Application of PCR assay for detection of bovine respiratory syncytial virus

By

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SUMMARY

In this study, culture and RT-PCR assays for detection of fusion protein (F) and glycoprotein genes (G) of bovine respiratory syncytial virus (BRSV) were used in 18 and 15 nasopharyngeal swabs from calves and camels, respectively. Also, the assays were used to test 16 and 13 necropsy samples developing severe respiratory lesions of lung tissues from calves and camels, respectively. The results indicated that, RT-PCR assays more sensitive than the culture method for detection of BRSV in clinical samples. A total of 2, 3 and 5 samples collected from calves were positive by culture, RT-PCR (F) and (G), respectively. On the other hand, 3, 1 and 2 samples collected from camels were positive by culture, RT-PCR (F) and (G), respectively. The results also revealed that the RT-PCR, using G gene as the target, was able to detect BRSV in clinical samples more sensitive than RT-PCR (F). In conclusion, an important application of RT-PCR (G) in the future could be useful for direct, rapid specific and sensitive testing of respiratory secretions, and/or lung tissues from cattle for the presence of BRSV to gather molecular epidemiological information.

INTRODUCTION

Bovine respiratory syncytial virus (BRSV), a pneumovirus in the family Paramyxoviridae, is an important cause of acute respiratory disease in post-weaning calves and feedlot cattle (El-Vander, 1996). The virus is one of the main viruses associated with respiratory infections in various animal species (El-Vander, 1996 and El-Hakim, 2003). Although most infections are asymptomatic, the high prevalence of seropositive cattle indicates that the rate of infection is high (El-Vander, 1996 and El-Hakim, 2003).

BRSV is widely distributed in many countries (Baker et al., 1986; van der Poel et al., 1993;
Paton et al., 1998; Uttenthal et al., 2000; Arns et al., 2003 and Snowder et al., 2006). The infection can cause great economic losses for the dairy and beef industry worldwide (Snowder et al., 2006). In Sudan, Dioli and Stimmelmayr (1992) concluded that respiratory syncytial virus (RSV) is one of major cause of respiratory infection in camel. In Egypt, the pathological picture of BRSV disease was observed by Abou-Elail (1992) and Hegazy et al. (1995). The virus was detected in cattle in different governorates (Ghoniem 1995; Saber et al., 1996; Sahar, 1998; El-Mokamer, 2002; Ghoniem, 2002 and El-Hakim, 2003). Shaker (2003) reported the detection of RSV antibodies in 9.8% of 580 tested camel sera in Egypt. Little, however, is known about the role of BRSV in respiratory disorders in Egyptian cattle and camels. In addition there is a lack of information on the real distribution and participation of this virus in Egyptian herds.

Detecting specific BRSV antibodies in serum could be used as a diagnostic method. However, the disadvantages of the serological methods are the time needed to obtain the results, and the fact that some infections are serologically undetectable (Collins et al., 1996 and Larsen, 2000). Traditionally, diagnosis of BRSV infection has centered on the “gold standard” technique of culture of the virus. However, cell culture methods slow often taking from 11 to 21 days or more before a result is available (Smith et al., 1975) and its sensitivity may be affected by the liability of the virus (Larsen, 2000). Because of these difficulties, isolation of BRSV is not recommended as a routine approach to diagnosis in many laboratories (Li et al., 2008), and the use of rapid, sensitive, and specific laboratory tests for the detection of the virus are desirable.

In this study, a rapid RT-PCR assay was used to detect BRSV in clinical samples from calves and camels with suspected respiratory infections. This assay may be useful in the diagnosis of BRSV, and in epidemiological studies of in Egyptian cattle and camels.

MATERIAL AND METHODS

Clinical samples:

Nasopharyngeal swabs from 18 and 15 calves and camels, respectively; as well as necropsy samples of affected lung tissues from 16 and 13 calves and camels, respectively were collected from slaughter houses in Cairo and Giza. Nasopharyngeal swabs were prepared as previously described by El-Vander (1996). A 0.5-gm portion of each tissue sample was stored at −70°C for subsequent analysis by reverse transcription polymerase chain reaction assay
(RT-PCR). The other portion was kept at -70°C till used for virus isolation.

**Virus isolation and identification:**

All samples were prepared and used for virus isolation on MDBK (Madin Darby bovine kidney) cells according to the method described by El-Mokamer (2002). Identification of isolated virus was confirmed by direct immunofluorescence technique. The direct FA conjugates used were obtained from Bio-X Diagnostics Company, Jemelle, Belgium.

**Extraction of RNA and nested RT-PCR assays:**

The viral RNA was extracted from each sample and from the positive control virus (kindly supplied by Maryland University, College Park, Microbiology Department, USA) using TRIzol (Gibco, USA) according to manufacture’s instructions. The extracted RNA pellet was dried and resuspended in 25 μl of water containing 0.1% diethylpyrocarbonate (DEPC; Sigma–Aldrich, St. Louis, MO, USA).

RT-PCR (F) was used to amplify a 481 bp fragment corresponding to part of the fusion protein (F) gene and RT-PCR (G) was to amplify a 371 bp fragment corresponding to part of the glycoprotein (G) gene of BRSV. The outer and inner primers were described by Vilcek et al., (1994). cDNA was synthesized using a commercial SuperScriptTM II Rnase H Reverse Transcriptase kit (InvitrogenTM, CA, USA). The final reaction volume of 50 μl consisted of 5 μl of 10X amplification buffer, 2 μl of each dNTP (10 mM); 10 pmol of each F gene outer primers (5´AAT CAA CAT GCA GTG CAG TTA G 3´ and 5´ TTT GGT CAT TCG TTA TAG GCA T 3´) or each G gene outer primers (CCA CCC TAG CAA TGA TAA CCT TGA C and AAG AGA GGA TGC TTT GCT GTG G ), 0.6 μl of the DNA Polymerase (2.5 U/μl; InvitrogenTM, CA, USA), 1 μl of MgCl2 (50 mM); 2 μl of cDNA and water to complete the final volume. After an initial denaturation at 94°C for 2 min, 25 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 1 min. The second PCR was done using 2 μl of the first PCR product, 10 pmol of each F gene inner primers (5´ GTG CAG TTA GTA GAG GTT ATC GTT GT 3´ and 5´ TAG TTC TTT AGA TCA AGT ACT TTG CT 3´) or each G gene inner primers (5´ CAT CAA TCC AAA GCA CCA CAC TGT C 3´and 5´ GCT AGT TCT GTG GTG GAT TGT TGT C 3´), for 35 cycles that included denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and elongation at 72°C for 1 min.
for 45 s and elongation at 72°C for 1 min. A final elongation step of 7 min was done in both PCR assays. Negative and positive controls were included in each run. The products were separately by electrophoresis in 2% agarose gel, stained with ethidium bromide, and detected under UV light.

**RESULTS**

All collected samples were submitted to BRSV detection by culture and RT-PCR (F) and (G). The number of positive results for each method, expressed percentage of the total number of samples tested. A total of 2 and 3 samples from cattle and camel, respectively were positive by culture; 3 and 1 samples were positive by RT-PCR (F) and 5 and 2 samples were positive by RT-PCR (G). The other 29 and 25 cattle and camel samples, respectively were negative for BRSV by one or more of the tests. (Table 1, Fig. 1 and Fig. 2).

The results also indicated that, 2 and 1 samples from cattle and camel, respectively were detected by the three methods; while, one of the positive BRSV results in both cattle and camel samples was acquired from a combination of only 2 methods (RT-PCR assays). Only, 2 of the 34 and 1 of the 28 BRSV-positive samples from cattle and camel, respectively were detected by one method RT-PCR (G).

Table (1): Results of detection of BRSV by culture and RT-PCR assays in clinical samples from cattle and camels.

<table>
<thead>
<tr>
<th>Method</th>
<th>Swabs</th>
<th>Tissue samples</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Cattle</td>
<td>Camel</td>
</tr>
<tr>
<td>Culture</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>1</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>RT-PCR (F)</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>RT-PCR (G)</td>
<td>3</td>
<td>15</td>
</tr>
</tbody>
</table>
Fig. (1): Ethidium bromide stained gel electrophoresis for RT-PCR (F) amplification for detection of BRSV F gene: Lane 1: 100 bp marker. Lane 2: Positive control. Lanes 3 and 4: Positive swab samples (cattle). Lane 5: Positive tissue sample (cattle). Lane 6: Positive swab sample (camel). Lane 7: Negative tissue sample (camel). Lane 8: Negative swab sample (cattle). Lane 9: Negative tissue (cattle). Lane 10: Negative control. The fragment length of positive samples is 481 bp.
DISCUSSION

In this study, virus isolation, RT-PCR (F) and (G) assays were used for detection of bovine respiratory syncytial virus (BRSV) in 18 and 15 nasopharyngeal swabs from calves and camels, respectively; as well as a in 16 and 13 necropsy samples developing severe respiratory lesions of lung tissues from calves and camels, respectively.

The RT-PCR assays revealed its potentiality in detecting more samples than virus isolation method. The virus isolation method could not detect any BRSV isolates that had not been previously detected by one of the other two methods. On the other hand, there were 1 and 3 BRSV cases detected by RT-PCR (F) and (G) assays, respectively not detected by virus isolation. This cave RT-PCR assays were clearly more sensitive than virus isolation method.

In the present study, culture is a slow procedure; it took from 3-13
days (median, 7 days) from collection of the samples to produce a result. On the other hand, RT-PCR was both sensitive and specific for BRSV. The major advantage of the RT-PCR over virus isolation is its speed. RT-PCR result is available within 8 to 24 h. Thus it is considered a rapid alternative direct detection assay for detection of BRSV. Similar conclusion was previously reported by West et al. (1998); Valarcher et al. (1999) and El-Hakim (2003).

The BRSV culture negative samples (all but one and three of which were negative by RT-PCR (F) and (G) assays, respectively). This may be due to loss of BRSV viability during transport, or infectivity could be compromised by the presence of antibodies which capable of blocking virus adsorption (Larsen, 2000).

On the other hand, both RT-PCR (F) and (G) assays could detect only one and 2 positive samples, respectively collected from camels out of 3 positive samples by culture. This result was found to be positive unlike the expected higher sensitivity of RT-PCR in comparison to culture for bovine specimens (West et al., 1998; Valarcher et al., 1999 and El-Hakim, 2003). This may be due to some difference in gene sequence of RSV. Considering the available data, this work is the first to implement the RT-PCR assay in detection of BRSV in clinical samples collected from camels. Further studies may be needed for improving detection of BRSV in camel clinical samples by RT-PCR assay.

The RT-PCR amplified a fragment the G gene in 3 and 1 nasopharyngeal swabs collected from cattle and camel, respectively, but in only two and 1 tissue samples. The RT-PCR amplified a fragment the F gene in 2 and 1 nasopharyngeal swabs collected from cattle and camel, respectively, but in only 1 and 0 tissue samples. These results revealed that, the RT-PCR, using G gene as the target, was able to detect BRSV in clinical samples more sensitive than RT-PCR which using F gene as the target. RT-PCR (G) was positive in 2 samples while negative in the other two tests (RT-PCR (F) and culture). According to these results and on the fact that positive results were obtained only with primers to G gene in Balb/C mouse tissue (Almeida et al., 2004), the G gene is the best target for RT-PCR in bovine and camel clinical samples.

Vilcek et al. (1994) and Belknap et al. (1995) previously concluded that in some cases, respiratory signs may persist, and there may be serological evidence of the virus however there is no detection of BRSV. In addition, BRSV may not be detected by RT-PCR, even
when isolated (West et al., 1998). These data suggest that virus detection may only occur during a short period that coincides with the acute phase of the respiratory disease. Valarcher et al. (1999) detected BRSV by an RT-PCR in bronchoalveolar lavage fluid of infected calves with moderate to severe clinical signs. Similarly, West et al. (1998) detected the virus in nasal swabs from infected calves, and found the PCR was a sensitive assay. On the other hand, others have been unable to detect BRSV by RT–PCR of the F gene (El-Vander, 1996).

The results also indicated that, the BRSV was detected by both RT-PCR assays in swabs samples more than in tissue samples. This finding may be due to that respiratory disease caused by BRSV enhances bacterial colonization and adherence, and alters the specific and non specific defense mechanisms of the respiratory tract (Larsen, 2000). He added that, the animal with signs of respiratory disease may have secondary infections by bacteria, the presence of which may inhibit the PCR reaction and make it difficult to detect in lung tissue.

In conclusion, an important application of RT-PCR (G) in the future will be the direct, rapid, specific and sensitive testing of respiratory secretions, and/or lung tissues from cattle for the presence of BRSV to gather more molecular epidemiological information.

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تطبيق استخدام اختبار البلمرة المتسلسل في الكشف عن فيروس الخلايا العاملة التنفسي
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الملخص العربي
في هذه الدراسة تم تجميع عدد 18 عينة من إفرازات الجهاز التنفسي العلوي من العجول البقرى والجمال على التوالي كما تم تجميع عدد 12 عينة من آنسجة الرئة من العجول البقرى والجمال على التوالي. تم عزل فيروس تجميع خلايا الجهاز التنفسي في العينات باستخدام ارتفاع النسبى كما تم الكشف عن الفيروس باستخدام اختبار البلمرة المتسلسل المعاد السكري (G) المعاد في الكشف عن وجود بروتينات التثاثم (F) والبروتين السكري فورد أظهرت النتائج أن اختبار البلمرة المتسلسل المعاد كان أكثر حساسية عن الزرع النسيجي في الكشف عن الفيروس في العينات المجمعة من العجول البقرى حيث تم الكشف عن الفيروس في عدد 6 و 5 عينات باستخدام اختبار البلمرة المتسلسل المعاد (G) ، (F) على الترتيب بينما تم عزل الفيروس باستخدام ارتفاع النسبى من عدد 2 عينات فقط. بينما في عينات الجمال أعطي الزرع النسيجي نتيجة إيجابية لعدد 3 عينات و تم الكشف عن الفيروس في عدد 1 و 2 عينات باستخدام اختبار البلمرة المتسلسل المعاد (G) ، (F) على الترتيب.

وستخصص من النتائج أن تطبيق اختبار البلمرة المتسلسل المعاد خاصة في الكشف عن فيروس تجميع خلايا الجهاز التنفسي في عينات الإفرازات التنفسي أو آنسجة الرئة من العجول البقرى يتميز بأنه مباشر وشخص وسريع كما يمكن استخدامه لمعرفة الوضع الوثائي للفيروس.

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