VIRUS ISOLATION ON CELL CULTURE AND PCR IDENTIFICATION OF INFECTIOUS BURSAL DISEASE IN MOROCCO

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KEYWORDS:
Infectious bursal disease virus, Virus isolation, Cell culture, RT-PCR.

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ABSTRACT

The aim of this first study in Morocco was at isolation of infectious bursal disease virus (IBDV) on cell culture, detection by RT-PCR and virological characterization. Out of 85 bursal samples analyzed with RT-PCR, 81 samples (95.29%) were positive. Histopathological analysis of the bursa revealed necrosis, presence of depleted follicles and some infiltration of heterophils, characteristic to previously reported in IBDV. RK-13, Vero, BHK21, BGM and CEF cells monolayers inoculated with 85 field bursal samples resulted in isolation of the virus after three successive passages. Distinct CPE characterized by rounding and clumping of cells, extensive cytoplasmic granularity and detachment of the cells was observed. As, RK-13 cell line was found to be most susceptible and likely Having a maximum ECP (90%), Followed by CEF avian primary cells (85%), BGM-70 cell lines (63%), Vero cell lines (45%) and BHK-21 cell lines (25%). The infectivity titers of adapted IBDV on cells: RK-13, CEF, BGM, Vero and BHK21, were found to be 7.60, 3.50, 2.50, 5.40, and 6.80 log10 TCDI50 / ml respectively at 72 hours post-infection. It is concluded that this RK-13 cell line had a higher growth potential and infectivity, can be used to produce infectious bursal disease vaccines using local isolates.
INTRODUCTION:
Infectious bursal disease (IBD) is a highly contagious and acute viral disease that is characterized by destruction of lymphoid cells in the bursa of fabricius causing severe immunosuppression [1, 2] and mortality generally at 3 to 6 weeks of age. It is economically important to the poultry industry worldwide, due to increased susceptibility to other diseases and negative interference with effective vaccination. IBDV is a double stranded RNA virus with bi-segmented genome and belongs to the genus Avibirnavirus of family Birnaviridae [3]. There are two distinct serotypes of the virus, but only serotype 1 viruses cause disease in poultry and six antigenic types are identified by virus neutralisation test. Although viral antigen has been detected in other organs within the first few hours of infection, the most extensive virus replication takes place primarily in the bursa of fabricius. Activated dividing B-lymphocytes that secrete IgM serve as target cells for the virus. Viral infection results in lymphoid depletion of B cells and the destruction of bursal tissues, leading to an increased susceptibility to other infectious diseases and poor immune response to vaccines. VP2 is considered to be the major host protective antigen and contains the major antigenic site responsible for eliciting neutralizing antibodies [4]. VP2 induces virus neutralizing antibodies that protect chickens from IBDV [5]. It is responsible for antigenic variation [6], tissue culture adaptation and virulence [7].

Study of virological characterization and molecular diagnosis of IBD will strengthen the antiviral therapy and disease control. The IBDV has been adapted to replicate and produce cytopathic effects (CPE) in primary cell cultures, including chicken bursa lymphoid cells, chicken embryo kidney cells and chicken embryo fibroblast cells (CEF) [8-10]. These cells produce low yield of virus and have limited growth properties [11]. The virulence of IBDV is lost during the adaptation on CEF cell culture but antigenicity is retained [8]. Therefore, there is a need for cell cultures that can produce higher yields of infectious virus required for experimental purposes. Mammalian continuous cells lines, including RK-13 derived from rabbit kidney [12], Vero cells derived from adult African green monkey kidney, BGM-70 derived from baby grivet monkey kidney [9], and BHK-21 cells derived from Baby hamster kidney, would be more suitable for IBDV isolation and for use in routine virus neutralization tests, with several advantages over the use of the primary cell cultures. These are easy to handle, maintain, and free from vertically transmitted extraneous viruses of avian origin [13]. If higher viral titers could be obtained, continuous cell line would be valuable and economical method of growing virus. It would be useful for laboratories that have limited access to specific-pathogen-free (SPF) embryos for CEFs or for SPF chicks as we have in Morocco.

The present study aims at RT-PCR based detection and isolation on cell culture of autochthon strains very virulent IBDV (vvIBDV) from field isolates. These autochthon strains responding to
the epidemiological Moroccan conditions could be used as references and challenge strains in the control of efficacy of vaccines against infectious bursal disease in Morocco and can be also used to produce infectious bursal disease vaccines.

MATERIALS AND METHODS

Field samples
During the period from July 2002 to May 2013, Four twenty five (85) bursal samples collected from field outbreaks of suspected IBD had been submitted to our laboratory for diagnostic of infectious bursal disease. All bursal samples were put on ice, divided into 25-g samples, and stored at −70°C until use.

Histopathological examination
The bursa of Fabricius (BF) samples were fixed in 10% neutral buffered formaldehyde, after embedding in paraffin blocks, sectioned at 2 mm, conducted at the veterinary pathology section. Slides for histopathology were stained with haematoxylin and eosin using routine methods, and were finally examined using a light microscope [14].

Preparation of clinical samples for RT-PCR and virus isolation.
The BF samples were homogenized in two volumes of minimal essential medium (pH 7.4) and clarified by centrifugation at 3000 g during 20 min. The supernatant was first filtrated once through a 0.75 mm paper filter (Millipore) and then twice through a 0.25 mm syringe filter (Corning). These suspensions were divided into two distinct aliquots: one was used for virus isolation, and the other was used for viral RNA extraction.

IBDV detection by RT-PCR
RNAs were extracted from the Bursal homogenates [one part of each bursa sample disrupting in sterile saline (1:1)] using QiAmp Viral RNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions. A set of primers were used for the RT-PCR reaction and for the subsequent sequence analysis using forward and reverse PCR primers for amplification of a 604 bp fragment within IBDV on VP2:

Forward primer: [TGT-AAA-ACG-ACG-GCC-AGT-GCA-TGC-GGT-ATG-TGA-GGC-TTG-GTG-AC]

Reverse primer: [CAG-GAA-ACA-GCT-ATG-ACC-GAA-TTC-GAT-CCT-GTT-GCC-ACT-CTT-TC] [15].

Briefly, the 50 µl reaction mixture contained 10 µl of extracted RNA, 10 µl of 5· RT-PCR buffer, 2 µl primer F, 2 µl primer R, 2 µl dNTP mix containing 400 µM each dATP, dGTP, dCTP, dTTP, and 2 µl of Qiagen One Step Enzyme Mix. A fragment of 604bp of the 50’-noncoding region was
amplified by PCR thermo cycling (DNA Engine Dyad, Biorad) as follows: 20 min at 50°C (RT reaction); 95°C for 15 min (initial PCR activation); 39 three-step cycles of 94 °C for 30 s (denaturation), 59°C for 40 s (annealing) and 72 °C for 1 min; then 72 °C for 10 min (final extension). After amplification, 5µl of PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide with final concentration of 0.5 µg/ml at 95 V for 30 min in 1 x TBE buffer, against GeneRuler 100 bp Plus DNA Ladder. Images of the gels were photographed on BioDoc Analyze Digital Systems (Gel Doc system, Biorad).

**Cell culture isolation and virus identification**

**Cell line and primary cell**

The primary cells and cell lines tested in this study were, Chicken Embryo Fibroblast (CEF) cultures prepared from 9-to-11 day old embryos of specific-pathogen-free (SPF) chicken eggs (VALO, Lohmann, Germany) by standard procedures. Baby hamster kidney (BHK-21), African green monkey kidney (Vero), rabbit kidney (RK-13) and Buffalo green monkey (BGM) cells were obtained from the America Center for Type Culture Collection (ATCC).

**Culture medium**

Primary CEFs were grown in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Glasgow, UK) supplemented with fetal calf serum (FCS; 2–10%; Gibco Invitrogen cell culture, Glasgow, UK).

Vero, BHK-21, RK-13 and BGM cells were maintained in MEM media (Gibco, Life Technologies, Grand Island, USA) supplemented with 10% heat-inactivated FCS 1% antibiotics–antimycotic and 1% L-glutamine.

**Culture conditions**

The cells were grown at 37°C in a humidified incubator set at 5% CO₂. Cells were subcultured after they formed a monolayer on the flask. The cells were detached by treating them with trypsin-EDTA.

**Virus isolation on cell culture**

For virus isolation, a 10% suspension was prepared in Cell culture medium containing 10% (v/v) FCS with 1 mg/mL of streptomycin sulphate, 0.4 mg/mL of gentamicin sulphate and 1000 UI/mL of penicillin. The suspension (0.2 mL) was inoculated in Chicken Embryo Fibroblast (CEF) cultures and four cell lines in 25 cm² flasks. Adsorb at 37°C for 30 minutes, wash twice with Earle's balanced salt solution and add maintenance medium to each flask. Observe daily for evidence of cytopathic effect (CPE). This is characterised by small round refractive cells. If no CPE
is observed after 6 days, freeze and thaw the cultures and inoculate the resulting lysate onto fresh cultures. This procedure may need to be repeated at least three times consecutively.

**Virus identification**

*Confirmation of the isolated virus by AGPT*

Once cytopathic effect was observed, the presence of viral antigens of IBD was tested in all samples by agar gel precipitation test (AGPT) using standard known positive and negative precipitating reference antisera against IBDV were obtained from Doorn Animal Health Research Institute[16,17].

*Polyacrylamide gel electrophoresis (PAGE) of viral RNA*

The viral RNAs from infected cultures were isolated by phenol: chloroform extraction followed by ethanol precipitation [18]. RNA was analyzed on 3.5% stacking gel and 7.5% separating gel using the discontinuous SDS-gel system of Laemmli et al, 1970 [19]. Electrophoresis was carried out for 18 h at 4°C at a constant current of 10 mA. The RNA bands were visualized by the silver staining method described by Herring et al. (1982) [20].

**Virus titration**

The virus titer was determined by calculating 50% end, as described by Reed and Muench, 1938 [21]. Prior to virus inoculation, all cells were grown to 70–80% confluence. Cell culture medium without FCS was used as virus dilution medium to obtain the desired titers. The serial 10-fold dilutions of each cell cultures-virus supernatants were prepared in growth medium without FCS and 50 µl of each dilution were transferred to each of the six wells of a 96-well microtiter plate (Titretek, UK), that contained the same volume of fresh cells suspensions (3.10⁵ cells/ml). After virus adsorption for 1 h at 37°C 100 µl of cell culture medium supplemented with 5% FCS was added to each well of the microtiter plate. Thereafter these plates were incubated at 37°C in 5% CO2 atmosphere for 7 days and examined by inverted microscope (Olympus Corp) and the virus titer was determined by the Reed and Muench method, 1938. The CPEs were stained with 1% crystal violet solution. The highest dilution of virus showing 50% CPEs was considered as end point to calculate TCID₅₀/ml.

**RESULTS**

**Histopathological analysis**

Bursa of Fabricus from diseased chicken with typical lesions showed depleted lymphoid follicles and atrophy with presence of vacuoles in the cortical and medullar portion and heterophils invasion (Fig.1).
Fig. 1. A: Bursa of Fabricius of a chicken uninfected control. normal Lymphoid follicles and normal interfollicular space. B: Bursa of Fabricus from diseased chicken with IBDV typical lesions showing necrosis and follicular depletion associated with severe follicle hemorrhage and edema of the interfollicular area. H & E, x 100.

**IBDV detection by RT-PCR**

Out of 85 bursal samples tested with RT-PCR, 81 samples (95, 29%) were positive (Table 1). All RT-PCR positive samples showed specific bands at 604 bp on agarose gel.

**Table 1. Results of detection of IBDV using RT-PCR**

<table>
<thead>
<tr>
<th>Place (city)</th>
<th>No. of cases</th>
<th>No. of positives</th>
<th>No. of negatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabat and Temara area</td>
<td>25</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Casablanca and Jadida area</td>
<td>20</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>Fès and Meknès area</td>
<td>12</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>Asila and Tanger area</td>
<td>13</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>Salé and Kenitra area</td>
<td>15</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Total No.</td>
<td>85</td>
<td>81 (95, 29%)</td>
<td>4 (4, 70%)</td>
</tr>
</tbody>
</table>

Describes the results of virus detection using RT-PCR, a, Percentage of positives, b, Percentage of negatives

**Virus isolation and confirmation**

Table 2 summarize the obtained results of virus isolation. After inoculation of organ homogenates in cell cultures, signs of cytopathic effect (CPE), consisting of appearance of swollen enlarged cells, slowdown of the growth rate and cell lysis were detected still on the third passage in the inoculated cells (Fig. 2). At the same time, non-infected control cells remained unaffected. These ECPS with
different degrees of severity were observed and characterized by different percentages (Fig. 3). Detection of IBDV in the infected cell culture supernatant was confirmed by AGPT analysis. The titration of IBD Virus in the cell cultures presented high virus titers after 72 hours post infection (Table 3).

**Table 2.** Results of virus isolation and confirmation by AGPT

<table>
<thead>
<tr>
<th>Cell Culture</th>
<th>Cytopathic effect of IBDV of field isolates</th>
<th>Results of AGPT</th>
<th>% of Cytopathic effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEF</td>
<td>++++</td>
<td>Pos</td>
<td>85%</td>
</tr>
<tr>
<td>BGM-70</td>
<td>+++</td>
<td>Pos</td>
<td>63%</td>
</tr>
<tr>
<td>Vero</td>
<td>++</td>
<td>Pos</td>
<td>45%</td>
</tr>
<tr>
<td>RK13</td>
<td>++++</td>
<td>Pos</td>
<td>90%</td>
</tr>
<tr>
<td>BHK21</td>
<td>+</td>
<td>Pos</td>
<td>25%</td>
</tr>
<tr>
<td>Negative control</td>
<td>_</td>
<td>Neg</td>
<td>0%</td>
</tr>
</tbody>
</table>

The results of virus isolation and confirmation by AGPT. +, Cytopathic effect positive; -; Cytopathic effect negative; AGPT, agar gel perciption test; Pos, positive; Neg, negative.

**Fig. 2.** (A) Uninfected CEF monolayers; (B) Cytopathic effect observed at 72 h post-infection in CEF cell monolayers infected by IBDV.
Table 3. Detection of viral RNA from cells cultures infected with IBDV and Infectivity titers of IBDV adapted in cells cultures.

<table>
<thead>
<tr>
<th>Propagation System</th>
<th>viral RNAs&lt;sup&gt;a&lt;/sup&gt; (IBDV)</th>
<th>DICT50/ml&lt;sup&gt;b&lt;/sup&gt;(log10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEF</td>
<td>+</td>
<td>6.80</td>
</tr>
<tr>
<td>BGM-70</td>
<td>+</td>
<td>5.40</td>
</tr>
<tr>
<td>Vero</td>
<td>+</td>
<td>3.50</td>
</tr>
<tr>
<td>RK13</td>
<td>+</td>
<td>7.60</td>
</tr>
<tr>
<td>BHK21</td>
<td>+</td>
<td>2.50</td>
</tr>
<tr>
<td>negative control</td>
<td>_</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

<sup>a</sup>Positive /negative (presence or absence of viral RNAs); <sup>b</sup>Median infectious dose, log10.

Fig. 3. Comparative cytopathic effect of local isolates of infectious bursal disease on different cell lines (RK13, BGM-70, Vero, BHK21) and CEF Cells.

DISCUSSION

The infectious bursal disease virus continuous to be a serious problem in Morocco as it does in other poultry producing countries all over the world. A preventive program is crucial to avoid virus spread and disease appearance [22, 23]. The clinical diagnosis of the acute forms of IBD relies on the observation of the symptoms and post-mortem examination of the pathognomonic lesions on the bursa of Fabricius. Clinical manifestations depend on the virulence, age of birds and the presence or absence of passive immunity [24, 25]. It was suggested that pathogenesis and viral multiplication is more pronounced in very virulent strains comparing to the milder strains [26].
The bursa is the preferred tissue to assess microscopic changes. There are, however, no marked differences between lesions induced by vvIBDV and other types of IBDV. Lesions become visible 24 h after infection and until day 3 are characterised by an increase in follicular lymphoid necrosis and acute inflammation. At day 3, all lymphoid follicles are affected with fewer lymphoid cells, more macrophages, bursal oedema and hyperaemia. Lymphoid depletion continues and, from day 5, cystic cavities filled with tissue debris develop in the medullary areas. In chicks infected with classical IBDV strains of low pathogenicity, regeneration of the bursa seen as repopulation with lymphocytes begins 8–21 days after infection. In the bursae of chicks infected with vvIBDV, as in those infected with more virulent classical IBDV strains, there is no recovery phase and chronic lesions develop from 3 weeks after infection. These lesions include scattered and irregularly repopulated lymphoid follicles separated by fibroblastic, interfollicular connective tissue. The bursal epithelium has a proliferative and mucin-containing glandular structure. Identification of the type of IBDV strain involved is not possible based on microscopic examination alone.

The present study demonstrated multifocal atrophy and depletion of follicles (Fig. 1), similar to the lesions described in chickens by Sharma et al. (1993) [27].

The definite identification of IBDV was obtained using RT-PCR which was known to be a sensitive test to detect the IBDV [28-32]. In this study 81 out of 85 field samples were positive by RT-PCR (Table 1).

A limitation of our study was the use of cell culture for initial IBDV identification. The isolation of IBDV in cell cultures is a laborious process requiring sometimes up to 10 passages until CPE appeared [33]. No cell line is capable of supporting the growth of all IBDV, and the use of other of different cells is recommended for IBDV isolation, [34, 35]; however, there is no consensus about which one(s) should be used. Some investigators use several cell lines, and we employed BHK21, RK13, BGM, Vero cells and CEF for routine isolation of IBD viruses in our study. However, detection and identification of IBDV occurred in primary CEF and cell lines used in this study but with percentages of different ECP, probably due to their greater susceptibility to IBD virus established. Although direct comparisons between cell lines and primary cells isolation are uncommon, a few studies indicate that cell lines support the growth of IBDV well and primary cells support the growth of IBDV well, similar to our study[36-38]. Utilization of other cell lines may have further increased the sensitivity in detecting IBD Virus [39-41], in particular those that may not have grown well on the cell lines used in our study.
The isolation of IBDV in cell cultures from target organs indicates an active virus replication. Nevertheless, the changes induced by IBDV in cell culture were identical to the CPEs described for other IBDV strains.

The Vero, BHK21, RK13 and BGM-70 cells were almost normal at 24 hours post-infection. The cells started to aggregate at 48 hours and rounding of cells started at 72 hours post-infection (Table 2). The complete rounding of infected cells was observed at 90 hours post-infection. The granulation of cells was seen at 100 hours post-infection (Fig. 2). Therefore in our experiments, IBDV infection produced a CPE characterized by a marked granulation of cell cytoplasm, particularly around the nucleus, and further resulted in cell rounding, followed by fragmentation of cells into small particles and finally detachment from the substrate, until eventually the entire monolayer was destructed during 3th passage. These findings are in agreement with those of Peilin et al. (1997) [41]. They observed complete CPEs of IBDV on Vero cell line at 65 to 72 hours of infection during 4th passage. Also, it has been reported that RK-13 cells replicated IBDV and exhibited CPE with virus titers similar to those of CEF cells who have demonstrated a high viral titer in this study. Likewise, Vero cells were also reported to propagate this virus; however, initial passages do not produce visible CPE [34]. Therefore, finding an alternative cell line was crucial for propagating IBDV with increased titer and it might be a stronger affinity of IBDV receptor, rather than the CEF cells [36]. Moreover, the early development of CPE in inoculated cell cultures (as early on the 3rd passage on cell cultures) suggests for a presence of high viral load. Expressed histopathological lesions, including depletion of the lymphoid cells in the bursa of fabricius indicate an active infection. Taken together, these facts definitively show that IBDV has played an important role in the etiopathogenesis.

AGPT employed on the harvested cell lysates infected confirmed the isolation of IBDV viruses (Table 2). Variability in results in AGPT was previously reported [16, 42]. The titration of IBDV in the cell cultures namely: RK-13, Vero, BHK21, BGM and CEF presented high virus titers (7.60, 3.50, 2.50, 5.40, and 6.80 log10 TCDI50 respectively) to IBDV after 72 hours post infection (Table 3). Kibenge et al, 1988 [34] reported similar findings, when they observed growth pattern of five strains of serotype 1 and 2 and variant strains of IBDV in Vero cells. CEF monolayer supernatant revealed the viral presence 6 h post-infection. Our results are similar to those obtained by other researchers as Tham et al. (1995) [28], who also detected the virus RNA 6 h post-infection by studying IBDV replication in Vero and CEF cells. Several mammalian continuous cell lines have been reported to be susceptible to the IBDV infection, and to support virus propagation with a distinct cytopathic effect (CPE) at low infectivity rate (Fig. 3).
CONCLUSION
In conclusion, these results suggested that field IBDVs could be rapidly adapted to replicate in vitro by serial passages in CEF, Vero, RK13, BGM and BHK21 cells. The use of a safe culture system can stimulate the innovation on biotechnology for vaccine industry. Such studies will provide justification for development of diagnostic options for a virus that can be a serious problem both in Morocco and throughout the world.

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CONFLICTS OF INTEREST
All authors declare that they have no conflict of interests related to the research presented in this paper.

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