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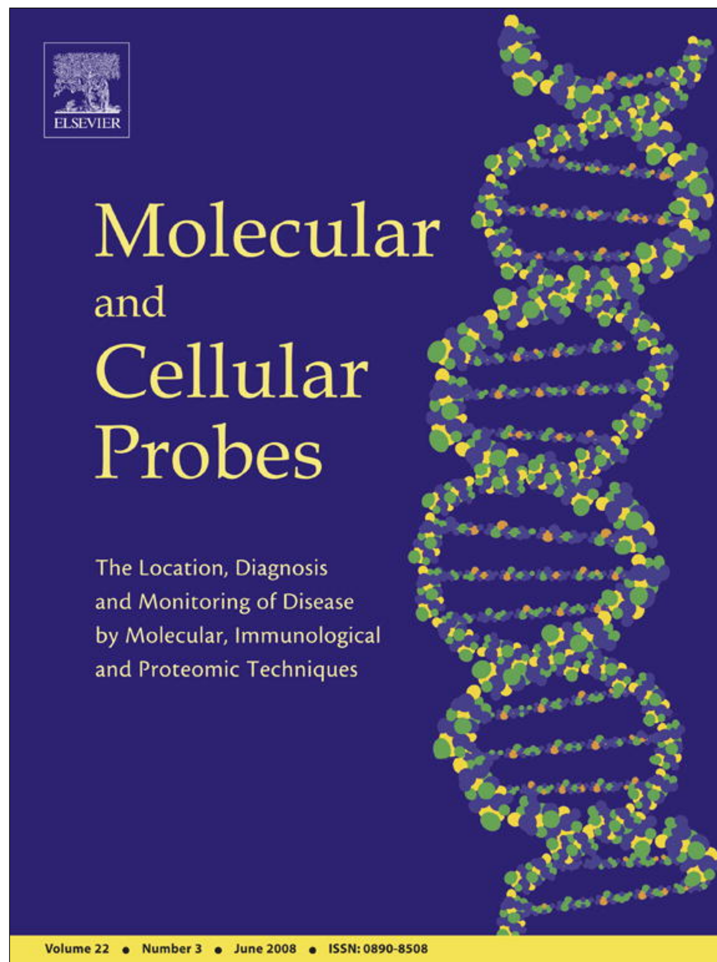

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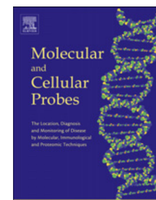

Short Communication

Simple procedures to obtain exogenous internal controls for use in RT-PCR detection of bovine pestiviruses

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Pestiviruses are ubiquitous pathogens of cattle and frequent adventitious viruses in biologicals. Furthermore, it has been suggested that these agents might be related to infantile gastroenteritis and microencephaly. Since the virus is highly prevalent in fetal bovine serum, the risk of contamination is high in most laboratories. Thus, the implementation of detection methods in all laboratories is of worth. Despite continuous surveillance, these agents have been detected in cell lines, fetal bovine serum, live and inactivated animal and human vaccines and interferon for human use. In this report, DNA and RNA internal controls (ICs) which can be implemented in laboratories with minimal equipment are described. The developed standards can be added before RNA purification, allowing to monitor all steps of the protocol (viral RNA extraction, reverse transcription and cDNA amplification). It is shown that inhibitory effects that could lead to decreased sensitivity can be minimized by controlling the amount of mimic molecules added to the samples. A method to avoid the problem of DNA traces present in in vitro transcribed RNA preparations is provided.

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The genus *Pestivirus* is classified in the Flaviviridae family, which also includes the genera *Hepacivirus* (Human Hepatitis C Virus, HCV) and Flavivirus. The pestiviruses that are able to infect bovines are mainly Bovine Viral Diarrhea Virus 1 (BVDV 1) and Bovine Viral Diarrhea 2 (BVDV 2) [1,2]. Bovine pestiviruses are ubiquitous pathogens in herds [3,4] and frequent contaminants of biologicals [5]. Despite continuous surveillance, these agents have been detected in cell lines [6], fetal bovine serum [7], live and inactivated animal and human vaccines [8–11] and interferon for human use [12]. Risk for animals is evident—inoculation can occur when contaminated veterinary products are used, for instance [13]— but attention should also be directed towards human health. The virus has been detected in preparations of human leucocytes [14]. Furthermore, it has been suggested that pestiviruses might be related to infantile gastroenteritis [15] and microencephaly [16]. Since the virus is a frequent contaminant in fetal bovine serum, risk of contamination is high in most laboratories. Thus, the implementation of detection methods in all laboratories is of worth.

The amplification of specific nucleic acid sequences has deserved an important position among the methods used in microbial diagnostic tests. The properties influencing the choice of this type of technique in diagnostic and research laboratories are sensitivity and easiness of implementation. Considerable attention has been focused on the problem of negative samples performing as positive due to cross-contamination (false positives). Another important problem of nucleic acid amplification is the occasional failure of reaction which results in false negatives [17–19]. Herein, DNA and RNA ICs which can be implemented in laboratories with minimal equipment are described. The oligonucleotides used for PCR were S. Vilcek's panpestivirus primers 324 and 326 [20], which are widely used to detect *Pestivirus* RNA (e.g. [21–24] and citations therein). This primer pair is directed towards conserved regions in the 5′ untranslated genomic region (5′UTR).

A modified 5′UTR sequence was introduced in a pGEM plasmid to obtain construction pMG1-5UTR(−) (Fig. 1A). This mimic molecule was obtained by means of a mutagenic primer (5′-TCA ACT CCA TGT GCC ATG TAC GCT GTA TCC GTA ACA GTC-3′) which, through RT-PCR amplification, allows to obtain a 238-bp amplicon; that is, a DNA molecule 50 bp shorter than the wild type product obtained after standard RT-PCR. This amplicon is flanked by complementary sequences to 324 and 326 primers. Fig. 1 shows a scheme of the corresponding construction and a picture of an agarose gel in which amplification products of wild type 288 bp and mutant 238 bp templates were electrophoresed.

To produce mimic RNA, the plasmid pMG1-5UTR(−) was linearized and used as template for RNA synthesis. After in vitro transcription, plasmid/RNA mixtures were treated with an RNase free DNase to eliminate the plasmid DNA. Despite the fact that

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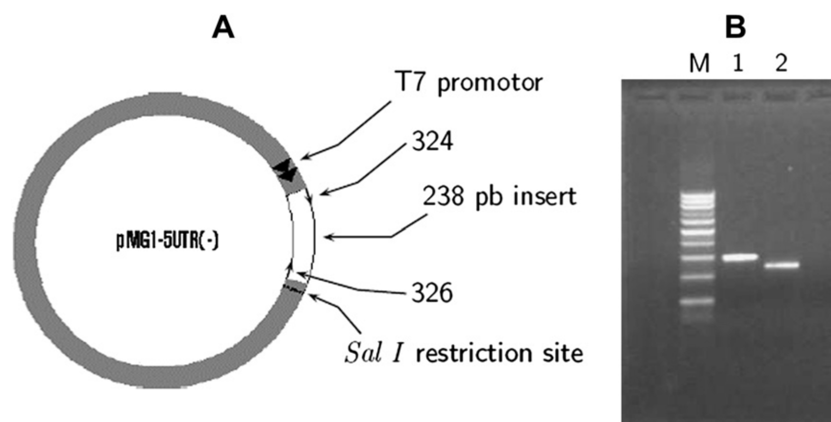

Fig. 1. A) Schematic representation of plasmid pMG1-5UTR(−). Relative locations of the target, T7 promoter and SalI restriction site are indicated. (B) Amplification products of wild type target (lane 1, 288 bp) and mimic DNA (lane 2, 238 bp); M: 1 kb ladder.

treatment with DNase appears to eliminate all plasmids, traces of DNA still persisted in mimic RNA preparations as demonstrated by direct PCR amplification. Although it was not particularly addressed in previous reports, we know through oral communications that many scientists and technicians have experienced problems in eliminating DNA from in vitro translated RNA preparations. That is, although plasmid DNA appears to be absent after analytical agarose gel electrophoresis, more sensitive techniques demonstrate that plasmid DNA still persist in the RNA preparations. The persistence of plasmid DNA is a serious problem if the standard is used for controlling the RNA extraction and reverse transcription steps of a diagnostic assay—if DNA traces are present, the standard would only provide PCR checkup. After several unsuccessful experiences, we solved this problem by serially diluting mimic RNA solutions until PCR amplification did not produce amplicons detectable in agarose gels. Remarkably, large amounts of amplification products were obtained by RT-PCR in dilutions much greater than those allowing for direct PCR amplification (Fig. 2). The mimic RNA was stored in a 10 mM vanadyl ribonucleoside complex solution. These RNA preparations were stable for up to 48 h at room temperature and upon several freeze/thaw cycles.

When performing a multi-target PCR (i.e. a PCR in which two or more templates are amplified with a single primer pair), inhibitions can occur if any of the templates is added in excess [25]. For this reason, and with the aim of minimizing inhibitory effects, DNA and RNA IC preparations were serially diluted until no product band was visualized after RT-PCR amplification. The greater dilution from which a sharp band was obtained when using 1 μl as template was selected as the working solution. We refer to this quantity of IC as a band forming unit. Both DNA and RNA IC batches have had to be diluted up to 1:10E5 (the exact dilution factor varies among particular DNA/RNA preparations) to have one band forming unit per microliter. Thus, very large amounts of the internal standard can be prepared from a single RNA or DNA batch. In contrast with titration, RNA quantification by spectrophotometry or agarose electrophoresis did not produce reproducible results (data not shown). This could be attributed to the fact that, especially when working with small amounts of molecules, little mass variations can correspond to large variations in the effective number of template molecules.

Sensitivity of the standard RT-PCR resulted to be around five plaque forming units (PFUs). To check if the addition of IC molecules leads to the detriment of performance, different amounts of virus ($4.35 \times$ 10E5 to 4.35 PFUs) were mixed with one band forming unit of either mimic DNA or mimic RNA and the mixtures were subjected to RNA extraction and RT-PCR. A decrease of sensitivity was not evident after electrophoresis of amplification products in agarose gels (data not shown). Brightwell et al. [25] have shown that when the amount of IC is optimal, the assay sensitivity is not compromised when the template is a bacterial DNA. Our results support the notion that these observations are also valid for RT-PCR amplification of viral RNA, both if DNA or RNA is used as IC. We also observed that inhibitory effects can be diminished moderately by adjusting the amounts of primers used in PCR (data not shown), a fact suggesting that a part of the inhibitory effect is related to oligonucleotide annealing dynamics.

Internal controls to be used in molecular detection of pestiviruses have been described previously. Heath and collaborators designed a probe which can be used in *Taq*Man nested reverse transcription-polymerase chain reaction tests [26]. Kim and Dubovi

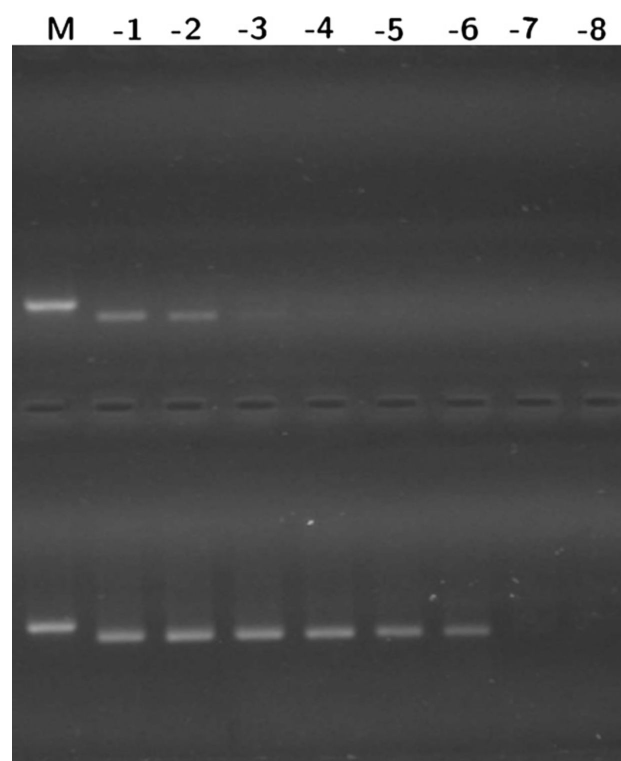

Fig. 2. Agarose gel electrophoresis of amplification products obtained using successive 1:10 dilutions of RNase treated mimic RNA as template. Lanes marked from −1 to −8 correspond to 1:10 to 1:10^8 dilutions. The upper part of the gel corresponds to direct PCR amplifications and the lower part corresponds to products of RT-PCR reactions. M: 288 bp marker.

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developed a one-step single-tube RT-duplex PCR method which includes an endogenous IC [27]. Universal internal standards for use in real-time PCR detection of Classical Swine Fever Virus have been described and validated in previous reports [28,29]. Our aim was to develop a method suitable for diagnostic laboratories with basic equipment. We avoided the use of endogenous controls, since this practice can influence the accuracy of RT-PCR results [30].

Given the high prevalence of pestiviruses in bovine serum, the most likely source of contamination is the cell cultures. The techniques described here were implemented with the aim of being used in these kind of samples. It is not impossible that the methods behave differently with other specimens (e.g. bovine serum, tissues, blood, semen, etc.). Thus, these ICs should be tested before use with other clinical samples.

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References

[1] Paton DJ, Sands JJ, Lowings JP, Smith JE, Ibata G, Edwards S. A proposed division of the pestivirus genus using monoclonal antibodies, supported by cross-neutralisation assays and genetic sequencing. Veterinary Research 1995;26:92–109.
[2] Becher P, Orlich M, Kosmidou A, König M, Baroth M, Thiel HJ. Genetic diversity of pestivirus, identification of novel groups and implications for classification. Virology 1999;262:64–71.
[3] Houe H, Baker JC, Maes RK. Prevalence of cattle persistently infected with bovine viral diarrhea virus in 20 dairy herds in two countries in central Michigan and comparison of prevalence of antibody positive cattle among herds with different infection and vaccination status. Journal of Veterinary Diagnostic Investigations 1995;7:321–6.
[4] Houe H. Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. Virology 1999;268:456–65.
[5] Nutall PA, Luther PD, Stot EJ. Viral contamination of bovine foetal serum and cell cultures. Nature 1977;266:835–7.
[6] Harasawa R, Mizusawa H. Demonstration and genotyping of pestivirus RNA from mammalian cell lines. Microbiology and Immunology 1995;39:979–85.
[7] Bolin SR, Ridpath JF. Prevalence of bovine viral diarrhea virus genotypes and antibody against those viral genotypes in fetal bovine serum. Journal of Veterinary Diagnostic Investigation 1998;10:135–9.
[8] Harasawa R. Adventitious pestivirus RNA in live virus vaccines against bovine and swine diseases. Vaccine 1995;13:100–3.
[9] Harasawa R. Comparative analysis of the 5′ non-coding region of pestivirus RNA detected from live virus vaccines. Journal of Veterinary Medical Science 1994;56:961–4.
[10] Giangaspero M, Vacirca G, Harasawa R, Buttner M, Panuccio A, De Giuli Morghen C, et al. Genotypes of pestivirus RNA detected in live virus vaccines for human use. Journal of Veterinary Medical Science 2001;63:723–33.
[11] Harasawa R, Tomiyama T. Evidence of pestivirus RNA in human virus vaccines. Journal of Clinical Microbiology 1994;32:1604–5.
[12] Harasawa R, Sasaki T. Sequence analysis of the 5′ untranslated region of pestivirus RNA demonstrated in interferon for human use. Biologicals 1995; 23:263–9.
[13] Ewnsvoort G, Terpstra C. Bovine viral diarrhoea virus infections in piglets born to sows vaccinated against swine fever with contaminated vaccine. Research in Veterinary Science 1988;45:143–8.
[14] Giangaspero M, Harasawa R. Genotypic analysis of the 5′-untranslated region of a pestivirus strain isolated from human leucocytes. Microbiology and Immunology 1997;41:829–34.
[15] Potts BJ, Sever JL, Tzan NR, Huddleston D, Elder GA. Possible role of pestiviruses in microencephaly. Lancet 1987:972–3.
[16] Yolken R, Dubovi E, Leicester F, Reid R. Infantile gastroenteritis associated with excretion of pestivirus antigens. Lancet 1989:517–20.
[17] Wilson I. Inhibition and facilitation of nucleic acid amplification. Applied and Environmental Microbiology 1997;63:3741–51.
[18] Belák S, Ballagi-Pordany A. Experiences on the application of the polymerase chain reaction in a diagnostic laboratory. Molecular and Cellular Probes 1993; 7:241–8.
[19] Legay V, Sailleau C, Dauphin G, Zientara S. Construction of an internal standard used in RT nested PCR for Borna disease virus RNA detection in biological samples. Veterinary Research 2000;31:565–72.
[20] Vilĉek S, Herring AJ, Nettleton PF, Lowings JP, Paton DJ. Pestiviruses isolated from pig, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. Archives of Virology 1994;136:309–23.
[21] Jones LR, Weber EL. Application of single strand conformational polymorphism to the study of bovine viral diarrhea virus isolates. Journal of Veterinary Diagnostic Investigation 2001;13:48–54.
[22] Jones LR, Zandomeni RO, Weber EL. Genetic typing of bovine viral diarrhea virus from Argentina. Veterinary Microbiology 2002;81:367–75.
[23] Jones LR, Zandomeni RO, Weber EL. Quasispecies in the 5′ untranslated genomic region of bovine viral diarrhea virus from a single individual. Journal of General Virology 2002;83:2161–8.
[24] Jones LR, Cigliano MM, Zandomeni RO, Weber EL. Phylogenetic analysis of bovine pestiviruses: testing the evolution of clinical symptoms. Cladistics 2004;20:443–53.
[25] Brightwell G, Pearce M, Leslie D. Development of internal controls for PCR detection of *Bacillus anthracis*. Molecular and Cellular Probes 1998;12:367–77.
[26] Heath GS, King DP, Turner JLE, Wakeley PR, Banks M. Use of an internal standard in a TaqMan nested reverse transcription-polymerase chain reaction for the detection of bovine viral diarrhoea virus. Veterinary Microbiology 2003;96:357–66.
[27] Kim SG, Dubovi EJ. A novel simple one-step single-tube RT-duplex PCR method with an internal control for detection of bovine viral diarrhoea virus in bulk milk, blood and follicular fluid samples. Biologicals 2003;31: 103–6.
[28] Hoffmann MA. Construction of an infectious chimeric classical swine fever virus containing the 5′UTR of bovine viral diarrhea virus, and its application as a universal internal positive control in real-time RT-PCR. Journal of Virological Methods 2003;114:77–90.
[29] Hoffmann B, Beer M, Schelp C, Schirrmeier H, Depner K. Validation of a realrime RT-PCR assay for sensitive and specific detection of classical swine fever. Journal of Virological Methods 2005;130:36–44.
[30] Selvey S, Thompson EW, Matthaei K, Lea RA, Irving MG, Griffiths LR. β-Actin—an unsuitable internal control for RT-PCR. Molecular and Cellular Probes 2001;15:307–11.