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Infection with *Leishmania* (*Leishmania*) *infantum* of 0 to 18-Month-Old Children Living in a Visceral Leishmaniasis-Endemic Area in Brazil

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Abstract. The diagnosis of *Leishmania* (*Leishmania*) *infantum* infection in children from birth may serve as a reference for the early identification of cases that would progress to classical visceral leishmaniasis (VL) in endemic areas. This study prospectively evaluated newborns of mothers living in the municipality of Paracatu, Minas Gerais, Brazil. The infants were followed up at 6-month intervals by clinical examination, serological tests (immunofluorescence [IIF] and enzyme-linked immunosorbent assay with rK39 [ELISA-rK39]) and polymerase chain reaction (PCR) until they had completed 18 months of age. A total of 166 pregnant women were included to evaluate the possible transfer of antibodies or even congenital transmission. Twenty-two of the women tested positive by IIF, four by ELISA-rK39, and one by PCR. Three infants of the 25 women with some positive test results were also positive in the first test (one by IIF, one by ELISA-rK39, and the third by ELISA-rK39 and PCR). One hundred and sixty infants were included in the study; of these, 43 had at least one positive sample over time. However, agreement between tests was low. Follow-up of children with a positive result in the tests studied revealed no progression to classical disease within a period of 18 months. In contrast, two children with negative IIF, PCR, and ELISA-rK39 results developed classical VL at 9 and 12 months of age. In conclusion, a positive test result was variable and sometimes temporary and agreement between tests was low. Therefore, the early diagnosis of *Leishmania* infection was not associated with the early identification of cases that would progress to classical VL in the endemic area studied.

INTRODUCTION

With an estimated global incidence of 500,000 new cases per year and more than 50,000 annual deaths, visceral leishmaniasis (VL) is one of the six endemic diseases regarded as major public health problems in the world. The disease occurs in 65 countries, with the largest number of cases (90%) being notified in Bangladesh, Brazil, Ethiopia, India, Nepal, and Sudan.[1] Brazil has witnessed a progressive increase in the number of VL cases despite the application of control strategies by the Ministry of Health. Between 1999 and 2008, the mean annual number of cases was 3,379 and the incidence was 1.9 cases per 100,000 inhabitants. The lethality rate of VL increased from 3.4% in 1994 to 5.5% in 2008, corresponding to an increase of 61.8%.[2]

The early diagnosis and treatment of human cases of VL, which is one of the cornerstones of the Brazilian Visceral Leishmaniasis Surveillance and Control Program,[3] have emerged as a challenge for clinicians. A delay in the diagnosis of VL frequently compromises the clinical evolution of patients and results in a more reserved prognosis caused by delays in the initiation of specific treatment.[4]

The serological tests commonly used in Brazil for the diagnosis of VL are indirect immunofluorescence (IIF) and enzyme immunoassays (ELISA) using crude antigen.[3] These methods show variable sensitivity of 83.3–98% and 50–100% and specificity of 88–100% and 75–100%, respectively.[5–12] The recombinant antigen K39 has also been used in both ELISA and immunochromatographic tests and has shown better performance than IIF and ELISA using crude antigen.[13–15] The tests cited were validated for patients with the classical clinical presentation of VL. However, there is no consensus on the test of choice for the early diagnosis of infection in the absence of clinical manifestations. Studies in which different methods were simultaneously applied to the same population revealed low agreement between results. In addition, individuals with positive tests may or may not progress to the disease, a fact impairing the selection of a test that will identify those who will eventually develop symptoms of classical VL.[12,16,17]

The polymerase chain reaction (PCR) has shown greater efficacy in identifying asymptomatic cases of VL than serological tests.[18] However, this technique also identified individuals living in endemic areas that had positive results, but did not progress to classical disease. In addition, disagreement between the results of serological tests and PCR has been frequently reported.[16,18,19]

The objective of this study was to evaluate newborns from a VL-endemic area and to determine the time when these infants are infected with *L.* (*L.*) *infantum*. Prospective evaluation should provide information to identify risk factors associated with the disease in recently infected children, and to determine the usefulness of diagnostic tests for predicting its clinical evolution.

SUBJECTS AND METHODS

Subjects. The municipality of Paracatu, Minas Gerais, was chosen for this study because it is an area of intense transmission of VL according to the Brazilian Ministry of Health, with the notification of 129 cases between 2003 and 2005. Based on available data that show an incidence of 1.5% (21 cases in 1,400 children < 18 months of age) were recruited from 160 pregnant mothers.

Considering the possible transfer of maternal antibodies to the infant, first the mothers were invited to participate in the study. For this purpose, pregnant women at a gestational age of more than 34 weeks were submitted to clinical evaluation and collection of peripheral blood. The women were recruited

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from different healthcare units of the municipality, proportionally to the number of inhabitants per neighborhood. Next, the link with the mother was established and the first blood sample was collected from the newborn during the heel prick test, which is part of the Brazilian Family Health Strategy. Subsequent samples were collected at intervals of 6 months over a total follow-up period of 18 months. Clinical evaluation was performed on the occasion of each blood collection.

Furthermore, at 6-month intervals, the researchers, in cooperation with the Health Department of the municipality, actively searched for all notified human cases of VL. This search was used to identify children lost to follow-up who eventually developed the disease.

Classical VL was defined as the presence of fever > 38.0°C for 1 week or longer, associated with hepatosplenomegaly (liver > 2 cm from the right costal margin) or splenomegaly (border of the spleen exceeding the left costal margin or Traube's space percussion) and pallor, accompanied by positive serology and/or a positive parasitological test. The prospective 6-month assessments were performed by infectologists participating in the project. The remaining clinical evaluations outside the 6-month intervals were performed by pediatricians of the municipal health services.

The samples for this study were obtained in accordance with the guidelines of Resolution 196/96 of the National Health Council, which regulate research involving humans.[20] The project was approved by the Ethics Committee of Universidade Federal do Triângulo Mineiro (Protocol No. 1038).

Methods. *Blood collection.* Two tubes of peripheral blood were collected from the pregnant women (N = 166), one containing EDTA for PCR and another dry tube for the separation of serum. The first blood sample of the newborns (N = 160) was only collected on filter paper. However, samples of whole blood and serum were collected in the second and following samplings, whenever possible. All blood samples were collected by trained professionals using standard protection equipment and disposable material. The samples were frozen at −20°C for up to 4 hours after collection and only thawed for processing in the laboratory.

Diagnosis of asymptomatic infection. The serological tests chosen for the diagnosis of asymptomatic infection were IIF and ELISA using recombinant antigen K39 (ELISA-rK39), methods commonly used for the serological diagnosis of VL in Brazil.[12,16] The PCR, which is considered to have a greater capacity of identifying infection, was also performed.[18] The methods are described below.

Indirect immunofluorescence test (IFT). The test was performed as described by Camargo[21] using *L.* (*L.*) *amazonensis* (MHOM/BR/60/BH6) promastigotes in the exponential growth phase cultured in liver infusion tryptose medium as antigen. Fluorescein isothiocyanate-labeled human immunoglobulin G (IgG) antiglobulin obtained from rabbit immune serum was used as conjugate (Biomanguinhos, Rio de Janeiro, Brazil). All samples showing positivity at a dilution of 1:80 were considered to be reactive.

Enzyme-linked immunosorbent assay with rK39 (ELISA-rK39). The assay was performed according to Burns,[22] with minimal modifications. Briefly, microassay plates (Nunc-Immuno Plate Brand Products, Roskilde, Denmark) were sensitized (50 µL/well) overnight at 4°C with rK39 antigen diluted (1 µg/mL) in coating buffer (15 mM Na_2HCO_3, 28 mM NaH_2CO_3, pH 9.6) followed by blocking by 2 hours at 37°C with phosphate buffered saline (PBS) added to 0.05% Tween 20 and 2% skim milk (PBS-T-Milk 2%). After three washes with PBS-T, 50 µL per well of the samples diluted to 1:100 in PBS-T-milk 1% were incubated for 1 hour at 37°C. Wells containing positive and negative control sera diluted 1:100 were included. New washes were proceeded and the plates were incubated 1 hour with 50 µL per well of anti-human IgG conjugated to peroxidase diluted 1:30.000 (Sigma Chemical Co., St. Louis, MO) in PBS-T-milk 1%. The plates were washed newly and 50 µL of substrate solution (TMB, Sigma Chemical Co.) were added into each well. The plates were incubated for 5 minutes in the dark and the reaction was stopped with 50 µL of 1 N sulphuric acid solution. The absorbance readings were measured at 450 nm in a microplate reader (Model 550; Bio-Rad Laboratories, Tokyo, Japan). The cut-off value was determined in each assay day by absorbance media from 15 negative samples plus three standard deviations. Furthermore, the reactivity index was calculated by dividing the mean absorbance of each sample by cut-off of the assay day. The samples with a reactivity index higher than 1.0 were considered ELISA-rk39 positive.

PCR assay. The extraction of total DNA from peripheral blood samples and from blood collected on filter paper was held with *GenomicPrep Blood Mini Spin Kit* (GE Healthcare, Buckinghamshire, UK) according to the recommendations of the manufacturer, with some modifications. For the extraction of blood samples collected on filter paper an area of 0.5 × 0.5 cm was removed with the aid of a scalpel to elution in 200 µL of 1X TE buffer (Promega, Madison, WI) and allowed at room temperature for 30 minutes. The primers used were previously described by Degrave and others[23] and designed to amplify a 120-basepair (bp) sequence from the conserved region of the kDNA minicircle of *Leishmania* spp. (150-GGGG/TAGGGGCGTTCTC/GCGAA and 152- C/GC/GC/GA/TCTATA/TTTACACCAACCCC).

For PCR amplification 1 µL of DNA samples from blood diluted 1:5 and from blood collected from the digital pulp in filter paper not diluted were used as template in a final volume of 10 µL containing 1.5 U of Platinum *Taq* DNA Polymerase (Invitrogen, São Paulo, Brazil), 1 µL PCR Buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl - Invitrogen), 0.6 µM of each primer, 2.0 mM $MgCl_2$, 1 U of Uracil N-glycosylase (UNG - Applied Biosystems, Foster City, CA), 400 µM dATP, dCTP, dGTP, and 800 µM of dUTP (Promega). The cycling program preceded by 10 minutes incubation at 37°C (UNG digestion period), 5 minutes incubation at 95°C followed by 37 cycles at 95°C for 30 sec, 60°C for 30 sec, and an elongation step at 72°C for 5 minutes. A positive control based on DNA from the reference strain MHOM/BR/74/PP75 was included in all assays. Negative control containing all components of the reaction mixture except DNA was also included in each PCR assay. For decreased contamination episodes, UNG was incorporated into the reaction and all procedures were processed in separated rooms, using sterilized materials in the laminar flux chamber. Under these laboratory conditions, the analytical sensitivity of PCR was 0.1 fg, determined using eight 10-fold serial dilutions of genomic DNA from the reference *L. (L.) infantum* strain (MHOM/BR/74/PP75).

Additionally, the human beta actin gene (*ACTB*) was PCR-amplified with the primers Aco1 and Aco2[24] in all samples and subjected to electrophoresis on a 6% polyacrylamide gel and analyzed by silver staining.

RESULTS

A total of 166 pregnant women without clinical signs and symptoms of VL were included in the study in March 2008. Of these, 22 (13.3%) tested positive by IIF, four (2.4%) by ELISA-rK39, and only one (0.6%) by PCR. During follow-up from March 2008 to December 2010, none of these women showed clinical progression to VL or received specific treatment. During the same period, 109 cases of the disease occurred in the municipality.

Analysis of the first sample collected from the children on filter paper revealed that only one (0.6%) of the 160 samples tested positive by IIF and two (1.3%) by ELISA-rK39, whereas six (3.8%) were positive by PCR. Because the frequency of positive serological tests was much higher among the mothers when compared with their children, we believe that the loss of reactivity might be related to the collection on filter paper. We therefore chose to collect serum samples from the newborns of the 22 mothers who tested positive by IIF. Three infants had a positive IIF result in serum, although the same test was negative when blood collected on filter paper was used. As a consequence, after the second collection, serum samples were obtained preferentially and blood sample on filter paper was restricted to cases that presented technical difficulties.

The children were followed up from birth until 18 months of age by clinical examination and blood collection at intervals of 6 months. Sample size calculation indicated a number of 134 children for evaluation. A larger number of children (N = 160) was included at birth, considering the difficulty of follow-up in the endemic area. This loss to follow-up occurred and 135 (100% of the calculated sample size) samples were processed at 6 months, 120 (89.5%) at 12 months, and 77 (57.5%) at 18 months. The main reason for dropout was that the mother considered the blood collection painful and unnecessary for her apparently healthy child.

Table 1

Results of PCR, indirect immunofluorescence, and ELISA-rK39 over time obtained for 43 children with at least one positive sample

	Mother			Newborn			6 months			12 months			18 months		
Test child	PCR	IIF	ELISA-rK39	PCR	IIF	ELISA- rK39	PCR	IIF	ELISA- rK39	PCR	IIF	ELISA- rK39	PCR	IIF	ELISA- rK39
2	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
6	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
8	–	–	–	+	–	–	–	–	+	–	–	+	–	+	+
10	–	–	+	–	–	–	NP	NP	NP	–	–	+	NP	NP	NP
11	–	–	–	–	–	–	+	–	–	–	–	–	NP	NP	NP
12	–	–	–	–	–	–	+	–	NP	–	–	–	–	–	–
14	–	–	–	–	–	–	+	–	NP	–	–	–	NP	NP	NP
15	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–
17	–	–	–	–	–	+	–	–	–	+	–	–	–	–	–
18	–	–	–	–	–	–	–	–	–	+	–	–	NP	NP	NP
24	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
31	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
34	–	–	–	–	–	–	–	–	+	–	–	+	NP	NP	NP
46	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
50	–	–	–	–	–	–	–	–	–	–	–	+	**NP**	**NP**	**NP**
57	–	–	–	–	–	–	NP	NP	NP	–	–	+	–	–	+
61	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
73	–	–	–	+	–	–	NP	NP	NP	NP	NP	NP	+	–	–
75	+	–	+	+	–	+	+	–	–	–	–	–	–	–	–
77	–	–	–	NP	–	NP	–	–	–	–	–	+	+	+	+
79	–	–	–	–	–	–	–	–	–	–	–	+	NP	NP	NP
91	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
96	–	–	–	–	–	–	–	–	–	+	–	–	NP	NP	NP
98	–	–	–	–	–	–	–	–	NP	+	–	–	–	–	–
99	–	–	–	–	+	–	–	–	–	–	–	+	–	–	+
101	–	–	–	–	+	–	–	–	–	NP	NP	NP	NP	NP	NP
104	–	–	–	–	+	–	–	–	–	NP	NP	NP	–	–	–
116	–	–	–	–	–	–	–	–	+	NP	NP	NP	NP	NP	NP
118	–	–	–	–	–	–	–	–	–	–	–	–	+	–	NP
120	–	–	–	–	–	–	NP	–	NP	–	–	–	–	–	+
121	–	–	–	–	–	–	–	–	–	–	–	–	+	–	NP
129	–	+	–	–	–	–	+	–	–	–	NP	NP	–	–	–
130	–	+	–	–	–	–	–	–	–	+	–	–	–	–	NP
133	–	+	–	–	–	–	NP	–	NP	+	–	–	–	–	–
135	–	–	–	–	–	–	–	–	–	+	–	–	NP	NP	NP
138	–	–	+	–	–	–	NP	NP	NP	+	–	–	NP	NP	NP
139	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–/+*
141	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+
142	–	+	–	–	–	–	NP	NP	NP	–	–	+	NP	NP	NP
144	–	–	–	–	–	–	–	–	NP	–	–	–	–	–	+
146	–	–	–	–	–	–	–	–	–	NP	NP	NP	–	–	+
155	–	–	–	+	–	–	+	–	NP	NP	NP	NP	NP	NP	NP
159	–	+	–	–	–	–	–	–	–	–	–	–	+	–	–

*The reactivity index is within the grey zone (< 1.1).

IIF = indirect immunofluorescence; PCR = polymerase chain reaction; NP = not performed; + = positive; – = negative. Numbers in bold indicate patients who received a clinical diagnosis and treatment of visceral leishmaniasis.

The results of the serological tests and PCR obtained for the children during follow-up showed the presence of seroconversion that was not accompanied by a positive PCR result and vice versa. In addition, the positive IIF, ELISA-rK39, and PCR results were not associated with the clinical presentation of VL and were often occasional results (Table 1). At the end of the study, 17 children 18 months of age had at least one leishmaniasis-positive test (Figure 1). All children were followed up clinically and none of them developed the disease.

All cases of VL notified in the municipality were evaluated and it was determined whether they belonged to the group of children under follow-up. Only two children of the study group had classical VL and received specific treatment during follow-up, one at 9 months of age and the other at 12 months of age. These notifications were made during periods when the researchers were not in the endemic area. As a consequence, the patients were characterized based on the information available in the notification forms and hospitalization records or directly obtained from the mothers. The summary of the cases is reported below using the case identification number of the forms.

Case No. 15. A 9-month-old boy was admitted with a 1-week history of fever accompanied by dry cough, pallor, and splenomegaly. The boy had been evaluated by an infectologist at birth and at 6 months of age and was asymptomatic. Physical examination was normal, and IIF and PCR were negative. The IIF was repeated on admission and the result was positive. The boy was treated for VL with *N*-methylglucamine antimonate (20 mg/kg/day) for 20 days. Progressive regression of signs and symptoms was observed and the child was asymptomatic at the end of treatment. At 12 months of age, the boy continued to test positive by IIF, but PCR and ELISA-rK39 were negative. Five months after the end of treatment (14 months of age), the boy was again admitted with intermittent fever for 30 days. He had received several antibiotic regimens without improvement of symptoms. Physical examination revealed pallor and hepatosplenomegaly. A new cycle of *N*-methylglucamine antimonate was prescribed using the same dose and duration as before and the boy was asymptomatic upon discharge, with complete remission of hepatosplenomegaly. The tests performed at 18 months of age showed positive IIF and negative PCR and ELISA-rK39. The patient remained without complications and the physical exam was normal (last assessment in March 2012).

Case No. 50. A 12-month-old girl was admitted with a 10-day history of fever, accompanied by unmeasured weight loss, pallor, and splenomegaly. The girl had been evaluated by an infectologist at birth and at 6 months of age and was asymptomatic. Physical examination was normal, and IIF and PCR were negative. On admission, the girl received *N*-methylglucamine antimonate (20 mg/kg/day) for 20 days. The girl was afebrile after 2 days of treatment and splenomegaly regressed. An infectologist clinically reassessed the patient immediately after the end of treatment at 13 months of age.

FIGURE 1. Results obtained for children and pregnant women over the study period. A positive result was defined when at least one of the diagnostic tests studied was positive and a negative result was defined when all tests were negative (polymerase chain reaction [PCR], indirect immunofluorescence, and ELISA-rK39).

The girl was asymptomatic, with physical examination showing no anomalies. On that occasion, IIF and PCR were negative and ELISA-rK39 was positive. The child was asymptomatic upon clinical reassessment and had not shown any complications since hospitalization (last assessment in March 2012).

DISCUSSION

The number of human VL cases has increased in Brazil over recent years, with an estimated number of 3,500 cases/year. In addition to the increase in notifications, urbanization of VL has been observed, and an increase in lethality rates. These rates have reached 15% in some regions of the country, even after the introduction of specific treatment. This lethality has been attributed to different factors such as drug toxicity, comorbidities, and delays in the diagnosis of the disease.[25,26] The signs and symptoms of VL resemble those of other diseases such as leukemia, malaria, and schistosomiasis mansoni. Complementary tests are therefore important for diagnostic definition. Several serological and molecular tests have been used for this purpose because of their relative ease of operation.

The objective of this study was to identify children infected with *L.* (*L.*) *infantum* by means of IIF, ELISA-rK39, and PCR. Follow-up would then permit the early detection of those children that would require specific treatment. We may thus be able to reduce the lethality of VL in Paracatu (14.3% in 2009), if the disease is identified before its progression to complications and death.

However, we observed that the diagnosis of seroconversion of IIF and/or ELISA rK39 and/or a positive PCR result was not useful for the early identification of cases that would progress to classical VL. These results agree with studies showing the presence of seropositivity or even positive PCR in the absence of progression to the disease.[17,18,27,28] In contrast, other authors suggested seroconversion to be associated with disease progression. In Brazil, Badaró and others (1986) and Evans and others (1992) identified individuals in VL-endemic areas who seroconverted by ELISA using promastigote antigen and progressed to classical VL during prospective follow-up.[8,29]

In India where VL is caused by *L. L. donovani*, Singh and others (2002) observed that a positive ELISA-rK39 test in VL contacts was associated with progression to clinical disease in 69% of cases over a period of one year.[30] Furthermore, in India and Nepal, Ostyn and others (2011) showed that the probability of progression to disease was higher among individuals with seroconversion of direct agglutination test, but the disease also occurred in previously seronegative individuals.[31]

It should be noted that the two children of this study developed the disease about 3 to 6 months after a negative result in the three tests. The fact that these children were exposed in an endemic area suggests that they contracted the disease during the period between assessments. Thus, the period of 6 months would be too long to permit early detection of infection. Another fact that calls attention is that children with clinically diagnosed VL presented conversion in only one of the three tests performed. In view of their immature immune system, children younger than 2 years of age may have difficulties in producing anti-*Leishmania* antibodies at levels that can be detected by the tests, a fact that would explain the positive result in only one of the tests. However, PCR has been indicated as a good alternative for the diagnosis in this age group,[32] which disagrees with the results obtained here.

Furthermore, seroconversion and a positive PCR result were not associated with the clinical signs or symptoms of VL. Agreement between the tests used was low and the positive results were frequently temporary, a fact also reported in other studies.[16–18] Negativation of the tests may indicate a self-limited infection not associated with persistence of the parasite. Taken together, the results suggest that detection of infection has no practical usefulness in the endemic area. Therefore, periodic follow-up of asymptomatic children in these regions by IIF, ELISA-rK39, and PCR does not seem to be justifiable as a prognostic tool of the disease.

Another aspect to be discussed is the early positive result of IIF, ELISA-rK39, and PCR observed in newborns. Positive serology might be associated with the transfer of antibodies from the mother to the child. However, a positive PCR result could indicate the possibility of asymptomatic vertical transmission of *Leishmania*, because the samples were collected within a maximum period of 5 days of life, a fact rendering vector transmission less likely. Vertical transmission has been reported in the literature, even from asymptomatic mothers carrying *Leishmania* to children who developed VL in a non-endemic area.[33–35] This transmission may play some role in the maintenance and dissemination of the disease in endemic areas of Brazil, even after the implementation of control measures.

One limitation of this study was the loss to follow-up of children included in the study. The most frequent reasons for dropout were a change in address and the fact that the mother felt sorry to submit her asymptomatic child to blood collection. However, because most children were located in the municipality and all cases of VL that had occurred during the period were confirmed, we believe that there were no cases of the disease other than those reported. Another limitation is related to the lack of homogeneity of the samples. Because the children were very young, technical difficulties were encountered on some occasions in the collection of blood samples by venipuncture. In this case, we preferred to obtain the sample on filter paper instead of not collecting any sample at all, which would increase sample loss even further. Finally, the lack of a parasitological diagnosis of cases notified and treated as VL in the municipality was also a limiting factor. Bone marrow aspiration was not performed because of the lack of trained professionals in the area. However, the signs and symptoms were highly suggestive of the disease and complete remission was observed after the initiation of specific treatment. In addition, antimonate would not act on other diseases that are part of the differential diagnosis.

In conclusion, the diagnosis of asymptomatic infection by IIF, ELISA-rK39, and PCR used in this study did not contribute to the early identification of cases that would progress to classical VL nor did it reduce the time necessary for its diagnosis. In addition, the results of these tests frequently do not agree and positive results may be temporary.

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