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AN OUTBREAK OF INFECTIOUS BOVINE RHINOTRACHEITIS (IBR) IN EGYPT

(With 2 Tables and 7 Figures)

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وباء مرض التهاب الأنف والقصبة الهوائية المعدى للأبقار فى مصر

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وباء مرض تنفسى وأعراض على العين تم ملاحظته حديثا (ابريل - يونيو٢٠٠٣) فى مصر. لمعرفة سبب هذا الوباء تم إجراء فحص إكلينيكي وفيروسى وسيرولوجى. لإجراء هذه الفحوص تم جمع عينة حليب مشتركة تمثل كل القطيع كما تم جمع مسحة أنفية وعينة حليب وعينة سيرم من كل حيوان من الحيوانات التى تم فحصها وعددها مائة بقرة. بعض هذه الحيوانات (٧٠بقرة) كانت تعانى من أعراض تنفسية أو تغيرات فى العين أو الاثنين معا وباقى الحيوانات (٣٠ بقرة) كانت سليمة ظاهريا. تم فحص زوج من عينات الحليب والسيرم للتعرف على الأجسام المضادة الخاصة بمرض التهاب الأنف والقصبة الهوائية المعدى للأبقار ولمعرفة التحول السيرولوجى نحو فيروس الهربز البقرى -١ المسبب لهذا المرض وذلك باستخدام اختبار الإليزا التنافسى بينما تم استخدام المسحات الأنفية لعزل الفيروس المسبب للمرض. تم إجراء اختبار الإليزا مرتين متتاليتين يفصل بينهما ثلاثة أسابيع. الفحص الإكلينيكي أظهر وجود أعراض تنفسية وتغيرات فى العين مع تباين فى هذه الأعراض واختلاف فى شدتها بين الحيوانات المصابة . تم عزل فيروس الهربز البقرى-١ والتعرف عليه فى ٧٤ حيوان. ٨٥ عينة سيرم كانت إيجابية للأجسام المضادة لفيروس الهربز البقرى-١ فى اختبار الإليزا الأول بينما أوضح اختبارالإليزا الثانى وجود ٨٨ عينة سيرم إيجابية. ٨٣ عينة حليب كانت إيجابية للأجسام المضادة لفيروس الهربز البقرى-١ فى اختبار الإليزا الأول بينما سجل اختبار الاليزا الثانى عدد ٨٥ عينة حليب إيجابية. عينة الحليب المشتركة الممثلة لكل القطيع كانت إيجابية مع اختبار الاليزا الأول والثانى. ٧٠ بقرة مصابة بمرض التهاب الأنف والقصبة الهواية المعدى للأبقار كانت تعانى من أعراض إكلينيكية بينما ١٨ بقرة مصابة (بينهم ٣ بقرات حديثة الشراء) كانت سليمة ظاهريا. هذه الدراسة أثبتت أن فيروس الهربز البقرى-١ يستطيع أن يلعب دورا هاما كمسبب لوباء مرض تنفسى يتميز بوجود تغيرات فى العين بالإضافة إلى الأعراض التنفسية الشديدة. كما أثبتت أن إدخال حيوانات جديدة سليمة ظاهريا إلى القطيع هو سبب دخول ونشر مرض التهاب الأنف والقصبة الهوائية المعدى للأبقار لهذا القطيع. أيضا أوضحت نتائج هذا العمل أن عزل الفيروس أقل حساسية من اختبار الاليزا الذى يعتبر حساس وعملى لتشخيص

مرض التهاب الأنف والقصبة الهوائية المعدى للأبقار سواء مع عينة السيرم أو عينة الحليب. من خلال هذه الدراسة نستطيع أن نسجل أن المسح الدورى المنتظم لعينة الحليب المشتركة الممثلة لكل القطيع في مزارع إنتاج الحليب وفحص عينات السيرم أو عينات الحليب الفردية أوكلاهما للحيوانات المشتراة حديثا باستخدام اختبار الأليزا تعتبر خطوات هامة جدا للسيطرة على مرض التهاب الأنف والقصبة الهوائية المعدى للأبقار فى مصر. هذه أول دراسة تتناول مرض التهاب الأنف والقصبة الهوائية المعدى للأبقار فى صعيد مصر كما أنها أول دراسة تستخدم عينات الحليب (التى يسهل جمعها والتعامل معها) لتشخيص هذا المرض فى مصر.

SUMMARY

Outbreak of respiratory tract and eye infections has recently (April-June, 2003) been observed in cattle in Egypt. To investigate the cause of this outbreak, clinical, virological and serological examinations were performed. Nasal swabs, bulk tank milk, and individual milk and serum samples were collected from 100 cattle, some of them were suffered from respiratory and/or ocular signs (70 cattle) and the remainder (30 cattle) was apparently healthy. Pair samples of sera and milk were tested for antibodies against IBR and for detection of seroconversion to IBR virus using blocking enzyme linked immunosorbent assay (ELISA) while nasal swabs were used for isolation of the causative virus. Blocking ELISA was performed two successive times three weeks apart. Clinical investigation showed great variation in signs and its severity among infected animals, varied from severe respiratory and/or ocular signs to mild affection. Bovine herpesvirus type1 (BHV-1) was isolated and identified in 74 animals. 85 serum samples were positive for BHV-1 virus antibodies in first blocking ELISA of serum while the second blocking ELISA of serum showed 88 positive samples. 83 milk samples were positive with first blocking ELISA of milk while second blocking ELISA recorded 85 positive milk samples. Bulk milk sample was positive in first and second ELISA. 70 out of 88 infected animals were suffered clinically while the remainder 18 infected animals were apparently healthy (three of them were recently purchased). This study proved that BHV-1 could play an important role as a cause of respiratory tract infection outbreak characterized by eye affections and severe respiratory signs, introduction of newly purchased apparently healthy animals is the cause of introduction of the infection into the herd. Also, results of this work showed that virus isolation is less sensitive than blocking ELISA which is sensitive and reliable test for diagnosis of IBR either in serum or milk samples. Through out this study we can recorded that regular screening of bulk milk sample in dairy farms using blocking

ELISA and examination of serum and/or individual milk samples of newly purchased animals by blocking ELISA before introduction into the herd are very important steps in control of IBR in Egypt. This is the first study investigated IBR infection in Upper Egypt and the first to use milk sample (which can be easily collected and handle) in diagnosis of IBR in Egypt.

Key words: Infectious bovine rhinotracheitis (IBR), Bovine herpesvirus-1 (BHV-1), Blocking ELISA, Virus isolation, bulk milk, cattle, Egypt

INTRODUCTION

Bovine respiratory disease is a leading cause of loss to cattle industry throughout the world (Ackermann and Brogden, 2000). About 5-20% of the young animals succumbed to acute or chronic pneumonia every year (Rusvai and Fodor, 1998).

IBR is an important cause of bovine respiratory disease (Graham *et al.*,1998;Martin *et al.*,1999), it is consider the main infectious disease initiating the *respiratory* disease in cattle (Castrucci *et al.*,1998) and IBR virus is one of the most important respiratory pathogens of domestic ruminants (Hanon and Pastoret, 1998; Rusvai and Fodor, 1998; Silva *et al.*,1999).

IBR is caused by bovine herpesvirus type 1 (BHV-1) which have been described as member of the genus Varicellovirus, within the subfamily Alphaherpesvirinae, belong family Herpesviridae (Vonk Noordegraaf *et al.*,1998 ; Ozherelkov *et al.*,2001;Van Drunen *et al.*,2001) .

Herpesviruses are widespread viruses, causing severe infections in both humans and animals (Turin *et al.*,1999). Cattle are the natural host of herpesviruses. Since now four different bovine viruses have been described as members of the family Herpesviridae. BHV-1 is the prototype of the bovine herpesviruses, it is the aetiological agent of a number of diseases and not only IBR (Straub, 2001). These diseases are, mild genital disease known as infectious pustular vulvovaginitis (IPV) and infectious balanoposthitis (IBP) (Trapp *et al.*,2003). These diseases are caused by strains with different genotype of BHV-1 (Peshev *et al.*,1998; Rijsewijk *et al.*,1999; Schynts *et al.*,1999).Recently, two genotypes of BHV-1 were recorded, BHV-1.1 (cause of IBR) and BHV-1.2 (cause of IPV).Therefore, infection of cattle with herpesviruses have clinical and diagnostic importance (Trapp *et al.*,2003).

IBR has a worldwide distribution (Leary and Splitter,1992 ;Vonk Noordegraaf *et al.*,1998; Mars *et al.*,1999) and many outbreaks were recorded in different countries at last few years (Dunbar *et al.*,1999; Boelaert *et al.*,2000; Paisley *et al.*,2001; Solis Calderon *et al.*, 2003) causing systemic, eye, respiratory and genital tract infections (Gilliam *et al.*,1993; Castrucci *et al.*,1995;Turin *et al.*,1999), as well as chronic wasting in some cases (Castrucci *et al.*,2000;De Kruif, 2001;Van Wuijckhuise *et al.*,2001). Cattle infected with IBR suffered from severe clinical signs including fever, depression, hyperventilation, dyspnea, nasal discharge and coughing (Stipkovits *et al.*,2000).

Eradication of IBR is extremely difficult because of their ability to establish latent and life-long infections (Turin *et al.*, 1999; Lemaire *et al.*, 2001;Meyer *et al.*, 2001). However, latency is only one tool that has evolved in herpesviruses to successfully infect their hosts. Such viruses display a wide panoply of genes and proteins that make it difficult to develop accurate diagnosis by traditional techniques (Turin *et al.*, 1999;Perler *et al.*,2000; Six *et al.*,2001).

ELISA system may be used successfully to detect seroconversion in serum pairs in outbreaks of respiratory disease (Graham *et al.*,1998).ELISA of milk and serum samples were used for diagnosis of BHV-1 (Kramp *et al.*,1994; Tekes *et al.*, 1999).

In Egypt, There is no adequate available data concerning IBR and its diagnosis especially during outbreaks whereas accurate diagnosis is very important and consider the first and most important step in control of the outbreak. Therefore, the objective of the present study was to investigate respiratory tract disease outbreak clinically, serologically and virologicaly to identify the cause of the outbreak. At the same time, comparison between different tests used in diagnosis in this study and selection of the most sensitive and suitable one was an important objective of the study.

MATERIAL and METHODS

Animals:

100 adult female cattle were used in the study. Some of them (70) were suffered from respiratory and/or ocular signs and the remainder (30) were apparently healthy (out of them, 5 animals were recently purchased). These animals were found in dairy farm at Sohag province, Upper Egypt.

Serum:

5ml blood was collected twice three weeks apart from each examined animal to obtain serum for blocking ELISA.

Milk:

(a) Individual milk:10ml milk was collected twice three weeks apart from each examined animal for blocking ELISA.

(b) Bulk milk:10ml milk was taken from milk tank (collected all milk of the farm) two times three weeks apart .

Bovine Rhinotracheitis virus gB Antibody Test Kit (IDEXX Laboratories. Inc.USA):

Used for blocking ELISA in serum and milk.

Nasal swab: One nasal swab was collected from each examined animals at the beginning of the outbreak for isolation of the virus.

Baby Hamster Kidney (BHK) tissue culture: For BHV-1 isolation.

Clinical examination

All animals used in this work examined clinically. The animals were examined once daily for three weeks. An evaluation system modified from that of Collie (1992) was used. Rectal temperature, respiratory rate, nasal or ocular discharge, cough, abnormal lung sounds, dyspnea, conjunctivitis and keratoconjunctivitis were evaluated and specific clinical signs were reported.

Detection of other viruses

To rule out unintended infection as a contributing factor in disease, the animals were tested for antibodies to RSV, PI3 and BVD viruses using blocking ELISA (Utenthal *et al.*,1996) two successive times one month apart.Any positive animal for antibodies to these viruses was excluded from the study.

Virus Isolation

Virus was isolated from nasal swabs using Baby Hamster Kidney (BHK) cell monolayers grown to near confluence in Minimum Essential Medium with 1% bovine fetal serum (GIBCO,Grand Island,NY) which were maintained for14 days in 5% Co_2 at 37°C and evaluated daily for cytopathic effect of the virus. Immunofluorescence assays with specific BHV-1antisera (GIBCO,Grand Island,NY) were used to confirm the identity of virus isolates.Isolation and confirmation of presence of BHV-1 were done according to Leary and Spliotter (1992) and Weigler *et al.*, (1997).

Blocking ELISA

All reagents were allowed to come to room temperature before use and mixed by vortexing.

(a) Blocking ELISA in serum

-Preparation of wash solution

The (10x) wash concentrate was brought to room temperature and mixed to ensure dissolution of any precipitated salts. The wash concentrate was diluted 1 to 10 with distilled/deionized water before use (e.g., 50 ml of wash concentrate + 450 ml of water per plate to be assayed).

-Test protocol

1- Antigen coated plates were obtained and the sample position was recorded in sheet.
2- 50 µl of reconstituted wash solution was added to each well of the plate.
3- 50 µl negative control was dispensed into appropriate duplicate wells (A1 and A2).
4- 50 µl positive control was dispensed into appropriate duplicate wells (A3 and A4).
5- 50 µl samples were dispensed into remaining wells.
6- The content of the microwells was mixed using microtiter plates shaker.
7- The plates were tightly sealed (to avoid any evaporation) and incubated at 37°C for
2 hours.
8- Liquid contents of all wells were aspirated in appropriate waste reservoir.
9- Each well was washed with approximately 300 µl of wash solution five times, liquid contents of each wells was aspirated after each wash Plate drying between washes and prior to the addition of conjugate was avoided by fast washing using automatic plate washer wellwash4 (Labsystems, A Thermo Bioanalysis Company, Research Technology Devision, Helsinki, FINLAND). Following the final aspiration, the residual wash fluid was firmly taped from each plate onto absorbent material.
10- 100 µl of IBR-gB specific monoclonal antibodies: HRPO (Horseradish Peroxidase) conjugate was dispensed into each well.
11- The plates were incubated for one hour at room temperature (20°C to 25°C).
12- Liquid contents of all wells were aspirated in appropriate waste reservoir.

13- Each well was washed with approximately 300 µl of wash solution five times, liquid contents of each wells was aspirated after each wash
14 -100 µl of TMB substrate solution was dispensed into each well.
15- The plates were incubated for 10 minutes at room temperature (20°C to 25°C) in darkness (timing was begun after filling of the first well).
16- 100 µl of stop solution was dispensed into each well to stop the reaction. The stop solution was added in the same order as the substrate solution was added.
17- The spectrophotometer was blanked on air.
18- The absorbance of the samples and controls was measured at 450 nm using multiskan (Labsystems, A Thermo Bioanalysis Company, Research Technology Devision, Helsinki, FINLAND).

(b) Blocking ELISA in milk (individual and bulk samples)

-Preparation of sample

Whole milk samples were centrifuged for 15 minutes at 2000 xg. Skim milk from underneath the fat layer was used as a milk sample.

-Test protocol

The same protocol as mentioned in serum except that: -

-There is no addition of reconstituted wash solution to each well (step No.2 in serum).

-Negative control, positive control and milk samples were added in a volume of 100 µl instead of 50 µl used in serum (steps 3,4 and 5 in serum).

-There is no need to mix the content of microwells in the ELISA plate before first incubation (step No. 6 in serum).

-First incubation of ELISA plate must be at 2°C to 7°C in refrigerator over night or for 12-18 hours (step No.7 in serum).

(c) Calculation of results of blocking ELISA in serum and milk

-For the assay to be valid, the negative control mean (NCX) must be greater than or equal to 0.750 optical density (OD). In addition, the positive control mean (PCX) must have a blocking-percentage of more than 80%.

-The presence or absence of antibody to IBR-gB is determined by the blocking percentage for each sample. The positive control has been standardized and represents a significant level of antibody to IBR-gB.

1-Calculation of negative control mean (NCX)

$$NCX = \frac{A1\ A\ (450) + A2\ A\ (450)}{2}$$

2-Calculation of positive control mean (PCX)

$$PCX = \frac{A3\ A\ (450) + A4\ A\ (450)}{2}$$

3-Calculation of blocking percentage for test sample

$$\text{Blocking \%} = \frac{\text{NCX A(450) – optical density (OD) of tested sample}}{\text{NCX A (450)}} \times 100$$

4-Calculation of blocking percentage for positive control

$$\text{Blocking \%} = \frac{\text{NCX A(450) – PCX A (450)}}{\text{NCX A (450)}} \times 100$$

(d)Interpretation of results

-Milk and serum samples with blocking % less than 45% are classified as negative for IBR antibodies.

-Milk and serum samples with blocking % greater than or equal 45% but less than 55% are considered suspect and must be retested.

-Milk and serum samples with blocking % of 55% and greater are considered positive for IBR antibodies.

Blocking ELISA was performed according to Van Dionkersgoed and Babiuk (1991); Kramp *et al.*, (1994) and manufacturer's instructions.

RESULTS

1-Clinical examination:

Clinical examination of examined animals exhibits wide variation in clinical signs among them. These signs were restricted to respiratory system and eye. The signs varied from mild fever up to severe respiratory signs with dyspnea, pneumonia and ocular signs.Results of clinical examination are summarized in table (1) and figures (1-7).

Table (1): Results of clinical examination.

Cough	Fever	Nasal Discharge	Dyspnea	Ocular Discharge	Corneal Opacity	Conjunctivitis	(A)	(B)	(C)
52	61	54	41	12	5	12	34	16	20

(A) = Animals with respiratory signs only.
(B) = Animals with ocular signs only.
(C) = Animals with respiratory and ocular signs.

2-Virus isolation:

BHV-1 was isolated and identified in 74 animals.

3-Blocking ELISA

(a) Blocking ELISA in serum

First examination: 85 serum samples were positive for BHV-1 antibodies.
Second examination: 88 serum samples were positive for BHV-1 antibodies.
(b) Blocking ELISA in individual milk
First examination: 83 individual milk samples were positive for BHV-1 antibodies.
Second examination: 85 individual milk samples were positive for BHV-1 antibodies
(c) Blocking ELISA in bulk milk
Bulk milk sample was positive in first and second blocking ELISA.
Results of laboratory investigations are summarized in table (2).

Table (2): Results of laboratory investigation.

Laboratory Investigation	Virus isolation		Blocking ELISA		
			Serum	Individual milk	*Bulk milk
No. of positive animals	74	First examination:	85	83	+
		Second examination:	88	85	+

*Bulk milk is one sample representative for the entire herd as it taken from the large tank collected all milk of the farm.

DISCUSSION

IBR is a major plague in zootechnics and commercial trade, because of its ability to spread through asymptomatic carrier animals and latency .At the same time many aspects of this disease and its causative virus are still incompletely known. All of these factors make it difficult to develop accurate diagnostic technique (Turin *et al.*,1999; Lemaire *et al.*,2001). Therefore, recent diagnostic techniques were used in this study to detect animals infected with IBR especially asymptomatic carrier or latently infected animals.

In this study, blocking ELISA was used to estimate level of antibodies to IBR in serum and milk samples. The health status of the animal infected with IBR could be represented by the level of serum antibodies to IBR (Fulton *et al.*, 2002). Antibodies to IBR were detected in the present investigation in serum and milk samples. Biuk-Rudan *et al.*, (1999) detected antibodies to IBR in serum samples and Pritchard *et al.*, (2002) reported that IBR virus infection antibodies could be estimated in milk samples.

BHV-1 cauld be detected in bulk milk but if the bulk milk sample is negative or only slightly positive, the next step is to test all the

individual animals (Frankena *et al.*,1997). In the present work, the bulk milk sample was strong positive in first and second blocking ELISA. This could be obvious as a result of presence of large number of positive individual milk samples in our study while if number of positive samples is low, the negative samples will diluted the positive one and bulk milk may appear negative or weak positive (Frankena *et al.*,1997).

Blocking ELISA detected seroconversion in serum pairs of 88 animals examined in the present study. Graham *et al.*, (1998) concluded that blocking ELISA system can be used successfully to detect seroconversion in serum pairs. The second examination of serum and milk with blocking ELISA recorded larger number of positive animals than the first one. The possible explanation of this result could be the conclusion of Frankena *et al.*, (1997) that the frequency distribution of the titre of antibodies against BHV-1 appeared to be bimodal. The second explanation derives from the type of test used, in a blocking ELISA monoclonal antibodies was used and it is known that the affinity of antibodies to a single epitope has a bimodal distribution. Therefore, serologic diagnosis of IBR requires paired serum sample collected 7-14 days apart (Leary and Splitter,1992).

Byrne *et al.*, (2001) isolated BHV-1 from cattle suffering from severe respiratory signs and they reported that IBR most frequently detected in respiratory tract infection outbreak, at the same time Rijsewijk *et al.*,(1999) concluded that BHV1 cause severe respiratory disease. Experimental infection with BHV-1 developed severe acute respiratory sign in a study of Castrucci *et al.*, (1998) who concluded that BHV-1 is one of the main infectious agent initiating the respiratory disease in cattle. BHV-1 was isolated from outbreak of respiratory tract infection investigated in the present work.

BHV-1 was isolated from nasal swabs of 74 cattle in our study, as the virus replicates in the nasal mucosa (Makoschey and Keil, 2000; Romera *et al.*,2000;Meyer *et al.*,2001). Castrucci *et al.*, (2002) concluded that cattle infected with IBR developed typical severe respiratory signs and excreted virus in their nasal secretions (Borchers *et al.*,2001). However in the present investigation, the virus was isolated from animals with respiratory signs and/or eye infections (70 cow) and from apparently healthy animals (18 cow). This could be explained by the tendency of the virus to establish latency in the infected animal (Meyer *et al.*,2001; Six *et al.*,2001). Therefore IBR may persist in closed herds and be introduced into uninfected herds through the addition of a latently infected animal (Leary and Splitter, 1992).

Virus isolation success rate is usually low, therefore, the most commonly used method in diagnosis of IBR is measuring the increasing of antibody titer (Leary and Splitter,1992).Blocking ELISA (in serum and milk samples) used in this investigation seemed to be more sensitive than virus isolation as it could detect 88 and 85 infected animals respectively while virus was isolated only from 74 animals.

Severe respiratory signs were recorded in large number of animals examined in our study. Rijsewijk *et al.*, (1999) reported severe respiratory signs caused by BHV-1. Stipkovits *et al.*, (2000) and Trapp *et al.*, (2003) concluded that BHV-1 causes severe respiratory disease with severe clinical signs including fever, depression, nasal discharge, hyperventilation and coughing.

Eye affections were observed in large number of infected animals examined in this study. Turin *et al.*,(1999) concluded that BHV-1 mainly cause systemic and eye infections. At the same time Leary and splitter (1992) and Brown *et al.*, (1998) reported conjunctivitis and keratoconjunctivitis accompanied to IBR infection.

No nervous signs were observed in the present investigation. Turin et al (1999) reported that BHV-1 is a neurotropic herpesvirus while Meyer *et al.*, (1996) and Meyer *et al.*,(1999) concluded that BHV-5 is the causative agent of fatal meningo-encephalitis, the authors added that BHV-5 is closely related to BHV-1 but a degree of difference was found between them. In the present investigated outbreak I found only BHV-1 and not BHV-5. Another explanation of absence of nervous signs in our work offered by Leary and Splitter (1992) who concluded that encephalitis is restricted only to very young animals, and our study investigated only adult animals.

Variation in clinical signs observed among infected animals may be referred to the strain difference. Rijsewijk *et al.*, (1999) reported the same explanation for variation in signs of IBR.

Results of this work proved that the cause of IBR outbreak was the introduction of newly purchased animals into the herd, as three out of five apparently healthy recently purchased animals were seropositive for IBR antibodies. BHV-1 was isolated from them while they were apparently healthy along the entire time of the work. The major route of introduction of IBR into a herd is the purchase of infected animals and the introduction of one or more seropositive animals would result in spreading of the infection inside the herd (Frankena *et al.*,1997).

Based on these results we can conclude that BHV-1 could play a role as a cause of respiratory tract outbreak with eye affections, the main

route of introduction of the infection into the farm is introduction of apparently healthy seropositive recently purchased animals. Therefore, examination of newly purchased animals using blocking ELISA in serum and/or milk samples together with regular examination of dairy farms for BHV-1 antibodies using blocking ELISA in bulk milk are very important steps in prevention and control of IBR in Egypt as blocking ELISA proved to be a sensitive and reliable test for diagnosis of IBR.

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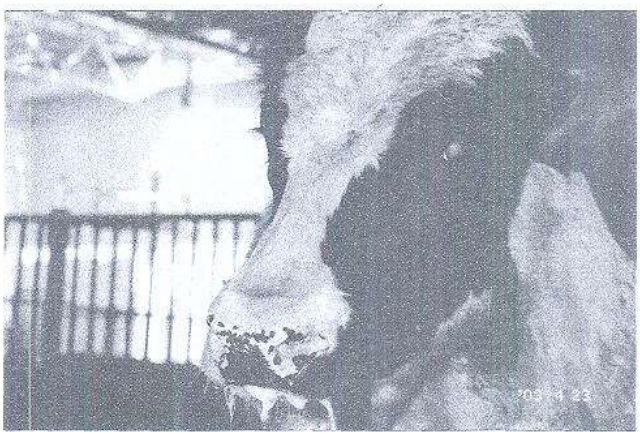
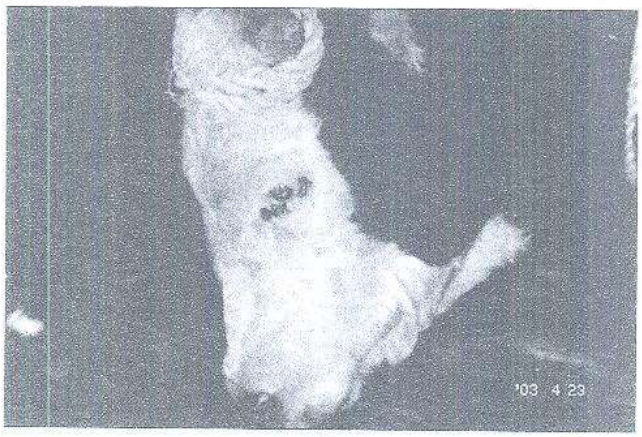
(1)



(2)

Figures 1 and 2 showed ocular signs only (conjunctivitis and partial opacity).



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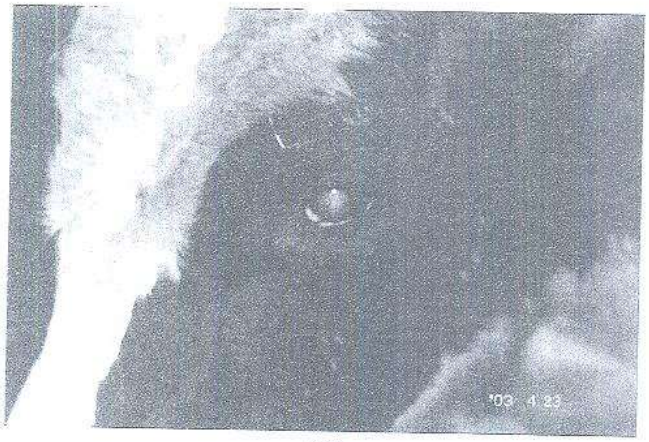

(4)

Figures 3 and 4 showed ocular signs only (conjunctivitis and partial opacity).

(5)

(6)

Figures 5 and 6 showed respiratory and ocular signs (unilateral nasal discharge and bilateral corneal opacity).

(7)

Figure 7 showed respiratory signs only (bilateral nasal discharge).