

Full Length Research Paper

Nested polymerase chain reaction (nPCR) based diagnosis of bovine leukemia virus in Panama

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The bovine leukemia virus is an exogenous retrovirus that causes enzootic bovine leukosis. The aim of this study was to apply and compare a diagnostic test in an outbreak of bovine leukemia virus by nested polymerase chain reaction (PCR) in a core conservation of native cattle Guaymí. From the results obtained by the three techniques used, the agar gel immunodiffusion (AGID) test detected 33 positive animals. The nested polymerase chain reaction (nPCR) tested blood and enzyme-linked immunosorbent assay (ELISA) detected more positive animals than AGID with 17 and 30%, respectively. Animals positive to the ELISA and AGID test but negative to nPCR could be attributed to the existence of animals with genotypes of BoLA-DRB3.2 of major histocompatibility complex class II alleles with favorable resistant to bovine leukaemia virus (BLV). The possibility of further studies on resistance against BLV can be done. It is concluded that the ELISA and nPCR are the diagnostic tests of option for BLV.

Key words: Biotechnology, bovine enzootic leukemia, electrophoresis, Guaymi.

INTRODUCTION

The bovine leukemia virus (BLV; family *Retroviridae*; subfamily *Orthoretrovirinae*, genus *Deltaretrovirus*) is an exogenous retrovirus that causes enzootic bovine leukosis (EBL), the most common malignancy of cattle worldwide (Schwartz et al., 1994; Dequiedt et al., 1999; Beyer et al., 2002; Moratorio et al., 2013). This virus is related to the human *T-lymphotropic* virus types 1, 2 and 3 (HTLV-1, -2 and -3) and to the primate *T-lymphotropic*

virus types 1, 2 and 3 (PTLV-1, -2 and -3) (Gelmann et al., 1983; Tanaka et al., 1990; Heneeman et al., 2012). Horizontal transmission of the infection occurs through the transfer of infected cells by direct contact, milk ingestion and possibly by hematophagous insects (Ferrer et al., 1979; Gillet et al., 2007). Vertical transmission (mother-child) via the uterus has also been demonstrated (Ferrer et al., 1979; Van der Maaten et al., 1981; Romero

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et al., 1983; Lassauzet et al., 1991). Dairy herds are more prone to infection due to the constant management of the animals and the frequent iatrogenic transmission caused by fomites such as milking equipment, surgery, equipment, needles and rectal palpation (Birgel Junior et al., 1995). Although the B lymphocyte is known to be the primary target of the virus, research has shown that monocytes, granulocytes, T cells and CD2⁺, CD3⁺, CD4⁺, CD8⁺ and γ/δ cells are also target cells of the virus (Williams et al., 1988; Stott et al., 1991; Schwartz et al., 1994; Mirsky et al., 1996). Another study by Panei et al. (2013) showed that B lymphocytes, CD5⁺ cells and IgM⁺ cells maintain the highest BLV proviral load, but in subclinical states, T lymphocytes, CD4⁺ cells and CD8⁺ cells are also observed as primary targets. BLV infection may remain clinically silent in the so-called aleukemic (AL) stage. However, in 30% of the infected animals, infection can be presented as a persistent lymphocytosis (PL), particularly with an increase in B lymphocytes. Between 1 and 5% of cases can be presented as B-cell lymphoma after a long latency period (Panei et al., 2013) caused by virus suppression (Kettmann et al., 1980), which is a possible strategy to evade the immune response and allow tumor development (Merimi et al., 2007). Certainly, the provirus-carrying B lymphocytes do not produce detectable levels of viral RNA or proteins (Lagarias and Radke, 1989; Jimba et al., 2012). Bovine leukemia virus (BLV) infection is associated with the natural emergence of B-cell tumors in bovine cattle and can be experimentally induced in sheep. Nevertheless, the complete understanding of how BLV induces tumorigenesis is still enigmatic, mainly because the majority of these tumor cells are positive for an integrated proviral genome of BLV, but they lack an abundant expression of transcriptomes or proteins encoded by RNA polymerase II (pol II) (Kettmann et al., 1985; Gaynor et al., 1996; Gillet et al., 2007).

According to Kettman et al. (1980), the lymphocytosis stage and tumor stage generally represent early and late disease stages, but these two pathological conditions do not necessarily affect the same herd, with separate responses as a result. This result suggests some genetic condition related to the response to the disease. Therefore, polymerase chain reaction (PCR), in addition to routine diagnostic tests such as agar gel immunodiffusion (AGID) and enzyme-linked immunosorbent assay (ELISA), is important for the diagnosis because PCR enables detection of the proviral genome integrated into the host genome (Panei et al., 2013). The three serological tests commonly used in the diagnosis of the disease are radioimmunoassay (RIA), AGID and ELISA. Dot Blot and PCR have been less widely used (Cockerell et al., 1992). Although AGID has been an indicator of BLV infection for decades and remains gold standard method in the Republic of Panama for the transport of animals inside the country, it has now

been shown to be an extremely insensitive test compared to newer techniques (Buehring et al., 2003). During the 1990s, many cattle that were negative for anti-BLV antibodies when tested by AGID were shown by immunoblot or ELISA to be positive (Walker et al., 1987; Have and Hoff, 1991; Grover and Guillemin, 1992). Have and Hoff (1991) found that ELISA was 50 to 100% more sensitive than AGID. In addition, Walker et al. (1987) and Grover and Guillemin (1992) showed that AGID failed in 53 and 70% of cases, respectively, in the detection of anti-BLV antibodies in immunoblot-positive cattle. Although RIA and ELISA are more sensitive than AGID, the latter has the advantage of being less expensive and easier to perform (Have and Hoff, 1991); however, the detection failure of AGID would be catastrophic, particularly for the bovine dairy industry, when animals are transported from areas with high prevalence of BLV to disease-free areas. ELISA is superior when there is a need to analyze many milk samples because it has the ability to detect low levels of anti-BLV antibodies (Nguyen and Maes, 1993). AGID cannot differentiate passively acquired antibodies (colostral) from those acquired by natural infection (Hugh-Jones, 1992). Another disadvantage of these techniques is that they cannot detect infected young animals or animals in the early stages of infection. PCR has been used for the early detection of EBL in animals less than six months old (Agresti et al., 1993; Kelly, 1993), thus avoiding the false positive reactions caused by passive immunoglobulin transfer through colostrum. Another advantage of PCR is its ability to detect the virus in immune-tolerant animals (Fechner et al., 1997). The BLV is one of the diseases reported by official authorities in most of their epidemiological reports (Real, 2008). However, there is no program to control and eradicate this disease. The aim of this study was to apply and compare nested PCR (nPCR) with serological tests ELISA and AGID in the diagnosis of an EBL outbreak of Guaymí creole cattle breed. This comparison would allow the selection of the test or diagnostic tests to be used in herds where the conservation of germplasm *in vivo* is the priority.

MATERIALS AND METHODS

The study was carried out in the laboratory of Agro-biotechnology at the Instituto de Investigación Agropecuaria de Panamá, Carretera Panamericana, km 214, Divisa, Provincia de Herrera. Five milliliter blood samples were drawn and placed into ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes. These samples were retrieved from a leukosis outbreak in five conservation nuclei of Guaymí creole bovine breeds. The samples were taken from 33 females and seven bulls that were positive with AGID test, the gold standard method of the Ministry of Agriculture laboratory, for the disease out of 96 animals that make up the original nucleus. ELISA, AGID and nPCR techniques were used for the analysis. For the AGID test, the PORQUIER® IDG kit for Bovine Leukosis was used, and the IDEXX Leukosis *Blocking* anti-gp51 ELISA test was used in the animal health laboratory of the

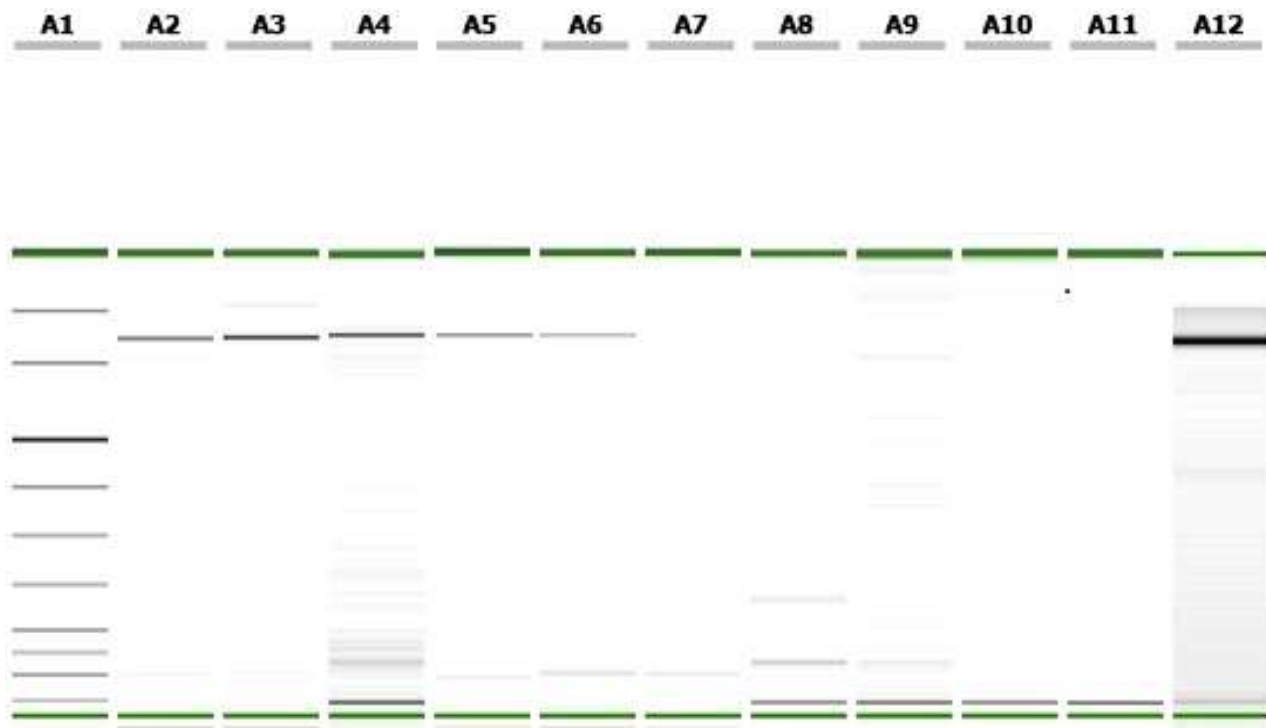


Figure 1. Capillary electrophoresis of Guaymi cattle (A1, marker; A2-A6, positive animals; A7-A10, negative animals; A11, negative control; A12, positive control).

Agricultural and Cattle Research Institute (IDIAP) Division. To apply the nPCR technique, a separation protocol of mononuclear cells (PBMCs) was first performed by adding three milliliters of Histopaque® 1077 Sigma-Aldrich in three milliliters of venous blood to an equal volume of phosphate-buffered saline (PBS), followed by centrifugation at 3500 *g* for 30 min. To extract the largest amount of white cells within each sample, a protocol was applied where two milliliters of the PBMC suspension was extracted and centrifuged at 3500 *g* for 10 min. The supernatant was discarded, and the pellet was stored in the bottom of the microtube for further processing. DNA extraction was performed using the commercial reagent QuickExtract™ from Epicenter and applying it to the PBMC isolate. This process consisted of the application of 500 μ l of the product, vortexing for 15 s and heating at 65°C for 6 min; then, the sample was vortexed again and the product was heated at 98°C for 2 mins. With this procedure, an average concentration of 218 ng/ μ l of genomic DNA was obtained.

nPCR was performed on the extracted DNA, in which a highly conserved region of the *env* gene coding for the *gp51* capsid protein was amplified. The protocol used was a modification of that proposed by Beier et al. (2001). The first reaction was performed using a final volume of 30 μ l, which included 70 to 100 ng of DNA, 0.5 Mm each of the Forward-*env*5032 (5'-TCTGTGCCAAGTCTCCCAGATA-3') and Reverse-*env*5608r (5'-AACAAACCTCTGGGGAGGGT-3') primers, 0.2 mM of each dNTP, 1X PCR buffer, 1.5 mM MgCl₂ and 1 U of Taq DNA polymerase. In the second reaction, three microliters of the PCR product from the first amplification was used as the DNA template, along with the same concentrations of the other reagents and the Forward-*env*5099 (5'-CCCACAAGGGCGGCCCGGTTT-3') and Reverse-*env*5521r (5'-GCGAGGCCGGTCCAGAGCTGG-3')

primers. The thermal profile included an initial denaturation step at 94°C for 9 min, followed by 35 cycles at 95°C for 30 s, 62°C for 30 s and 72°C for 1 min and ending with a final extension at 72°C for 4 min. In the second reaction, the amplification conditions were the same, except that the annealing temperature was increased to 70°C (Licursi et al., 2003). Identification of the animals that were positive for the provirus was performed using a QIAgen® capillary electrophoresis analyzer of DNA fragments. The presence of a band of 444 base pairs indicates that the provirus is present in the animal, using a QX DNA Size Marker 25 to 500 bp (50 μ l) v2.0 from QIAgen®.

To determine the parameters of sensitivity, specificity and concordance (*kappa* value) of nPCR, AGID and ELISA contingency tables (2x2) were used with the WinEpi 2.0 computer program (de Blas et al., 2006). All analyses were performed with a 95% confidence level.

RESULTS AND DISCUSSION

The band of 444 base pairs can be observed in the fragment analyzer consistent with the BLV *env* gene. Of the 40 animals tested by nPCR, 32 animals were positive (80%) and eight animals (20%) were negative (Figure 1).

The results observed are similar to those reported by Beyer et al. (2002), who used PBMC separation and the *env* gene to amplify the BLV provirus. Similarly, Alfaro et al. (2012) used nPCR to detect the BLV provirus; however, they used the gene of the long terminal repeat

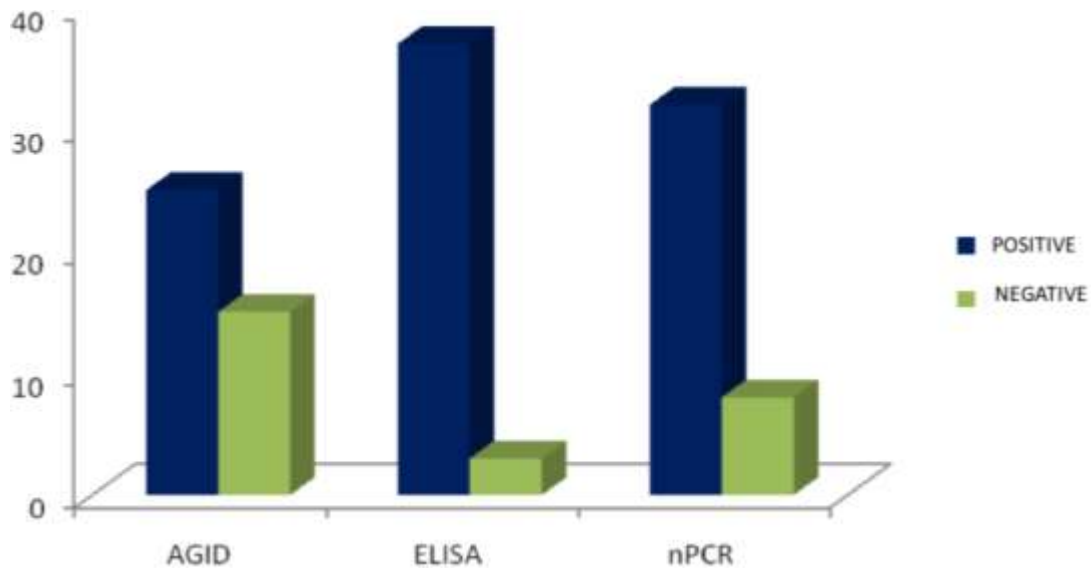


Figure 2. Analysis of the number of positive and negative animals identified with each of the AGID, ELISA and PCR tests in Guaymí cattle.

region (LTR). In the present study, the fragments analyzer was used with a cartridge of 12 capillaries, thus replacing the agarose gels that had been used in the laboratory in recent years. With this change of technology, fewer reagents were used, and less time was spent on the analysis. For each 96-well plate, the equipment use required 48 min, unlike with the agar gel, which required 2 h per run and did not include the time required to add the loading gel to the amplicons and the base pair marker as well as load the samples on the gel. The nPCR technique has also been compared by Lew et al. (2004) and Heenemann et al. (2012) with the real-time PCR technique, with advantage being observed in one methodology over the other in the detection of the provirus, optimized qPCR can detect less than 10 copies. Nevertheless, Jimba et al. (2012) used primers with the Coordination of Common Motif (COCOMO) algorithm to measure the proviral load of new and known variants in BLV-infected animals and were able to detect lower proviral loads than any other currently used PCR techniques. Clearly, real-time PCR has greater advantages relative to nPCR; it is a shorter procedure with a reduced risk of cross-contamination and provides a quantitative assessment of viral load. However, nPCR is still very useful and requires, such as real-time PCR, the use of other immunological techniques such as ELISA since there are variations in the kinetics of the proviral load and in the responses of individuals to the virus. These differences are attributable to the different BoLA-DRB3 genotypes that these animals possess (Jimba et al., 2012). On the other hand, Buehring et al. (2014) observed a decrease in the gag, pol and env sequences

in BLV-positive samples and in the presence of LTR and Tax sequences, the lack of gag, pol, and env sequences in some of the BLV-positive panel samples and the presence of LTR and tax sequences in all of them is consistent with results reported for the closely related HTLV-1, particularly when leukemia progressively increases (Buehring et al., 2014). These deletion mechanisms are postulated as closely related to the virus evasion from the host immune response, so it would be expected that, thereafter, primers of the LTR and Tax sequences should be used for better accuracy in the diagnosis.

From the results obtained from these three techniques, the standard test of the Republic of Panama, AGID, detected a lower number of positive animals than ELISA and nPCR in blood samples (Figure 2).

A comparison of this study with Felmer et al. (2006), Fernandes et al. (2005), Buehring et al. (2003) and Lamas et al. (2012) showed similar results, indicating that AGID has a lower capacity to detect positive animals compared to PCR and ELISA, which would represent a potential risk for the spread of virus within the Republic by allowing the passage of infected animals.

Of the 40 animals analyzed, AGID detected 25 positive animals, equivalent to 63% of the total number of animals (Table 1). On the other hand, nPCR identified 80% of the BLV-positive animals, 17% more than AGID: Four nPCR-positive animals were negative for AGID, and 11 of the 15 AGID-negative animals were positive for nPCR. These results are similar to those observed by Gregory et al. (2004) in Brazil and Felmer et al. (2006) in Chile who reported 63% of BLV-positive animals, having also used

Table 1. Analysis of sensitivity, specificity and concordance of blood PCR in relation to AGID of Guaymí creole.

Test	Result	PCR		Total
		+	-	
AGID	+	21	4	25
	-	11	4	15
Total		32	8	40

Sensitivity: 66%; specificity: 50%; concordance: *kappa* 0.12.

Table 2. Analysis of sensitivity, specificity and concordance of blood PCR in relation to ELISA of Guaymí creole.

Test	Result	PCR		Total
		+	-	
ELISA	+	30	7	37
	-	2	1	3
Total		32	8	40

Sensitivity: 94%; specificity: 13%; concordance: *kappa* 0.082.

AGID, the technique that detected the least number of positive animals. The concordance between the two tests was 63%, whereas the kappa statistic was 0.12, which is considered weak. The diagnostic sensitivity was 66%, whereas the specificity was 50% attributable to AGID.

Of the 40 animals tested, ELISA detected 37 positive animals, equivalent to 93% of the total number of animals (Table 2). On the other hand, ELISA detected 30% more positive animals than AGID and 13% more than nPCR: seven ELISA-positive animals were negative for PCR, and two of the three ELISA-negative animals were positive for nPCR. The concordance between the two tests was 78%, whereas the kappa statistic was 0.082, which was considered weak. The diagnostic sensitivity was 94%, whereas the specificity was 13%. When comparing AGID and ELISA, a kappa value of 0.238 was considered weak but significant, with a sensitivity of 68% and a specificity of 100%.

These results differ from those observed by Felmer et al. (2006) in Chile who reported similar results for nPCR and ELISA in terms of the diagnostic capacity; however, Gregory et al. (2004), in Brazil, reported variable results. ELISA, in the present study, was able to identify 93% positive animals, and the two nPCR tests, which followed the methodology of Ballagi-Pordany et al. (1992) and used the modified test of Beier et al. (2001), were able to identify 13 and 90% of positive animals.

The present study evaluated the use of a modified nPCR method proposed by Beier et al. (2001), as a direct test and compared its results with AGID and ELISA as indirect tests for the detection of BLV in a group of Guaymí creole bovine breeds in a conservation program. According to the results, blood PCR and serum ELISA

detected a higher number of positive animals (17 to 30%) than AGID. All serum ELISA-negative samples were also negative for AGID. However, four PCR-negative samples were identified as positive with AGID, and seven nPCR-negative samples were identified as positive with ELISA, which could have several possible explanations. The most common reasons for these discrepancies have been discussed by Eaves et al. (1994), who attributed them to the absence of lymphocytes in blood, and by Marsolais et al. (1994), who attributed them to variations in the nucleotide sequences and a decrease in the sequences of the gag, env and pol genes as an effect of the evasion of the immune system, which was reported by Buehring et al. (2014) and which, in turn, could prevent the recognition of primers at the time of banding in some viral strains or virus restrictions to the lymphoid organs as reported by Klintevall et al. (1994). However, the amount of PBMC used from the collected blood (2 ml) and the methodology applied increased the availability of lymphocytes; at the same time, nPCR was more effective than other traditional PCR methodologies. The effectiveness of nPCR has been compared with real-time PCR by Lew et al. (2012) and Heenemann et al. (2012). The animals that were positive for nPCR but negative for serological tests indicate the possibility of finding immune-tolerant or low-immune-response animals (Fechner et al., 1997; Jimba et al., 2012), although the virus itself is known to have mechanisms for evading the host immune response (Merimi et al., 2007), which is not surprising. Another striking result in the present study is the low kappa values observed in the three tests analyzed, a result that differs from those observed by Felmer et al. (2006) and Lamas et al. (2012), who found

a moderate statistical correlation between the tests used (Cerda and Villarroel, 2008). However, Jimba et al. (2012), when analyzing the same serological tests (AGID and ELISA) with the PCR tests, found a low correlation among the tests and attributed this result to the differences between humoral and viral kinetics in experimentally infected animals. Likewise, animals that were positive for immunological tests (AGID and ELISA) but negative for nPCR could be the result of animals with BoLA-DRB3.2 genotypes of the major histocompatibility complex type II, which are favorable for BLV resistance (Lewin et al., 1988; Aida 2001; Esteban et al.; 2009; Jimba et al., 2012). There are several authors that describe alleles of the BoLA gene that could be associated to the resistance or susceptibility to BLV dissemination; as an example, Nikbakht et al. (2016) in Holstein cattle, Miyasaka et al. (2013) in Black Japanese cattle.

On the other hand, most of the authors are not conclusive about the results, and suggest the presence of some other genetic or epigenetic factors that could influence the viral spread in infected animals (Juliarena et al., 2013; Ohno et al., 2015; Farias et al., 2016).

The breed used in the BLV test in the present study, the Guaymí, is one of the two Panamanian creole breeds, descendants of races brought by the Spaniards beginning in the 15th century and on which genetic diversity studies have previously been conducted (Villalobos et al., 2010; Delgado et al., 2012; Martínez et al., 2012; Ginja et al., 2013). Studies have shown that creole breeds present various disease resistance genes (Mirsky et al., 1998; Martínez et al., 2005), so that it is possible that some of the animals may be carriers of disease-resistance genes and could be included in BLV control and eradication programs through genetic marker-assisted selection (Esteban et al., 2009). Although it has not been demonstrated in this population, the hypothesis is raised that it possesses favorable genes of DRB3.2 that can be used in programs of crossing that includes tolerance to diseases. Therefore, other studies should be carried out where this hypothesis is demonstrated.

Conclusions

The following conclusions are drawn from the study:

1. The use of the AGID technique for the diagnosis of EBL should be discontinued.
2. nPCR and ELISA should be the techniques of choice for the diagnosis of EBL for the conservation of nucleus of Guaymí creole bovine breeds; the use of these techniques in the official laboratories of the Republic of Panama is recommended.
3. Genotyping studies should be carried out to demonstrate the hypothesis that Guaymí creole cattle

population possess disease resistant genes

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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