and veterinary importance. Disadvantages of classical techniques for serological diagnosis of RVF include health risk to laboratory personnel, restrictions for their use outside endemic areas and inability to distinguish between different classes of immunoglobulins (Paweska *et al.*, 2003a). So, there is a need to recent technique which can overcome all these disadvantages. Therefore, I used RT-PCR as a recent technique for diagnosis of RVF.

In seven samples examined in the present study, viral genome was detected by RT-PCR but no virus was isolated. This observation might be explained by the fact that storage and transport conditions were not adequate for the rescue of infectious virus although nucleic acid was demonstrable by the persistence of RVF RNA after the clearance of the virus from the blood (Sall *et al.*, 2002). I think that the persistence of RVFV RNA after the clearance of the virus from blood is the accurate and accepted reason for failure of virus isolation in detection of these seven samples because in this work, my trial for isolation of RVFV was performed without transportation or storage of samples. However, this finding emphasize a significant advantage of RT-PCR over VI particularly during outbreaks when fast and sensitive assay is required. High sensitivity of RT-PCR in diagnosis of RVF has been recorded (Weidmann *et al.*, 2007).

Results of the present work proved that RT-PCR is more sensitive than ELISA and virus isolation. RT-PCR more sensitive than vero cell cultures for virus detection beside that there were no false positive and the procedure followed with 2 particular chosen primers gave consistently clear band of the PCR products on agarose gel without nested PCR being necessary (Jup *et al.*, 2000). RT-PCR was found more sensitive than virus isolation method (Sall *et al.*, 2001).

In addition to its ability to detect positive samples that gave negative result with VI and ELISA, RT-PCR used in this study could detect RVFV genome early. RT-PCR can be used reliably for RVF diagnosis early in the disease (Sall *et al.*, 2002). VI performed in this work could also identify early infection of the disease. Since RT-PCR is more rapid and sensitive, I recommend using of this technique as a first line diagnostic method for RVFV when a suspected outbreak occurs. Repeated occurrence of RVF outbreaks specially with circumstances observed in the last outbreak investigated in this study proved that RVF

became endemic in KSA so, it will be the first RVF endemic country outside Africa continent.

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