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CLINICAL AND DIAGNOSTIC STUDIES ON RESPIRATORY SYNCYTIAL VIRUS (RSV) INFECTION IN CATTLE AND BUFFALOES

(With 3 Tables and 2 Figures)

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دراسات إكلينيكية و تشخيصية للإصابة بالفيروس التنفسى المتضخم فى الأبقار والجاموس

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تم فحص ٥٠ بقرة و ٥٠ جاموسة للكشف عن الإصابة بالفيروس التنفسى المتضخم. هذه الدراسة أجريت للتعرف على الصورة الإكلينيكية هذا المرض فى الأبقار والجاموس وللمقارنة بين الطرق المختلفة للتشخيص لاختيار أفضلها من ناحية الحساسية والدقة والسرعة. تم فحص الحيوانات من الناحية الإكلينيكية والسيرولوجية (باستخدام اختبار الاليزا) والتكنولوجيا الحيوية (باستخدام التفاعل التلمرى المتسلسل).بالنسبة لاختبار الإليزا تم فحص الحيوانات مرتين متتاليتين يفصل بينهما شهر. الفحص الإكلينيكي أظهر تباين شديد فى الأعراض بين الحيوانات المصابة. الأعراض الإكلينيكية كانت شديدة فى العجول بينما تراوحت بين المتوسطة إلى الخفيفة أو الغير ظاهرة فى الحيوانات الكبيرة ما عدا ٤ بقرات وجاموستين أظهروا أعراض شديدة تشبه الأعراض التى سجلت فى العجول.تم عزل الفيروس والتعرف عليه فى ١٢ بقرة (٢٤%) و٨ جاموسة (١٦%). فى اختبار الإليزا الأول تم تشخيص المرض فى ١٣ بقرة (٢٦%) و١٠ جاموسة (٢٠%) ولم تتغير هذه النتيجة فى اختبار الإليزا الثانى. باستعمال التفاعل التلمرى المتسلسل تم التعرف على الحامض النووى للفيروس التنفسى المتضخم فى ١١ بقرة (٣٨%) و ١٤ جاموسة (٢٨%). الحيوانات التى أعطت نتائج إيجابية مع عزل الفيروس و مع اختبار الإليزا كانت إيجابية مع اختبار التفاعل التلمرى المتسلسل. نتائج هذا العمل أوضحت أنه لا يمكن تشخيص هذا المرض من الفحص الإكلينيكي فقط ولكن من الضرورى استخدم التشخيص المعملى خاصة فى الحيوانات الكبيرة حيث أن كثير من الحيوانات التى ثبت إصابتها بالفحص المعملى كانت سليمة ظاهريا مما يمثل خطورة شديدة من الناحية الوبائية. كما أثبتت نتائج هذا البحث أن اختبار التفاعل التيمرى المتسلسل أكثر حساسية من العزل الفيروسى ومن اختبار الإليزا كما أن اختبار الإليزا أشد حساسية من العزل. كما يمتاز التفاعل التلمرى المتسلسل بالسرعة والدقة حيث أمكن الحصول على نتائج دقيقة خلال ٥ ساعات من جمع العينات ولهذا نوصى باستعماله لتشخيص هذا الفيروس بدلا من الطرق التقليدية. من خلال هذه النتائج يمكننا القول أن الفيروس المتضخم التنفسى يصيب الأبقار والجاموس فى مصر ويلعب دور هام كمسبب

رئيسى لأمراض الجهاز التنفسى لذلك يجب وضعه فى الاعتبار عند التعامل مع أى وباء لمرض تنفسى . هذه أول دراسة تتناول إصابة الجاموس بالفيروس المتضخم التنفسى فى مصر كما أنها أول دراسة تتناول تشخيص هذا الفيروس باستعمال تقنية تعتمد على الهندسة الوراثية (التفاعل التبلمرى المتسلسل) فى مصر.

SUMMARY

50 cattle and 50 buffaloes were investigated for respiratory syncytial virus (RSV) infection. Clinical, serological (using indirect enzyme linked immunosorbent assay " ELISA"), virological (using virus isolation) and biotechnological (using reverse transcription polymerase chain reaction "RT-PCR") examinations were performed. Clinical examination revealed great variations in clinical signs among infected animals. Clinical signs were severe in calves while adult cattle and buffaloes showed only mild to moderate signs except 4 cattle and 2 buffaloes suffered from severe signs similar to that observed in calves. The virus was isolated and identified in 12 cattle and 8 buffaloes. 13 cattle (26%) and 10 buffaloes (20%) were positive with first indirect ELISA and the same result was observed with the second indirect ELISA. By using RT-PCR, RS viral nucleic acid was detected in 19 cattle (38%) and 14 buffaloes (28%). The animals, which gave positive result with VIRUS isolation and indirect ELISA, were positive with RT-PCR. Results of the present work proved that clinical examination is not enough for diagnosis of RSV infection but laboratory investigation is very important specially in adult animals as large number of positive animals were apparently healthy, this is very dangerous from epidemiological point of view. RT-PCR technique seemed to be more sensitive than virus isolation and ELISA. At the same time this technique fast and accurate as we could obtained the results within 5 hours from collection of samples, therefore, we recommend using of this technique in diagnosis of RSV. From our results, we can record that RSV infects cattle and buffaloes in Egypt and plays an important role as main cause of respiratory tract disease and must put in consideration in dealing with any respiratory tract disease outbreak. This is the first study investigate RSV infection in buffaloes and the first to use RT-PCR technique in diagnosis of this virus in Egypt.

Key words: *Respiratory syncytial virus (RSV), RT-PCR, ELISA, virus isolation, Buffaloes, Egypt*

INTRODUCTION

Respiratory tract disease complex has a major economic impact on the beef industry in terms of losses from morbidity, mortality, and reduced efficiency of beef production (Brodrsen and Kelling, (1998). It remains the most important health problem in both dairy and fattening calves (Uttenthal *et al.*, 1996).

Bovine respiratory syncytial virus (BRSV) is a major respiratory tract pathogen of cattle and is one of the few viruses that can cause respiratory tract disease without significant interaction with other pathogens (Duncan and Potgieter, 1993). It has been established as an important viral component in the bovine respiratory tract disease complex (Baker *et al.*, 1997) and has an important role in the pathogenesis of pneumonia in cattle, especially in calves and yearlings (Scott *et al.*, 1996; Baker *et al.*, 1997)

This virus is a member of the genus pneumovirus in the subfamily pneumovirinae of the family paramyxoviridae (McIntosh, and Chanock, 1990) and was first isolated from cattle with acute respiratory disease in Switzerland and Japan in 1970 (Inaba *et al.*, 1970;Paccaud and Jacquier, 1970). Since that time, BRSV has emerged as an important and unique cause of respiratory tract disease of cattle in many countries (Baker *et al.*,1986a; Burgu *et al.*,1990). The viral genome is a linear, single-stranded negative-sense RNA (Viuff *et al.*, 1996 ; Van Der Poel *et al.*, 1999).

In calves, bovine respiratory syncytial virus (BRSV) induces acute interstitial pneumonia with alveolitis and bronchitis (Collie,1992). Signs of BRSV in older cattle are mostly moderate or inapparent (Baker *et al.*, 1986b; Van Der Poel *et al.*, 1993) but severe respiratory tract disease as a result of infection by this virus in adult dairy cattle has been reported (Elvander,1996 ; Woldeneskel *et al.*,2002).

Outbreaks of BRSV infection can develop without clinical evidence of the disease (Van Der Poel *et al.*, 1993) therefore the laboratory investigation for detection of BRSV and consequently its control is very important (Walsh *et al.*,2001).

Several laboratory methods were used to establish the diagnosis of BRSV infection including virus isolation and serologic procedures (Baker *et al.*,1986a ; Ploeger *et al.*,1986).Recently, diagnosis of BRSV has been done by using reverse transcription polymerase chain reaction (RT-PCR) as an excellent application of molecular biology in diagnosis (Walsh *et al.*,2001).

There is no adequate report on the presence and impact of BRSV infection in cattle and buffaloes in Egypt and the potential losses due to this infection may have not been recognized in the past, therefore the study reported here was done to establish existence of BRSV in Egypt using virus isolation, indirect ELISA and polymerase chain reaction. Comparison between different methods used in diagnosis of infection in this study and selection of most sensitive and rapid one was an important aim of the study.

MATERIAL and METHODS

Animals:

50 cattle (44 female and 6 male) and 50 buffaloes (45 female and 5 male) with age ranged from 6 months to 5 years were used in the study. Some of these animals were suffered from respiratory signs. These animals were found in farms at Assiut and Sohage provinces, Upper Egypt.

Serum:

5 ml blood was collected twice one month apart from each examined animal to obtain serum for indirect ELISA.

Nasopharyngeal swabs: One swab from caudal aspect of nasopharynx was collected from each examined animal for virus isolation.

Nasal swab:

One nasal swab was collected from each examined animal for extraction of viral RNA.

1- Evaluation of clinical signs:

All animals used in this study examined clinically. The animals that gave positive results with laboratory investigation were examined once daily for two weeks. An evaluation system modified from that of Collie (1992) was used, specific clinical signs were reported to convey a more detailed picture of the infected animals. Rectal temperature, respiratory rate, nasal or ocular discharge, cough, abnormal lung sounds and dyspnea were evaluated.Nasal discharge was defined as any discharge from one or both nostrils and characterized as serous, mucoid or mucopurulent . Ocular discharge defined as any discharge from one or both eyes and characterized as serous, mucoid or mucopurulent. Cough was defined as an unelicited cough heard during the examination process or elicited on light palpation of the trachea. Dyspnea was defined as forced expiration (expiration with abdominal effort) , open-mouth breathing or both. (Woolums *et al.*,1999)

2- Virus isolation:

Swab specimens were obtained from the caudal aspect of nasopharynx using sterile cotton–tipped swabs. After a specimen was obtained from one nostril of examined animal , the swab specimen was placed in a tube containing 2 ml of Eagle's minimal essential media and transport to the laboratory on ice. Media from the tube was filtered through 0.2 µm filter. Primary bovine turbinate cells growing in multiple-chamber slides were inoculated with the filtrate. Slides were incubated (37°C in 5% Co2) and observed for cytopathic effect (CPE). When CPE was apparent (generally 7-10 days after inoculation), slides were fixed with acetone, stained with fluorescein-conjugated anti–BRSV and observed for intracellular virus. Slides were scored as positive or negative for virus (Woolums *et al.*, 1999). All reagents used in isolation were obtained from GIBCO, Grand Island, NY, which supply all requirements of Viral Diagnostic Lab., Ministry of agriculture, KSA where the isolation done.

3- Detection of other viruses:

To rule out unintended infection as a contributing factor in disease, the animals were tested for antibodies to IBR, PI3 and BVD viruses using blocking ELISA (Uttenthal *et al.*,1996) two successive times one month apart.

Any animal proved to be positive for antibodies to IBR, PI3 and BVD viruses was excluded from this study.

4- Indirect ELISA

The BRSV-specific IgG in serum was determined by use of a double antibody sandwich ELISA as described by Van Der Poel et al (1999). Briefly, BRSV antigen was bound to the wells of microtitration plates that had been coated with a mixture of BRSV fusion protein-specific monoclonal antibodies. The BRSV-specific antibodies in serum, which react with BRSV antigen was detected by addition of rabbit anti-bovine immunoglobulin conjugated with horseradish peroxidase. Serial twofold dilutions of sera were tested, starting from 1:80. Substrate solution consisted of 10mM sodium phosphate buffer (pH 6.8), 0.1 mM EDTA, 0.1 5-aminosalicylic acid, and freshly added 0.005% H2O2. Mixture were allowed to incubate overnight at 4°C. Color development (absorbance) was measured by use of a spectrophotometer (Multiskan EX Version 1.1, Labsystems) at a wave length of 450nm.

Indirect ELISA was performed two times one month apart for each animal.

5- RT-PCR

Synthetic oligonucleotide primer:

The primer for BRSV (Pharmacia Biotech.) was designed by comparison of published sequences of BRSV (Harmon *et al.*,2001). More specifications of this primer are listed in Table (1).

Table 1: Specification of used primer.

Primer	Sequence5` - 3`	Expected virus	Position
P89	GTCACGTAGCTCATCGAGAGCTAG	RSV	3224 – 3247

RNA extraction:

Total RNA was extracted with TRIzol Reagent (Gibco BRL). 100 µl sample (nasal swab extracted in phosphate buffered saline) was mixed with 900 µl TRIzol Reagent. The samples were incubated for 5 min at room temperature, addition of equal amount chloroform, precipitation of RNA with isopropanol and washing of RNA with 75% ethanol. The purified RNA was resuspended in 20 µl Rnase free-water and redissolved by incubating for 10 min at 55°C

Reverse transcription:

First strand cDNA synthesis was performed for 15 min at 37°C in a 10 µl reaction mix containing the followings:- (1) 50 mM Tris-Hcl (pH 8.3)), (2) 75mM KCl, (3)3 mM MgCl2, (4) 10 mM dithiothretiol, 0.125 mM (each) dNTP, (5) 5 µM (each) downstream primer, (6) 100 units Moloney murine leukemia virus reverse transcriptase (Gibco BRL) and (7) 0.5 µl RNA. This was followed by heating for 5 min at 95°C, in order to denature RNA-cDNA hybrid. Samples were chilled and stored at -20°C.

PCR amplification:

A PCR mix (10 µl) consists of the followings:- 10mM Tris-Hcl (pH 8.8), 50mM KCl, 1.5mM MgCl2, 0.1% Triton X100, 0.05 mM (each) dNTP, 0.6 µM (each) primer, 0.25 units Taq polymerase (Promega) and 2% cDNA reaction mix. Amplification and reverse transcription were performed on a gene Amp CR system 9600 (Perkin Elmer) using program as follow: (1) I min at 95°C (2) 15 sec at 95°C (3) I min at 60°C (4) 6 min at 60°C, repeating steps (2) and (3) for 35 cycles.

Gel electrophoresis:

The samples were electrophoresed at 100 V for 30 min in TAE buffer on 1.5 % agarose gels, stained with ethidium bromide (0.6 µg/ ml). DNA molecular weight marker type 100bP ladder (GibcoBRL) was applied to identify the size of the PCR products. Negative control for

RT-PCR were performed by adding of distilled water instead of the primers.

RT-PCR was performed as described by (Walsh *et al.*,2001).

RESULTS

1-Clinical examination:

Clinical examinations of investigated animals showed great variation in clinical signs among infected animals. These signs varied from slight rise of rectal temperature up to severe pneumonia. Calves were the most affected animals while adult cattle and buffaloes exhibit only mild to moderate signs, except 4 cattle and 2 buffalo that suffered from severe pneumonia similar to that observed in infected calves. In details, four calves showed persistent fever over 40°C, profuse mucoid nasal discharge, strong dry cough, abnormal lung sound and dyspnea. Two calves showed persistent fever over 40°C ,mucoid ocular discharge and weak dry cough. One calf showed persistent fever, profuse serous ocular discharge and strong dry cough. Three adult cattle showed persistent fever, serous nasal discharge, strong dry cough, abnormal lung sound and dyspnea, one adult cattle showed persistent fever, scant mucopurulent nasal discharge and abnormal lung sound. Concerning buffaloes, three buffalo calves showed persistent fever over 40°C, mucopurulent nasal discharge, strong dry cough, abnormal lung sound and dyspnea. One buffalo calf showed persistent fever, mucoid nasal discharge, mucopurulent ocular discharge, abnormal lung sound and dyspnea. Two adult buffaloes showed persistent fever, mucoid nasal discharge, strong dry cough and abnormal lung sound and two adult buffalo showed only mucoid nasal discharge. Large number of examined animals (8 cattle and 6 buffaloes) showed no clinical signs while was positive for laboratory investigations. Results of clinical examination are summarized in Table (2).

Table 2: Results of clinical examination.

	Persistent fever	Nasal discharge S	M	MP	Ocular discharge S	M	MP	Cough SD	WD	Abnormal lung sounds (Crackles or Wheezes)	Dyspnea	No signs
Cattle	11	3	4	1	1	2	-	8	2	8	7	8
Buffalo	6	-	5	3	-	-	1	5	-	6	4	6

S = Serous M = Mucoid MP = Mucopurulent SD = Strong dry WD = Weak dry

2-Virus isolation:

RSV was isolated and identified (through detection of characteristic CPE and fluorescein staining) in 12 cattle (24%) and 8 buffaloes (16%).

3-Indirect ELISA:

First examination: 13 cattle (26%) and 10 buffaloes (20%) were positive for RSV antibodies.

(b) Second examination: the same result like first examination without change.

4-RT-PCR:

Respiratory syncytial viral nucleic acid was detected in 19 cattle (38%) and 11 buffaloes (22%).

Results of virus isolation, indirect ELISA and RT-PCR are summarized in Table (3). Results of RT-PCR are shown in Figs. 1 and 2.

Table 3: Results of laboratory investigation.

	Virus isolation	Indirect ELISA	RT-PCR
Cattle	12	13	19
Buffalo	8	10	14

DISCUSSION

BRSV is one of the primary agents in the pathogenesis of respiratory tract disease (Baker *et al.*, 1997; Gershwin *et al.*,1998; Martin *et al.*, 1999). Severe respiratory disease with clinical signs including dyspnea along with coughing, , nasal discharge, increased respiratory rate and rectal temperature of greater than 40°C have been recorded in calves examined in the present study. An excellent models for BRSV infection including severe respiratory disease in calves have been published (Lotthammer and Ehrles, 1990; Belknap *et al.*,1995; Brodersen and kelling, 1998).

Our results showed wide varieties in clinical signs among infected animals. The outcome of a BRSV infection can be influenced by several factors (Elvander, 1996). Dyspnea was observed mainly in calves used in our study. Woolums *et al.*, (1999) recognized dyspnea in several infected calves. Dyspnea might be related to the presence of edema and primary emphysema followed by inflammation of the lungs. The main cause of this change is destruction of mucociliary apparatus by the viral action in respiratory epithelia (Kimman *et al.*, 1989; Redondo *et al.*, 1994; Otto *et al.*, 1996).

Cattle infected with BRSV have only mild signs of the disease including various combinations of nasal discharge, cough, variable pyrexia and rarely abnormal lung sounds (Woolums *et al.*, 1999). Cattle and buffaloes in our work showed mild form of the disease. However 4 cattle and 2 buffaloes exhibited severe clinical signs similar to that observed in calves. Severe respiratory tract infection with BRSV may develop in adult cattle (Elvander,1996). These variations in disease severity among infected animals could be explained by the fact that animal exposed to the same infection more than one time may have some resistant to the infection and even if infected suffer only from mild signs, adult animals used in our study may have previous exposure to RSV with exception of 4 cattle and 2 buffalo together with calves may have not previous exposure to the virus. Some authors (Baker *et al.*, 1992; Woolums *et al.*, 1999) tried to produce explanation for the cause of difference in severity of the disease among infected animals based on strain variation but they couldn't prove that and they concluded that strain variation has not been definitively identified as a determinant of disease severity in RSV infection. Large number of infected cattle and buffaloes examined in this study didn't record fever above 39.5°C. The same result have been reported by Scott *et al.* (1996). The disease may induce no clinical signs in infected animals (Van Der Poel *et al.*,1993). Our data, in addition to those of Van Der Poel *et al.* (1993) and Scott *et al.* (1996) indicate that routine clinical examination of animals is not sufficient for diagnosis of BRSV

Diagnosis of BRSV in living animals based on virus isolation and serological examinations, but both techniques have many disadvantages (Kimman *et al.*, 1986; Baker *et al.*, 1997)

In this study a rapid method, based on molecular biology and amplification of BRSV genome has been used to establish an accurate, sensitive and rapid test for diagnosis of BRSV. RT-PCR is rapid and it is more sensitive than traditional techniques used for diagnosis of RSV (Walsh *et al.*, 2001).

In conventional diagnostic work a demonstration of the virus requires lung tissue which is only available post mortem (Uttenthal *et al.*,1996). In the present study we overcome this disadvantage by using nasopharyngeal swabs taken from the living animals as a source for virus isolation (Woolums *et al.*, 1999). However, RT-PCR is more sensitive than virus isolation and could detect positive cases that virus isolation failed to detect it. At the same time virus isolation on cell culture is a cumbersome method and ineffective in field specimens

which are not fresh (Kimman *et al.*, 1986). Another disadvantage of virus isolation is that BRSV may only be detected in the acute stage of the disease (Uttenthal *et al.*, 1996), while RT-PCR technique could detect the infection at any stage (Walsh *et al.*, 2001)

ELISA seemed to be more sensitive than virus isolation through the result of our study. Serum antibodies remain for longer after the acute infection while virus isolation available only in acute stage (Kimman *et al.*, 1989; Kimman and Westenbrink, 1990).In the other hand indirect ELISA couldn't used in calves less than four months age as the passive immunity in calves (derived through ingestion of colostrum) remain detectable in an average of 99 days (Baker *et al.*, 1985; Baker *et al.*, 1989), whereas diagnosis of BRSV in this age is very important (Kimman *et al.*, 1988; Van Der Poel *et al.*, 1994). RT- PCR can be performed at any age (Viuff *et al.*, 1996 ; Harmon *et al.*, 2001). At the same time ELISA requires pair samples ,at least two weeks apart between collections, (Kimman *et al.*, 1986) while RT-PCR requires only one sample. Our result reported higher sensitivity of RT-PCR than ELISA did.

RT-PCR used in the present study improves the ability to detect BRSV in respiratory samples and should improve the ability to rapidly diagnose BRSV infection in cattle and buffaloes. This is very important in control of such infection and has a very important value in any epidemiological study concerning BRSV infection in Egypt in the future, because this sensitive technique can detect positive animals that couldn't be detected by virus isolation or serology especially apparently healthy animals which play great role in epidemiology of RSV infection

This study proved that BRSV infection is found in both cattle and buffaloes in Egypt .To my knowledge, the studies reported here are the first to investigate BRSV in buffaloes and the first to use RT-PCR in diagnosis of this virus in Egypt.

From results of this investigation we can conclude that BRSV plays an important role as a cause (alone, without presence of other viruses) of respiratory tract disease in cattle and buffaloes in Egypt and must put in consideration in investigation of any respiratory tract disease outbreak especially in calves, and before planning of any control program of this problem in Egypt .RT-PCR is very sensitive and rapid technique in diagnosis of BRSV infection and have many advantages over the traditional methods of diagnosis (serology and virus isolation), therefore this technique should replace the conventional diagnostic techniques especially in epidemiological studies.

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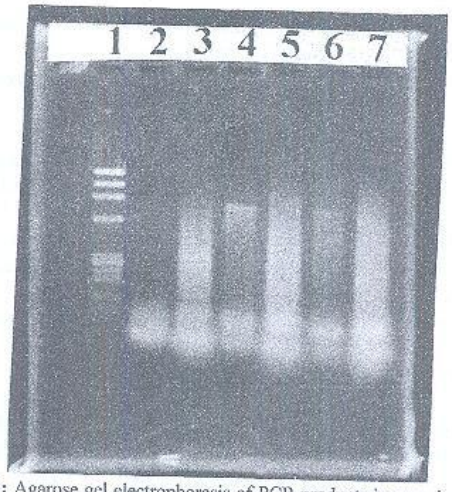

Fig. 1: Agarose gel electrophoresis of PCR products in examined cattle. Lane 1 (100 base pair DNA ladder), Lanes 2,3,5,7 (negative results) and lanes 4,6 (positive results).

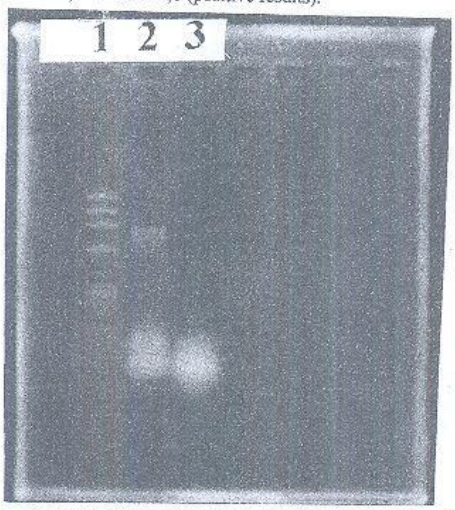

Fig. 2: Agarose gel electrophoresis of PCR products in examined buffaloes. Lane 1 (100 base pair DNA ladder), Lane 2 (positive result) and lane 3 (negative result).