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Infectious Bovine Rhinotracheitis in a Cattle Farm at Sharkia Governorate with Special Reference to its Effect on T lymphocytes

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Abstract

This study was applied on a cattle farm of Holstein cows at Sharkia Governorate for the isolation and identification of Bovine herpesvirus type-1 (BoHV-1) and to examine its effect on T lymphocytes. The results of clinical examination revealed that there were respiratory disorders in 30 out of 150 (20%) of cattle including elevated body temperature (40 to 42°C), nasal and ocular discharges, some animals developed severe rhinitis, conjunctivitis, corneal opacity, cough and diarrhea. Out of 30 nasal swabs, 15 swabs were positive for virus isolation as indicated by cytopothic effect (CPE) on MDBK cells. Only 11 of the 15 isolates were confirmed by virus neutralization test (VNT) as BoHV-1 isolates. In addition, only 3 out of 4 BoHV-1 isolates were detected by PCR. Peripheral blood T lymphocytes (PBTL) were analyzed using electron microscopy and comet assay to examine the effect of BoHV-1 on lymphocytes. Electron micrographs of T lymphocytes revealed peripheral condensation of chromatin and fragmentation of the nucleus end of the cell leading to the formation of apoptoic bodies. Comet assay denoted fragmentation of cellular DNA. It could be concluded that BoHV-1 can infect T lymphocytes of cattle, causing directly and indirectly apoptosis which subsequently lead to suppression of cell-mediated immunity, enhancing establishment of latency and increasing the probability for secondary bacterial infection.

Key words: BoHV-1, Cattle, PCR, EM and Comet Assay

Introduction

Bovine herpes virus-1 (BoHV-1) is one of the most important viral infections of cattle that cause severe respiratory infection, conjunctivitis, abortion, vulvovaginitis and balanopostitis. BoHV-1 was classified as a member of family Herpesviridae and subfamily Alphaherpesvirinae [1]. According to the clinical manifestation, bovine herpes viruses were classified into three subtypes. BoHV-1.1 causes primarily respiratory infection (Infectious Bovine Rhinotracheitis), BoHV-1.2 induces genital infections (IVP/ IPB) and BoHV-1.3 causes primarily neurological infections and this classification is based on molecular epidemiological studies on BoHV-1 strains [2]. BoHV-1 produces long latency in ganglionia of the peripheral nervous system following initial mucosal epithelia infection [3].

BoHV-1 can spread to other susceptible animals after natural infection or corticosteroid-induced stress [4]. Infection with BoHV-1 decreases the circulating T lymphocytes [5,6]. The outcome of virus infection depends on virus-cell interactions. Programmed cell death, as part of the host-cell defense mechanisms, may reduce virus growth and its spread within the host. Viruses block or delay apoptosis by specific virus progeny produced, or they use it as a strategy to get released from the cell [7]. Apoptosis is characterized by chromatin condensation and nuclear fragmentation [8]. The aims of this study were the isolation and identification of BoHV-1 from suspected cattle and examining the effect of BoHV-1 on T lymphocytes.

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Material and Methods

Animals

A total number of 150 cattle of both sexes and 1 to 4 years old were subjected to examination in the present study. These animals were of Holstein breed and belonged to private farm in Sharkia Governorate, which had not been vaccinated against IBR. All animals were clinically examined according to Radostits *et al.* [9]. Nasal swabs were collected from clinically infected animals and used for viral isolation and identification by virus neutralization test (VNT) and PCR.

Heparinzed blood samples were collected from 10 selected cows (8 cows clinically suspected to be infected with IBR and 2 apparently health cows were used as a negative control) for collection of T lymphocytes that were subjected to examination by transmission electron microscopy and comet assay.

Samples

Swabs

Thirty sterile cotton swabs were inserted into the nasal cavity to collect discharges, soaked immediately in 2 mL of transport media and kept on ice and transported as early as possible to the laboratory. The swabs were centrifuged at 3000 rpm for 30 min at 4°C. The supernatants were collected, labeled and stored at -70 °C for virological examination.

Blood samples

Five mL of heparinized blood were taken from the selected 10 cows, layered over Ficoll-Histopaque (Sigma) and centrifuged at 3000 g for 15 min. Lymphocytes at the interphase were collected and washed three times with RPMI 1640 medium (Gibco). T lymphocytes were obtained using Nylon wool column as previously described [10]. T lymphocytes were then examined by electron microscopy and comet assay.

Control sera

Reference BoHV-1 positive and negative sera supplied by the Central Veterinary Laboratory, Uk, were used for VNT.

Reference BoHV-1

Reference BoHV-1 was supplied by the Central Veterinary Laboratory, UK.

Tissue Culture

Madin Darby Bovine Kidney (MDBK) cell culture was obtained from Virology Department, Animal Health Research Institute, Dokki, Egypt, for virus isolation.

Virus isolation and identification

All samples were propagated for three passages on MDBK cells for virus isolation [11]. Inoculated cultures were tested for BoHV-1 by the presence of cytopathic effect and then they were confirmed and identified with VNT using the reference BoHV-1 positive and negative sera [12].

Detection of viral nucleic acid by Polymerase chain reaction (PCR)

Four samples (3 samples showed clear CPE and VNT positive and one sample showed CPE and negative VNT) were examined by PCR for the detection of BoHV-1 DNA.

Twenty-four hours confluent MDBK cells sheet infected with reference BoHV-1 and the isolated virus were freezed and thawed 3 times, then centrifuged at 3000 rpm for 5 min at 4°C to remove cell debris and extraction of viral DNA was then carried out [13]. Extracted DNA was amplified using primer sequences based on the sequence of BoHV-1 glycoprotein III (gpIII) gene [14]. The sequence of oligonucleotides are:

P1: 5`–CTG CTG TTC GTA GCC CAC AAC G-3`, P2 5`-TGT GAC TTG GTG CCC ATG TCGC-3`.

For DNA amplification, a mixture containing 2 units of *Taq* polymerase, 5 uL PCR buffer, 0.2 mmol deoxinucleotides, 0.1 pmol from each primer and 5 uL of extracted DNA was prepared. The PCR mixture was subjected to 38 cycles of amplification in a thermal cycler. The cycling conditions were as follows, denaturation, 1 min at 95°C, primer annealing, 1 min at 60°C, and extension, 1 min at 72°C. Final extension cycle for 10 minutes at 72°C was applied. A negative and positive controls were included and treated as above [15]. Following amplification, 10 uL of the PCR product was mixed with 2 uL of 6X

loading buffer and taken for electrophoreses on 2% (wt/vol) a garose gel. A visible band of 173 bp being sized by DNA molecular marker was considered as positive sample.

Electron microscopy

T lymphocytes were harvested and processed for electron microscopy and ultrathin sections were examined with EM according to the method previously described [8,16].

Comet assay

Harvested T lymphocytes were processed for comet assay which is an electrophoresis technique that detects DNA damage and repair in individual cells [17]. The damage is represented by an increase of the migration of DNA in an agarose matrix under electrophoresis condition which when viewed under the microscope, the nucleus has the appearance of comet with the head (nuclear intact material) and a tail (containing damaged DNA fragment). The length and fragment content of the comet tail is directly proportional to the degree of DNA damage.

Results

Thirty out of 150 examined cows (20%) suffered from different clinical manifestations of respiratory disorders, including, elevated body temperature (40 to 42°C), nasal and ocular discharges in all clinically infected cows (no=30). Some cows developed severe rhinitis, conjunctivitis, corneal opacity, cough and diarrhea (Figure 1).

Figure 1: Cattle showing symptoms of nasal discharges (Right) and ocular discharges and corneal opacity (left).

Viral isolation and identification

Out of 30 nasal swabs, 15 swabs were positive for virus isolation as indicated by cytopothic effect (CPE) on MDBK cells which were examined daily for the presence of CPE. After 3 successive passages, the positive samples showed the characteristic grape like clusters of cells which are rounded and aggregated together in a separate manner after 24 to 48 h post inoculation and gradually increased till 70 to 80% of the sheet which then completely detached (Figure 2). These isolates were confirmed by VNT using reference positive serum for BoHV-1 and only 11 of the 15 isolates were confirmed as BoHV-1 isolates.

PCR

Four samples (3 samples showed clear CPE and VNT positive and one sample showed only CPE) were examined by PCR for confirmation of BoHV-1. The result revealed that only 3 BoHV-1 isolates were detected by PCR (Figure 3).

EM

EM revealed that peripheral blood T lymphocytes from non-infected animals have no marginally condensed chromatin (Figure 4-A) and T lymphocytes of infected animals, in ultrathin sections showed morphological characters seen during apoptosis. There were chromatin condensation, zeiosis of the plasma membrane (Figure 4-B) and fragmented nucleus (apoptotic bodies) (Figure 4-C).

Comet assay

Cells on each slide were analyzed microscopically and undamaged cells (T lymphocytes) without tail (Figure 5-A) were detected in non infected animals. Cells (T lymphocytes) with a clear tail (Figure 5-B) were detected in BoHV-1 infected ones.

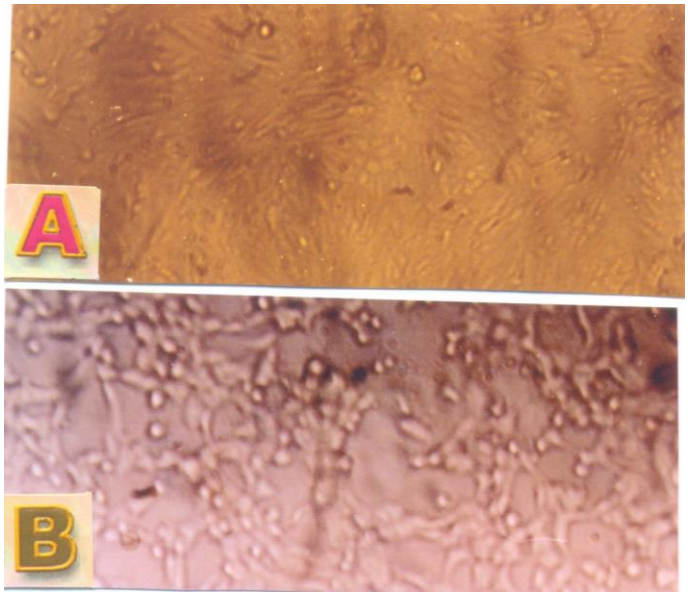


Figure (2): A: Non infected (control) complete sheet of MDBK cells (Mag. 40 x), B: Characteristic CPE of BoHV-1 isolates post inoculation on MDBK cells in the form of grape like appearance, rounding and cell detachment (Mag. 40 x).

Discussion

Bovine herpes virus-1 (BoHV-1), a member of Alpha herpes Virinae subfamily, is the etiological agent of infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis. It causes initial respiratory infection and can predispose secondary bacterial infections which lead to severe pneumonia and death if not treated [18,19].

The results of clinical examination revealed that there were respiratory disorders in 30 out of 150 cattle (20%) including elevated body temperature (40 to 42°C), nasal and ocular discharges, severe rhinitis, conjunctivitis, corneal opacity, cough and diarrhea. Nearly similar signs were recorded in previous studies [20,21].

In Egypt, there is no vaccination program on a national scale and on examined herd against BoHV-1, so the isolation of BoHV-1 from animals showing respiratory symptoms gave a suspicious of natural infection. Further identification of the isolated cytopathic viral agents was carried out to confirm the role of BoHV-1 as a causative agent for the previously mentioned disorders. This result was concordant with those observed by Saha *et al.* [22].

The characteristic CPE was observed in the form of grape like aggregation of the MDBK cells 24-48 h post inoculation which lead to suspicion for the presence of BoHV-1. Ibrahim *et al.* [23] reported that BoHV-1 developed CPE 24 h post inoculation. Moreover, IBR virus produced in MDBK cells were 10-times higher as compared to other cell cultures [24].

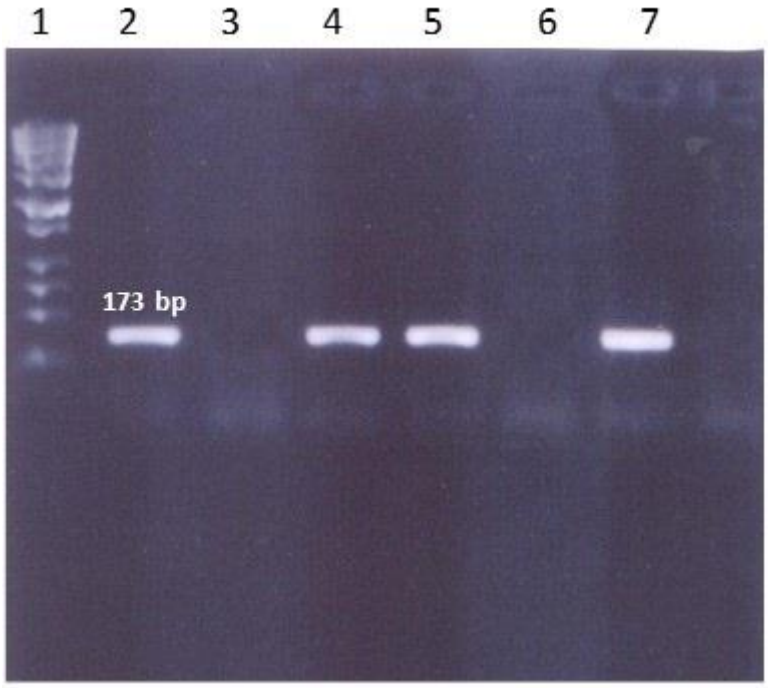
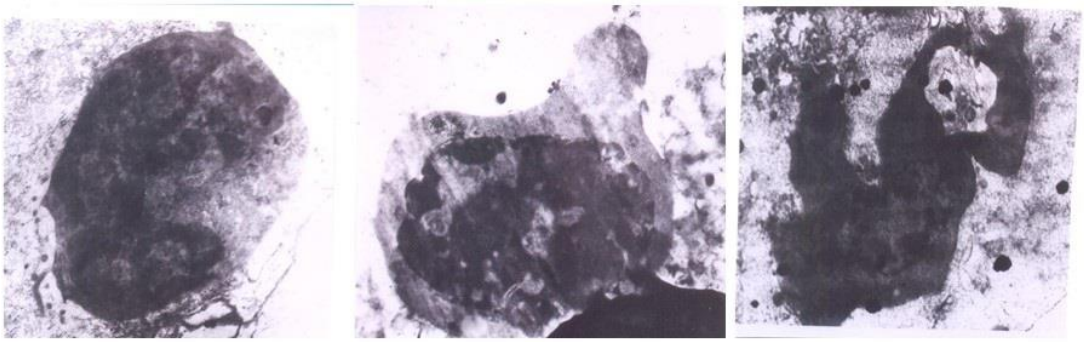


Figure (3): Lane 1: Marker. lane 2: positive control of reference strains of BoHV-1. Lane 3 negative control. Lane 4, 5 &7 positive samples showed amplified visible band of 173bp. Lane 6 negative sample.

The VNT is one of the conventional serological methods used for detection of BoHV-1 infection [25]. The results of VNT revealed that 11 out of 15 BoHV-1 isolates were positive. Our results are similar to those reported by Mahmoud *et al*. [26] who stated that 23 out of 136 vaginal, nasal and ocular swabs, from a herd of cattle and buffaloes, suffering from respiratory and genital disorders at Giza Governorate, were suspected IBR viral isolates (gave clear CPE on MDBK). Only 10 isolates were identified by fluorescent antibody technique (FAT).

During the past decade PCR have been developed for detection of BoHV-1 [27]. PCR is more sensitive and rapid than identification by conventional techniques [15]. PCR application requires a very minute amount of BoHV-1 DNA / sample to produce large number of DNA copies to be easily detected by electrophoresis [28]. The results revealed that there was a complete agreement between PCR and VNT, as all 3 isolates which were positive by VNT were positive also by PCR. While the one positive isolate by virus isolation in MDBK cell culture was negative by VNT and PCR. This result was in agreement with El-Bagoury *et al.* [29] who emphasized that PCR was superior to immune-detection using IFA after BoHV-1 isolation procedure in cell culture from suspected cattle and buffalo nasal swabs. They added that PCR was a sensitive, discriminative and rapid tool for detection of BoHV-1 infections, without confusion with other ruminant herpesviruses.



A B C

Figure 4: A: Electron micrographs of peripheral blood T lymphocyte (PBTL) from the negative control (21.000x). B: Electron micrograph of apoptotic PBTL showing peripheral condensation of charomatin and blebbing of the plasma membrane. (14.000x). C: Electron micrograph of PBTL showing apoptotic bodies (arrows) around the apoptotic T cell of infected animal with BoHV-1 (14.000x).

Peripheral blood T lymphocytes (PBTL) of the proved infected animals with BoHV-1 examined with EM, showed extensive peripheral chromatin condensations and the formation of apoptotic bodies (containing repackaged portions of the fragmented nucleus). Also, T lymphocytes were subjected to comet assay to denote fragmentation of cellular DNA. Chromatin fragmentation results from activation of a calcium-dependent endonuclease that fragments cellular genomic DNA into oligomers of 180-200 base pairs multiples [30]. The PBTL of non-infected animals showed undamaged cells without tail. While, PBTL of the confirmed BoHV-1 infected animals showed fragmented DNA out of nucleus that lead to a characteristic comet like appearance with fluorescent staining of the DNA. During lysis process, small fragments of damaged DNA were separated on electrophoresis. Our findings are in agreement with Bock *et al.* [17] who also stated that comet assay is sensitive and rapid method for the detection and quantitation of DNA damage.

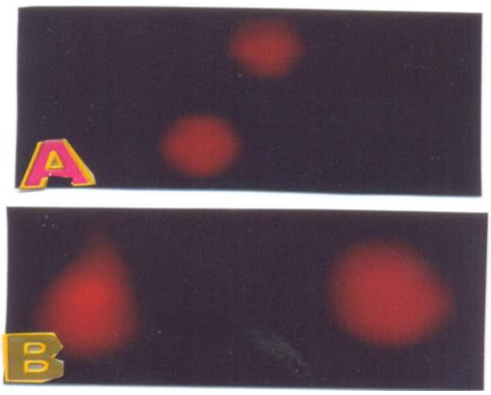


Figure 5: A: PBTL of BoHV-1 non infected animals. The cells were undamaged without tail. B: PBTL of BoHV-1 infected animals, showed migration of unwound or fragmented DNA out of the nucleus, resulting in a characteristic, comet like appearance that can be visualized microscopically.

Several viruses have been found to induce apoptosis, transient lymphocytopenia and immunosuppression. Infection of T lymphocytes with BoHV-1 lead to an increase in apoptosis [31]. Therefore, BoHV-1 infection induced apoptosis in T lymphocytes specially $CD4^+$ T cells resulting in immunosuppression and increase the probability of secondary bacterial infections. Moreover, reduction in $CD4^+$T cells as a result of virus infection and apoptosis decreases signals provided by these cells to B lymphocytes for their differentiation and production of antibodies [4].

Conclusion

It could be included that BoHV-1 infection, directly and indirectly, induced apoptosis of T lymphocytes and lead to immunosuppression. Further studies are recommended to determine the percent of apoptotic cells and to know the relation between apoptosis and BoHV-1 latency.

Conflict of interest

The authors declare no conflict of interest.

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الملخص العربي

مرض التهاب الأنف الرغامي في مزرعة أبقار بمحافظة الشرقية وبالأخص تأثيرة على الخلايا الليمفاوية التائية (T)

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تم اجراء هذه الدراسة على مزرعة أبقار هوليشين بمحافظة الشرقية لدراسة مرض التهات الأنف الرغامي ومحاولة عزله وتصنيفه واختبار مدى تأثيره على الخلايا الليمفاوية التائية (T) حيث أظهرت نتائج الفحص الإكلينيكي وجود أعراض تنفسية على ٣٠ من اجمالي ١٥٠ بقرة وكانت الأعراض ارتفاع درجة الحرارة وارتشاح والتهاب الأنف والأعين وبعض الحالات بها عتامة على العين وكحة . وقد تم أخذ عدد ٣٠ مسحة أنفية من الأبقار المصابة وكذلك ١٠عدد عينات دم (٨ من حيوانات مصابة وعدد ٢ من الحيوانات المخالطة) . وأظهرت النتائج عزل فيروس الهيربس البقري-١ من ١٥ عينة وتم وتصنيف ١١ عزلة منها بواسطة اخيبار التعادل الفيروسي وأيضاً تم التعرف على ٣ من ٤ عزلة بواسطة اختبار التفاعل المتسلسل PCR. وتضمنت هذه الدراسة أيضاً فصل الخلايا الليمفاوية النوع –T من دم الأبقار وفحصها بالميكروسكوب الإلكتروني الذي أوضح خواص الموت المبرمج بهذه الخلايا مثل تكثف الكروماتين حول الغشاء النووي ووجود امتدادات للغشاء البلازمي، تلاها فصل أجزاء من النواة والخلية (أجسام أبوبتوتيه)، كما تم عمل اختيار المذنب لهذه الخلايا للكشف عن تجزئة الحمض النووي DNA بالخلية. ونستخلص من نتائج هذه الدراسة أن فيروس الهيربس البقري-١ يصب الخلايا الليمفاوية النوع – T للأبقار ويستطيع أحداث موت مبرمج لها، وتثبيط الجهاز المناعي ويتيح الفرصة لبقاء الفيروس وقد يزيد من إمكانية الإصابة الثانوية بالبكتريا.