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# Comparison of Conventional Agarose Gel Based RT-PCR with Agar Gel Immunodiffusion assay for the Diagnosis of Infectious Bursal Disease virus in Chickens in Morocco

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## Abstract

The objective of this research was to compare the reverse transcription polymerase chain reaction (RT-PCR) and agar gel immunodiffusion test (AGID) in terms of sensitivity and specificity for the detection of Infectious Bursal disease virus (IBDV) in broiler chickens. 85 samples of Bursa of Fabricius were collected from poultry flocks of Morocco during the period 1991 to 2015. IBDV was isolated from suspected bursa samples. The 604-bp region of hypervariable VP2 gene of IBDV was amplified by using specific primers. Agar gel precipitation test revealed the presence of IBDV in 60 (70, 58%) field infected bursal samples and RT-PCR revealed the IBDV in 81 (95, 29%) samples. RT-PCR is more accurate, sensitive and specific method for rapid detection of infectious bursal disease virus from field samples. In conclusion, the RT-PCR assay described herein can be used to rapidly distinguish IBDV from other pathogens, which is important for diagnosis and control of infectious bursitis outbreaks in Morocco.

**Key words**: Infectious Bursal Disease Virus (IBDV), AGID, specificity, Sensitivity, RT-PCR

## Introduction

Infectious bursal disease (IBD) is a highly contagious acute viral disease of young chickens of 3-6 weeks old that causes a fatality or immunosuppression by damaging bursa of Fabricius and impaired growth of young chickens, which results significant economic losses in the poultry industry. The causal agent of IBD is infectious bursal disease virus (IBDV), a non-enveloped double stranded RNA (dsRNA) virus belonging to the family *Birnaviridae* [1]. IBDV strains have been classified into two distinct serotypes 1, pathogenic and 2, non-pathogenic. The viral genome is composed of two segments of double-stranded RNA, segments A and B, being 3.4 and 2.9 kb, respectively [2]. This disease represents a major problem in Morocco [3] as it does in other poultry producing countries all over the world. The confirmative diagnosis of clinical and subclinical cases of IBD is very important for formulating effective strategy for control of the infection.

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Various assays have been reported for detection of the virus with different levels of sensitivity and specificity [4], and their comparison in terms of sensitivity has always been found relevant to arrive at proper diagnostic protocols. Conventionally, detection of IBDV from field samples is performed serologically by enzyme-linked immunosorbent assay (ELISA), immunohistochemistry (IHC) and virus neutralization (VN) tests [5]. Another serological method used to detect antibodies to IBDV is the AGID test. This test is rapid but insensitive. It does not detect serotypic differences and measures primarily group-specific soluble antigens [6]. Accordingly, a sensitive, specific and rapid method is desirable to detect even small amounts of virus early in infection. Reverse-transcriptase-polymerase chain reaction (RT-PCR) has been applied successfully to diagnosis of avian infectious bursal disease virus by detecting the viral RNA directly from a clinical sample or from virus isolated in a laboratory host system [7, 8, 9]. Moreover, RT-PCR coupled with restriction fragment length polymorphism (RFLP) can be used for genetic characterization of IBDV to

determine the serotypes and pathotypes [10, 11, 12]. The purpose of this study was to compare the sensitivity and specificity of agar gel immunodiffusion test (AGID) with reverse transcriptase-polymerase chain reaction (RT-PCR) for the detection of infectious bursal disease virus (IBDV).

## Material and Methods

**IBD viruses**

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 .These samples were tested by two method, agar gel immunodiffusion test and reverse transcriptase-polymerase chain reaction for the detection of infectious bursal disease virus. A brief description of the origin of the local samples is shown in Table 1.Faragher 52/70 strain was used as the standard control [13].

**Table1 IBD viruses detected in Morocco from suspected outbreaks of field**

| IBD virus | Place (city) | Age (days) | Vaccine status |
|---|---|---|---|
| 25 bursa of Fabricius | Rabat and Temara | between 30 and 46 | + |
| 20 bursa of Fabricius | Casablanca and Jadida | between 22 and 42 | + |
| 12 bursa of Fabricius | Fès and Meknès | NK | NK |
| 13 bursa of Fabricius | Asila and Tanger | NK | NK |
| 15 bursa of Fabricius | Salé and Kenitra | between 22 and 40 | NK |

NK : not known]

**Clinical diagnosis of the viral disease**

Clinical diagnosis was made on the basis of clinical history from the responsible persons of the farms, recorded clinical signs and gross lesions of affected chickens.

**Preparation of bursa homogenates and hyper-immune serum**

The preparation of bursa homogenates and the Hyper-immune serum against IBDV were performed following the procedure described by OIE [14].

**IBDV detection by Agar gel immunodiffusion test (AGIDT)**

The test was performed following the procedures described by OIE [14].Briefly, the central well of a glass slide coated with melted agarose gel was loaded with known hyperimmune sera against IBDV and peripheral wells with reference antigen of IBDV and bursal suspensions. Slides were kept in moist chamber for 48-72 hours at 40C and observed for antigen antibody reaction in the form of appearance of precipitation lines in between the central and peripheral wells.

**IBDV detection by reverse transcription polymerase chain reaction (RT-PCR)**

The RT-PCR was performed following the instructions of the one-step Superscript III® RT-PCR commercial kit (Invitrogen™). Briefly, 5µl viral RNA plus 2 µl dimethyl sulphoxide was used as a template for amplification in a 50 µl reaction containing 20 pmol all primers (U3 /L3) and 1.6 mM $MgCl_2$. All PCR conditions were the same as described above; however, the amount of mixture RT-TAQ DNA polymerase platinum enzymes was calculated as 2.5 u/50 µl reaction. A total of 10 µl PCR products was electrophoresced at 100 V for 1 h in 2 % agarose gel in 1×Tris-borate ethylenediamine tetraacetic acid buffer and visualized by ethidium bromide staining and an ultraviolet transluminator. Gel images were captured using a Kodak DC290 digital camera and ADOBE 6.0 software.

**Sensitivity and specificity studies**

The sensitivity was calculated diluting the IBDV RNA until $10^8$ RNA/µl. The specificity of both RT-PCRs was tested by addition of other common avian RNA virus (Newcastle disease virus: NDV La Sota strain).

**Statistical analysis**

Data obtained from the two tests were analyzed by analysis of variance (ANOVA). Duncan's Multiple Range Test was applied for multiple means comparisons.

## Results

**Clinical findings and post mortem changes**

In most of the affected flocks, the birds revealed the signs and symptoms of depression, anorexia, ruffled feathers, inability to move followed by death. In almost all the flocks, the postmortem lesions were observed in bursa of Fabricius. The changes in bursa in acute form of the disease included edematous and swollen bursa, presence of gelatinous exudates around bursa, with hemorrhages.

**AGID test using hyper-immune serum against IBDV**

Preliminary AGID method indicated the precipitation line, which was formed between IBDV-infected tissue samples and homologous antiserum due to antigen and antibody reaction within 24- 48 hr. No line was formed between the negative control and the antiserum. Among the 85 Suspected field samples, 60 (70, 58%) samples were found positive for IBDV using the AGID test (Table 2).

**Detection of fragments from bursal-tissue specimens by RT-PCR**

Infectious bursal disease virus could be detected in 81 (95, 29%) of the 85 field samples and in the viral strain F52/70 used as positive control RT-PCR (Table2). The PCR amplicons yielded a single

specific band of 604 bp on ethidium bromide stained 2% agarose gel without any amplification in the negative control. The specificity studies indicated that the specific 604 bp PCR product was not amplified from 500 ng of RNA from Newcastle Disease (NDV) virus.

**Comparative sensitivity of RT-PCR and AGID for the detection of IBDV**

Both tests showed a greater number of positive samples for different IBDV field isolates. The results obtained with RT -PCR and AGID in IBDV detection in samples tested are summarized in Table 2. The statistical test shows that: Fobs <Fth, this demonstrates that the two methods did not differ significantly (p> 0.05) in terms of sensitivity for the detection of positive and negative samples. However, the analysis of variance allows parallel comparison of means, taking into account a possible interaction effect. The negative aspect of it is that the test is global. If we conclude that there is no significant difference in the sensitivity of both tests used in this study, it would be necessary to conduct conventional tests, comparing averages pair wise, to really confirm this result. We applied Duncan's test to the results obtained to make multiple comparisons of averages (fig.1).The analysis of variance did not determine the existence of a significant difference between the sensitivity of the two tests: IDG and RT-PCR. Thus, the paired comparison of means, using the test Duncan has, shows a gradual and uniform proportion to the evaluation of sensitivity of the two tests used in this study confirms the results obtained by the test of variance single factor.

**Table 2 Comparative sensitivity of RT-PCR and AGIP for the detection of IBDV from different Field Isolates in Morocco**

| | Serological Test (AGIDI)a | | Molecular Test (RT- PCR) b | | Variance analysis | |
|---|---|---|---|---|---|---|
| | positive cases | % of positive cases | positive cases | % of positive cases | Fobs | Fth |
| Positive control | + | 100% | + | 100% | 2,25 | 4,54 |
| Negative Control | _ | 0% | _ | 0% | | |
| | | | | | Fobs< Fth = F0, 95 | |
| 85 field Samples | 60c+ * | 70,58% * | 81c+ ** | 95,29% ** | | |

**a**: Agar gel immunodiffusion test for IBDV ; **b**: Reverse Transcription Polymerase Chain Reaction (RT-PCR) ; **c**: cases ; Columns with different number of star for each test indicating that these tests did not differ significantly (P> 0.05) from each other in detection of samples as positive or negative.

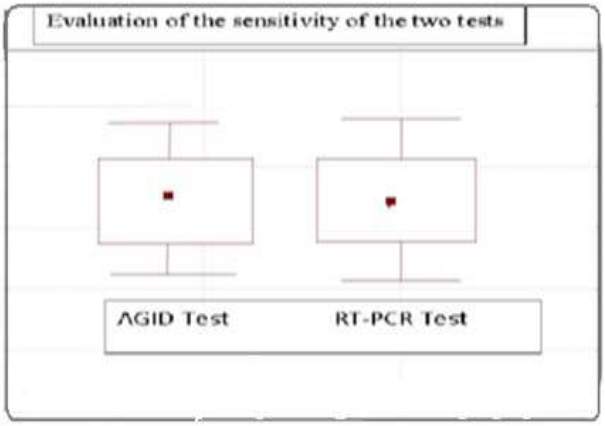


**Fig.1 Multiple comparisons of averages according to Duncan's test**

## Discussion

Infectious Bursal Disease (IBD) is a worldwide problem in poultry industry. Very little information is available about field isolates originally recovered in Morocco. Further studies on these field isolates are necessary to determine their biology, ecology and molecular epidemiology [3]. The IBDV field isolates from chickens used in this study represented a range of topotype viruses, isolated from a diverse geographic location in Morocco.

The confirmatory diagnosis of IBD, in Morocco, is traditionally performed with serological methods such as agar gel immunodiffusion (AGIP) test, the enzyme-linked immunosorbent assay (ELISA) test, virus neutralization (VN), immunoperoxidase staining, or immunofluorescence of bursal sections [3, 15]. Some of these techniques are however less sensitive and time consuming. Thus, they may not detect low-level infection in tissues. Many efforts have been made to establish rapid, sensitive and useful tests to diagnose IBDV in affected birds [16, 17]. A variety of molecular methods, particularly the conventional RT-PCR, has been developed for IBDV diagnosis [18, 19]. These methods have the advantages of being more rapid, more sensitive, and less laborious than ELISA or AGID assays, in part because they do not require the preparation of virus[20].Similarly, several researches have also reported of the increased sensitivity and reduction in time required for IBDV diagnosis using the RT-PCR technique. By this technique the tedious process of isolation and identification is eliminated as clinical

samples can directly be used and there is no need to grow the virus even if it is in low levels and the loss of virus infectivity does not affect the RT-PCR as indicated by Mittal et al (2005) [21].It also has the added advantage of detected organisms in samples that have not been properly stored.

In the present study, the classical AGID essay was compared with the molecular RT- PCR in an attempt to determine the feasibility of using PCR for rapid and accurate diagnosis of the IBDV in Morocco. The RT-PCR (using bursa samples from field outbreaks) was used successfully to amplify the IBDV VP2 gene region with oligonucleotide primers U3/L3 selected from highly conserved region. These oligonucleotide primers successfully directed the synthesis of IBDV genome sequence, yielding amplified fragment of 604 bp. The RT-PCR is found to be more sensitive since it detected 95, 29% of tested samples in comparison to AGID which detected 70.58% of tested samples. This result was confirmed by the assessment of the statistical analysis of data which showed that F observed (F0.05) = 2.25, which is lower than the critical value (Fth =4, 54), indicating that the methods did not differ significantly ($p > 0.05$) from each other in detection of samples as positive or negative.

Of the same, the RT-PCR evaluated in this work proved to be specific and no cross-amplification of non-IBDV RNA virus was observed. The AGID test is highly specific for IBDV precipitins in that no false positive reaction occurs, but it is less sensitive and may give false negative as indicated by other reports [22]. Classical methods have disadvantages like lacking of the ability to detect low levels of IBDV in tissues.

Currently, the RT-PCR is the molecular method more frequently used in the diagnosis of IBD. However, this test is relatively expensive since it requires costly reagents like RNA extraction kit, RT-PCR kit, agarose and buffer for electrophoresis and gel documentation system to visualize the RT-PCR product and record the results. This high cost is not affordable to most small poultry producers in the Morocco at present. To the well organized large poultry farms that seek reliable and quick tests, the PCR is an option, because of the intensive production system and the high inputs invested. Efforts should be continued to reduce the cost of performing the PCR in Morocco through standardization of the test for each disease and the use of locally prepared reagents.

## Conclusion

The RT-PCR assay described herein is a sensitive, specific, efficient, and cost-effective method that can be used to rapidly distinguish IBDV from other pathogens, which is important for diagnosis and control of avian infectious bursal disease in Morocco.

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