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Original article

Construction of an internal standard used in RT nested PCR for Borna Disease Virus RNA detection in biological samples

Vincent LEGAY, Corinne SAILLEAU, Gwenaëlle DAUPHIN, Stéphan ZIENTARA*

Agence Française de Sécurité Sanitaire des Aliments d'Alfort,
22 rue Pierre Curie, 94704 Maisons-Alfort Cedex, France

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Abstract – The highly neurotropic Borna Disease Virus (BDV), which belongs to the *Mononegavirales* order – *Bornaviridae* family – is generally detected using the RT-nested-PCR. If false positive results (often caused by laboratory contaminations) can be avoided, some false negative results which are mostly due to inhibitory effects of some reaction components and/or to sample preparation errors, can occur. Thus, in order to control the RT-PCR sample, an RNA internal standard molecule named "mimic" was constructed with the same primer recognition sites as the viral nucleic acids, flanking a heterologous DNA fragment of distinct molecular weight. Because of their different sizes, the mimic and viral PCR products can be easily discriminated by agarose gel electrophoresis. The co-amplification of both BDV and mimic RNA was performed on infected cells and on biological tissues such as the brain and blood, commonly known to contain PCR inhibitor components. After mimic sensitivity studies were achieved (2.5 fg of "p40 RNA mimic" and 0.25 fg of "p24 RNA mimic"), the competitive amplification reaction between both BDV and mimic RNA was performed on these tissues. The results confirmed that nervous tissue has an inhibitory effect on RT-PCR, which supports the necessity of BDV detection by a higher sensitive method such as RT nested PCR. Moreover, these results confirmed the interest of an internal standard for BDV RNA detection in biological samples.

Borna virus / PCR / internal standard / biological tissue

Résumé – Construction d'un témoin interne de RT-PCR nichée pour la détection du génome du virus de la maladie de Borna dans des tissus biologiques. Le génome du virus de la maladie de Borna (« Borna disease Virus » – BDV), du fait des propriétés biologiques de ce virus, n'est généralement détectable que par la méthode RT-PCR nichée. Si les résultats « faux-positifs » peuvent être évités par des conditions de manipulation très strictes, les résultats « faux-négatifs » demeurent bien

* Correspondence and reprints
Tel.: (33) 1 49 77 13 12; fax: (33) 1 49 77 13 13; e-mail: s.zientara@afssa.fr

souvent une source d'erreurs biaisant les résultats de la détection de génome viral. Ainsi, pour contrôler la RT-PCR dans chaque tube réactionnel, un témoin interne ARN appelé « mimic » a été développé. Cette molécule comprend les sites de reconnaissance des amorces utilisées pour l'amplification du génome du virus Borna et un fragment génomique de séquence et de taille différentes de celles du BDV. Les fragments amplifiés de BDV ou de « mimic » peuvent ainsi être facilement différenciés par un gel d'électrophorèse. La réaction de co-amplification a été testée tout d'abord à partir d'ARN extraits de surnageants de cultures cellulaires infectées par le virus Borna, puis à partir d'ARN extraits d'échantillons biologiques tels que des cerveaux et des prélèvements sanguins. La sensibilité de la détection par RT-PCR (2.5 fg « d'ARN mimic p40 » et 0.25 fg « d'ARN mimic p24 ») des deux molécules a ainsi été étudiée et les résultats obtenus ont confirmé une inhibition de la RT-PCR réalisée dans les tissus nerveux. Cette conclusion montre d'une part la nécessité d'utiliser une méthode plus sensible que la simple RT-PCR telle que la RT-PCR nichée, et d'autre part l'intérêt du témoin interne de réaction lors de la recherche du génome du virus de la maladie de Borna dans des tissus biologiques.

virus Borna / PCR / témoin interne / tissu biologique

1. INTRODUCTION

Borna Disease (BD) is an infectious immunopathological disease of the central nervous system (CNS) characterized by behavioral abnormalities. Its etiological agent has been defined as a nonsegmented, negative sense, single-stranded RNA virus [8]. Because of its genome organisation and its nuclear replication and transcription sites, this virus which belongs to the *Mononegavirales* order has been described as the unique member of the *Bornaviridae* family [9]. The geographical distribution of BDV is uncertain but infections have been reported in Central and Northern Europe, North America, Japan, Iran and Israel [5, 10]. Its natural hosts are classically described as horses and sheep but infections in donkeys, cattle, rabbits, cats and ostriches have also been described [10]. If serological data are abundant about BDV infections in various animals and humans presenting neurological disorders [6, 12, 13, 15, 17], BDV RNA detection by in vitro genomic amplification is one of the most efficient methods to detect viral infection. To detect the virus genome in infected tissues, it is often necessary to use the Polymerase Chain Reaction (PCR) method in two rounds of amplifications, i.e. nested PCR. Because of the high sensitivity of this method, a frequent problem is laboratory-contamination inducing false positive results [3]. When taking care about manipulation conditions, that is to say by using UV treatments, specific materials and rooms, most of these risks can be avoided. But sometimes, false-negative results may also occur because of inhibitory effects due to error in sample preparation or to tissue specificities [18]. As reported by Barker et al. [2] or Holodniy et al. [11], RT-PCR achieved on biological tissues such as brain or blood can be inhibited by various components including intrinsic factors such as haemoglobin in blood or extrinsic factors such as the RNA extraction step in the brain. It is then essential to control the efficiency of the RT-PCR in such samples with an internal standard molecule to discriminate technical and biological problems. To avoid the risks of false negative results, some standard molecules indicating that the reaction really succeeded can be used. These molecules can be amplified in other reaction tubes run in parallel (external standards) as described by Trapnell in 1993 [16], or in the same tube (internal standards). This RNA internal standard, named "the mimic", has the same primer recognition sites as the test template but its sequence and size differ from the test template [1]. It is then easy to distinguish the mimic from the template by agarose gel electrophoresis. Therefore, we constructed two RT-PCR internal controls containing the African Horse Sickness Virus

partial segment 2 of serotype 3 sequence and the primer recognition sites of two BDV targets: the p40 genomic region coding the nucleoprotein, and the p24 genomic region coding the phosphoprotein known as a co-factor of the BDV polymerase [4]. Since it contains another genomic fragment between the primer sites, this molecule cannot form a heteroduplex with the test template. Owing to this method, loss of sensitivity or efficiency of the amplification can be encountered.

2. MATERIALS AND METHODS

2.1. Mimic preparation

The mimic was produced by Mimic-Producing PCR (MP-PCR) [1] (as presented in Fig. 1) on a PCR product of the African Horse Sickness Virus (AHSV) – an *Orbivirus* – partial segment 2 of serotype 3 with the mimic primers. These specific primers, shown in Figure 1, were synthesized by Genome Express (Zone Astec, Grenoble, France) and contained AHSV segment 2 specific primers [14] (Tab. I) and BDV internal and external primers [15] described in Table I and the T_7 promoter sequence (ATT AAT ACG ACT CAC TAT AGG) [1] at the extreme 5' end of the upstream primer. Thus, the forward p40 mimic primer was a 82 bp fragment, the reverse p40 mimic primer was a 62 bp fragment, the forward p24 mimic primer was a 79 bp fragment and the reverse p24 mimic primer was a 58 bp fragment. This T_7 promoter was used to create an RNA template in the in vitro transcription reaction. The Mimic Producing PCR was carried out in a volume of 100 µL containing 86 µL of bidistilled water, 10 µL of Taq Buffer (10×), 0.5 µL of forward end reverse mimic primers (20 µM), 0.8 µL of a dNTP mix (100 µM) and 1.5 µL of *Taq* DNA polymerase (Roche, Meylan, France). The reaction samples were overlaid with 100 µL of mineral oil (Sigma-Aldrich, Saint Quentin

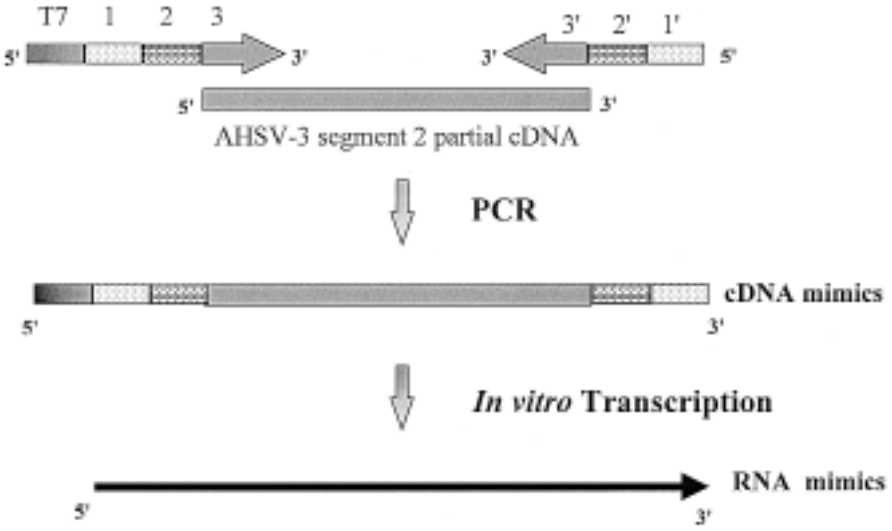

Figure 1. Schematic representation of the two mimics construction steps. T7: T7 promoter sequence; 1: forward external BDV primers; 2: forward internal BDV primers; 3: forward AHSV primers; 3': reverse AHSV primers; 2': reverse internal BDV primers; 1': reverse external BDV primers.

Table I. Sequences and location of the Borna Disease Virus and African Horse Sickness Virus primers.

Virus	Gene	PCR round	Primers	Sequence	Location in genome	References
BDV	p40	1st	BV 259 F	5'-TTCATACAGTAACGCCCAGC-3'	259–278	[15]
			BV 829 R	5'-GCAACTACAGGGATTGTAAGGG-3'	829–808	[15]
		2nd	BV 277 F	5'-GCCTTGTGTTTCTATGTTTGC-3'	277–297	[15]
			BV 805 R	5-'GCATCCATACATTCTGCGAG-3'	805–766	[15]
	p24	1st	BV 1387 F	5'-TGACCCAACCAGTAGACCA-3'	1387–1405	[15]
			BV 1865 F	5'-GTCCCATTCATCCGTTGTC-3'	1865–1847	[15]
		2nd	BV 1443 F	5'-TCAGACCCAGACCAGCGAA-3'	1443–1461	[15]
			BV 1834 R	5'-AGCTGGGGATAAATGCGCG-3'	1834–1816	[15]
AHSV	segment 2	1st	P 20 F	5'-GTTTAATTCACCATGGCGTC-3'	1–20	[14]
			3 N 228 R	5'-AATCTGTTCATAAATCATTC-3'	228–209	[14]

F: forward; R: reverse; 1st: first amplification round; 2nd: second amplification round.

Fallavier, France) and incubated in a thermocycler Crocodile II (Appligene Oncor, Illkirch, France) according to the following protocol: 40 cycles of 94 °C for 1 min, 45 °C for 1 min, 72 °C for 2 min, and one step of terminal extension at 72 °C for 10 min. The MP-PCR produced a 320 bp long product containing T7 promoter, p40 or p24 forward primers at one end of the molecule and p24 or p40 reverse primers at the other end. Between the BDV sequences, the molecule contained AHSV cDNA partial segment 2 of serotype 3 sequence (228 bp) [14]. This MP-PCR product was concentrated and purified from agarose gel using the Roche (Meylan, France) purification kit. The purified cDNA mimics (p40 mimic and p24 mimic) were then quantified by spectrophotometry and 3.4 µg of these were used for in vitro transcription to produce the RNA mimic.

2.2. In vitro transcription

The reaction was carried out in a volume of 20 µL using the Ambion transcription kit (Clinisciences, Montrouge, France). The reaction mixture contained 2 µL of Reaction Buffer 2×, 10 µL of Ribonucleotide Mix (2×), 6 µL of linearized template cDNA mimic, 2 µL of Enzyme mix (10×). The transcription reaction was performed at 37 °C for 1 h. After the transcription reaction was complete, the template DNA was degraded by addition of 1 µL of RNase-free DNase I for 15 min at 37 °C. The reaction was stopped by adding 115 µL of Nuclease free dH_20 and 15 µL of Ammonium Acetate stop solution. The transcribed RNA quantities were estimated by spectrophotometry. These stock solutions were diluted 10^1 to 10^{10} times and 1 µL of the highest positive dilution was used in the RT-PCR test as an internal standard.

2.3. RT-PCR with mimics and BDV RNA

BDV RNA was extracted from infected VERO cells, or from tissues such as the brain (limbic system region) and blood containing various dilutions of infected VERO cells using the isothiocyanate/acid phenol method [7]. The RNA pellets were dried and resuspended in a 20 µL volume of DEPC treated water.

The reverse transcription and the first round of the amplification were performed using a single tube RT-PCR method (st RT-PCR) with the Qiagen one step RT-PCR kit (Qiagen, Courtabœuf, France). The reaction

was carried out in a 50 µL reaction volume using the p40 and p24 internal BDV specific primers (Tab. I). The reaction mixture contained 21 µL RNase free water, 10 µL 5× Qiagen one step RT-PCR Buffer (Courtabœuf, France) (1X), 2 µL of dNTP mix (10 µL of each dNTP), 10 µL of 5× Q solution, 1.5 µL of each external primer, 2 µL of Qiagen one step RT-PCR enzyme mix and 1 µL of RNA matrix (BDV, mimics, or BDV and mimics). The samples were overlaid with 50 µL of mineral oil (Sigma) and incubated in a thermocycler Crocodile II (Appligene Oncor, Illkirch, France) according to the following protocol: RT step for 32 min at 50 °C and 15 min at 95 °C, amplification step of 40 cycles for 1 min at 94 °C, followed by 1 min at 57 °C and 1 min at 72 °C, and the terminal elongation step for 10 min at 72 °C. The second amplification round was achieved using 1 µL of the RT-PCR product and the nested set of BDV specific internal primers previously described. Amplification was achieved using the same amplification conditions as in the first PCR, in a 50 µL reaction volume containing 43 µL of bidistilled water, 5 µL of Taq Buffer (10×), 0.4 µL of each primer (20 µM), 0.4 µL of a dNTP mix (100 µM) and 1.25 U per reaction of *Taq* DNA polymerase (Roche, Meylan, France).

3. RESULTS

The MP-PCR was achieved by amplification of the AHSV cDNA partial segment 2 of serotype 3 using the mimic primers and produced a 372 bp cDNA fragment of the p40 mimic or a 365 bp cDNA fragment of the p24 mimic.

The amounts of RNA mimics obtained after in vitro transcription were estimated by spectrophotometry for 2.5 µg of each RNA mimic template (the p40 and the p24 RNA mimics) were obtained. The RNA mimic prepared dilutions (10^{-1} to 10^{-10}) were tested by single tube RT-PCR which produced the cDNA mimic fragments of 351 bp for the p40 mimic and 344 bp for the p24 mimic. These fragments were about 309 bp for the p40 mimic and 306 bp for the p24 mimic using the RT nested PCR.

The highest RT-PCR positive dilutions, corresponding to 2.5 fg of the p40 mimic RNA template (dilution 10^{-9}) and 0.25 fg of p24 mimic RNA template (dilution 10^{-10}) (data not shown) were used in a competition test with various dilutions of purified BDV RNA template (from 1 ng to 100 fg) as shown in Figure 2. The competitive RT-PCR assay between RNA mimics (constant concentration as determined before) and diluted purified BDV RNA revealed a sensitivity of approximately 1.4×10^6 copies of BDV RNA (corresponding to a 598 bp fragment) using the p40 primers as shown in Figure 2, and of approximately 1.4×10^5 copies of BDV RNA (corresponding to a 429 bp fragment) using the p24 primers (data not shown). These results were in agreement with those obtained by single-tube RT-PCR on BDV RNA alone (without mimic). These results were conforted when using the RT nested PCR which revealed an equal sensitivity with RT nested PCR achieved on BDV RNA alone (140 BDV copies – corresponding to a 528 bp fragment – with the p40 primers and 14 BDV copies – corresponding to a 391 bp fragment – with the p24 primers). In conclusion, there was no significant competition between the mimic and the viral BDV RNA using the mimic RNA concentration as previously determined. These dilutions of RNA mimics (10^{-9} for the p40 RNA mimic – 2.5 fg – and 10^{-10} for the p24 RNA mimic – 0.25 fg) were then used as internal standards in the RT-PCR tests.

We then achieved the RT-PCR detection of BDV RNA extracted from biological tissues (nervous tissue and blood). To develop the technique, different dilutions of BDV infected VERO cells were added to brains and blood. The sensitivity of the RT-PCR detection of the RNA extracted from these samples was compared to the sensitivity of such a detection with the BDV RNA

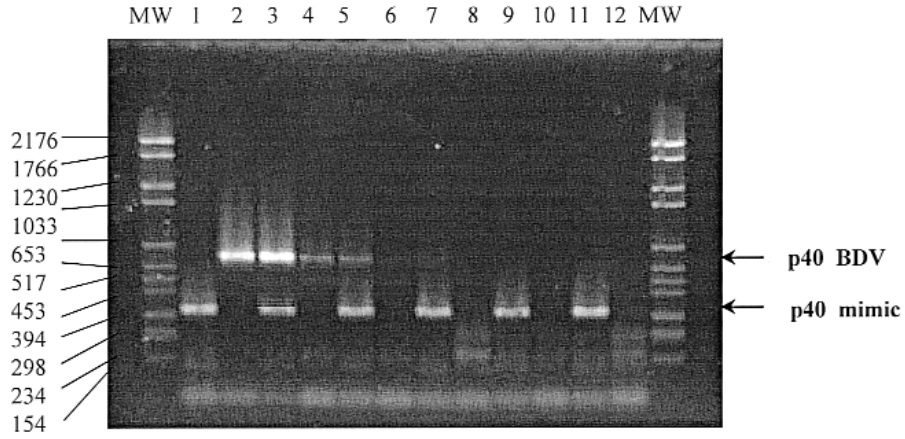

Figure 2. RT-PCR with various purified BDV RNA dilutions and constant p40 mimic RNA concentration (2.5 fg) on a 2% agarose gel. MW: molecular weight; lane 1: p40 RNA mimic; lane 2: 0.1 ng of purified BDV RNA; lane 3: p40 RNA mimic and 0.1 ng of purified BDV RNA; lane 4: 100 pg of purified BDV RNA ; lane 5: p40 RNA mimic and 100 pg of purified BDV RNA; lane 6: 10 pg of purified BDV RNA; lane 7: p40 RNA mimic and 10 pg of purified BDV RNA; lane 8: 1 pg of purified BDV RNA; lane 9: : p40 RNA mimic and 1 pg of purified BDV RNA; lane 10: 0.1 pg of purified BDV RNA; lane 11: : p40 RNA mimic and 0.1 pg of purified BDV RNA; lane 12: negative control (without any RNA template).

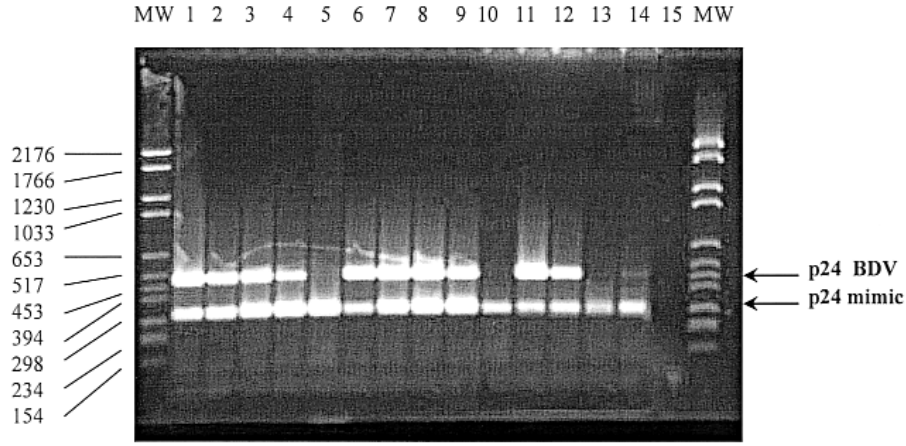

Figure 3. RT-PCR test with both BDV and mimic RNA in biological samples (2% agarose gel). MW: molecular weight; lanes 1 to 4: decreased dilutions of BDV infected VERO cells; lane 5: BDV infected VERO cells; lanes 6 to 9: decreased dilutions of BDV infected VERO cells in blood; lane 10: non infected VERO cells in blood; lanes 11 to 14: decreased dilutions of BDV infected VERO cells in brain; lane 15: non infected VERO cells in brain.

extracted from VERO infected cells. The results (Fig. 3) showed a decrease of the detection sensitivity for RNA extracted from brain samples. These data confirmed that the nervous tissue has an inhibitory effect on the RT-PCR detection of both mimics and BDV RNA. No inhibitory effect was observed with blood samples. These results confirmed the necessity to use the RT nested PCR to detect BDV RNA from such tissues.

4. DISCUSSION

The PCR assay is one of the most efficient and useful techniques for nucleic acid detection. Because of the reaction conditions, it is fundamental to take care of the manipulations in order to avoid contamination risks and false-positive results. The use of this method for the detection of nucleic acids in tissue samples such as brain and blood is confronted to both endogenous (reaction conditions) and exogenous (biological tissue properties) inhibitory factors [18].

The specimens may inhibit the reaction by altering the pH, Mg^{2+} or other ion concentrations and the viscosity and DNA polymerase enzyme activity [1]. False-negative results may also be the outcome of errors in the sample preparation or reaction setup. Considering the problem of false-negative results, it is advisable to use control molecules, i.e. external or internal standards. External standards have the advantage of easy applicability but in most cases, the high concentration of these standards can be a source of contamination for the detection test and can induce false-positive results. However, they provide only general information on the influence of the chemical ingredients and cycling profiles of the reactions. These standards are not suitable to reveal the PCR efficiency in the individual reaction samples. By using mimics as internal standards, the assay can be controlled in each sample [1]. The p40 and p24 mimics, achieved with the BDV primer recognitions sites and the African Horse Sickness Virus partial cDNA, present two major advantages. Firstly, there is no evidence of significant identity between BDV and AHSV genomic sequences which can exclude risks of heteroduplex formation. Secondly, the mimic fragment is shorter than the PCR product (160 bp shorter than the p40 PCR product and 100 bp shorter than the p24 PCR product), so the discrimination of the amplicons by electrophoresis can be easily confirmed. Because of the shorter size of the mimic fragment, high dilutions are required to prevent that the mimic outnumbers the test template and to avoid risks of competition between the two RNA templates. These conditions were observed when the highest positive dilutions of mimic RNA were used in parallel with the BDV RNA in RT-PCR. The sensitivity of the detection was conserved with one or both templates and just a decrease of the intensity of the signal on the electrophoresis gel was observed when BDV and mimic RNA were present together. Such results revealed no significant competition between the test template and the internal standard which confirmed the efficiency of such reaction control. Compared to external standards, this control is more reliable, because it can detect inhibition or reveal loss of sensitivity in each reaction sample as obtained with the brain but not blood. In the cases where neither the RNA mimic nor the test template were amplified, it can only be concluded that the reaction was negative. Considering the neurotropism of the Borna Disease Virus and the inhibitory factors due to molecules like haemoglobin or lipids [18] present in such tissues (brain and blood), the use of such internal standards seems to be fundamental in virus detection. As previously described, these biological tissues have inhibitory effects on the genomic amplification which are twisted using the RT nested PCR method. The use of mimics can avoid the risks of false-negative conclusions in BDV RNA detection by RT nested PCR in diagnostics or epidemiological studies.

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