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Short communication

Neospora caninum abortion in a Malayan tapir (*Tapirus indicus*)

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ABSTRACT

A captive 17-year old female Malayan tapir (*Tapirus indicus)* aborted a fetus with a crown rump length of 19 cm in early pregnancy. The fetus showed an early state of mummification. Histologically, a multifocal mononuclear encephalitis, myocarditis and periportal hepatitis was present indicating a possible protozoal cause of abortion. Although immunohistologically, *Neospora (N.) caninum* antigen could not be demonstrated, *N. caninum* DNA was detected by Polymerase Chain Reaction (PCR) in brain, heart, liver and lung of the fetus. *N. caninum* DNA was extracted from the aborted fetus and the microsatellite marker MS10 was amplified by PCR and sequenced. The obtained MS10 microsatellite pattern has not been described in Germany yet. Nevertheless, the MS10 pattern was very similar to those reported for *N. caninum* isolated from dogs and cattle in Germany. Because of the histological pattern and extent of the lesions, neosporosis was suspected as the cause of fetal death and abortion. This case report describes for the first time transplacental transmission of *N. caninum* and abortion due to neosporosis in a tapir.

1. Introduction

The Malayan tapir (*Tapirus indicus*) is the largest of the four extant tapir species (Baird's tapir, Lowland tapir, Mountain tapir und Malayan tapir) and the only extant old world species living in the rainforests of Southeast Asia. Phylogenetically, tapirs are an ancient group of herbivorous mammals. Due to habitat destruction and poaching the Malayan tapir is categorized as "endangered" on the IUCN red list of threatened animal species (Naveda et al., 2011). Together with horses and rhinoceroses, tapirs belong to the order of odd toed ungulates (Perissodactyla).

N. caninum is a globally distributed cyst-forming apicomplexan protozoan parasite closely related to *Toxoplasma gondii*. Definitive hosts are dogs (McAllister et al., 1998), coyotes (Gondim et al., 2004), dingoes (King et al., 2010) and gray wolves (Dubey et al., 2011). They excrete *N. caninum* oocysts within their feces. Natural intermediate hosts are several species of mammals, including ruminants, canines and horses (Dubey et al., 2007). They become infected either by ingestion of contaminated water or food or vertically by transplacental infection. In cattle, the transplacental route of infection is highly efficient resulting in fetal transmission rates as high as 95% (Donahoe et al., 2015). The parasite has emerged as a major pathogen for cattle, but also affects sheep and goats causing abortions, stillbirth and perinatal death (Dubey et al., 2007) *N. caninum* associated abortion and stillbirths are also reported in some wild and zoo ruminants (Donahoe et al., 2015) and transplacental transmission of *N. caninum* was very recently confirmed for the first time in moose (*Alces alces*) (Schlieben et al., 2017). In odd toed ungulates, *N. caninum* infections have hitherto only been reported in rhinoceroses (Sangster et al., 2010; Sommanustweechai et al., 2010; Williams et al., 2002) and horses. In a southern white rhinoceros it was identified as the cause of an abortion (Sangster et al., 2010). Horses can be infected by *N. caninum* and by another *Neospora* species, *N. hughesi*, which differs from *N. caninum* ultrastructurally, immunologically and on molecular base (Marsh et al., 1998). Whereas *N. caninum* in horses is associated with reproductive disorders like abortion and neonatal death (Dubey and Porterfield, 1990; Pitel et al., 2003), *N. hughesi* is the second most important cause of equine protozoal myeloencephalitis (EPM) next to *Sarcocystis neurona* (Reed et al., 2016).

Here we report for the first time a *N. caninum* associated abortion in another odd toed ungulate animal species, the Malayan tapir (*Tapirus indicus*).

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2. Material and methods

2.1. History

In Dortmund zoo, a 17-year-old Malayan tapir aborted a male fetus of 19 cm crown rump length (CRL) in November 2013. The female tapir was born in captivity in Oklahoma Zoo, kept at Munich Zoo before she came to Dortmund in 2000. She had three healthy calves previously in 2003, 2007, and 2009 before the abortion occurred. The cow and the bull are the only Malayan tapirs in Dortmund zoo and are living together as a breeding pair since the year 2000. The bull was aged 14 years when the abortion occurred. Both were accommodated in a rainforest house together with a group of Sumatran orangutans.

2.2. Necropsy and sampling

The fetus was submitted without placenta for necropsy to the Chemisches und Veterinäruntersuchungsamt Westfalen (CVUA), a North Rhine-Westfalian State Veterinary laboratory in Arnsberg, Germany in November 2013. A complete post mortem examination was performed and samples of brain, lung, heart, liver were fixed in 4% neutral buffered formalin for histological and immunohistological examination. Native samples of stomach content, lung and liver were taken for routine cultural bacteriological examination. Additionally, lung samples were used for PCRs to exclude infection with *Chlamydia* spp. (Kaltenböck et al., 1997) and *Coxiella burnettii* (Schrader et al., 2000). Samples of spleen, lung and brain were used to rule out infection with equine herpesvirus by direct immunofluorescence staining technique on frozen tissue sections. Brain, lung, heart and liver samples were taken to examine for DNA of *Toxoplasma (T.) gondii* and *N. caninum* by PCR.

Serum samples of the tapir cow and bull were provided by the zoo veterinarian in March and September 2014, respectively.

2.3. Histology and immunohistology

Formalin-fixed tissue samples were routinely processed for histology and embedded in paraffin wax. Sections were cut at 4 µm and stained with hematoxylin and eosin (H & E) for microscopic examination. The immunohistochemical examination was performed as described previously (Peters et al., 2000). Briefly, the tissue sections were deparaffinized. After incubation in isopropanol and 96% [v/v] ethanol, endogenous peroxidase was quenched with 0.5% [v/v] hydrogen peroxide in methanol for 30 min. After rehydration in 75% [v/v] and 50% [v/v] ethanol the slides were rinsed in phosphate buffered saline (PBS) and incubated with 0.05% [v/v] pronase E (Merck, Darmstadt, Germany) for 20 min at 37 °C. After another rinse in TRIS-PBS, sections were blocked in TRIS-PBS containing 20% [v/v] normal goat serum and were incubated (45 min, 37 °C) with a rabbit antiserum (1:500 in PBS) developed against *N. caninum* (NC-1) tachyzoites (Schares et al., 1997). Antibodies were detected using the avidin-biotin-peroxidase complex method with a commercial kit (Vectastain ELITE ABC Kit, Vector Laboratories, USA) as described by the manufacturer. 3,3-diaminobenzidine tetrahydrochloride (0.05 mg/ml in 0.05 M TRIS PBS, pH 7,6) and 0.03% [v/v] hydroxide peroxide were used for color development. The enzyme reaction was stopped after 5 min by thorough rinsing in tap water. The sections were counterstained with hematoxylin, dehydrated and mounted. Substitution of the primary antibody by normal rabbit antiserum was used as negative control.

2.4. Toxoplasma gondii and Neospora caninum PCR

To detect *T. gondii* and *N. caninum* DNA, conventional end-point PCRs were performed as described (Legnani et al., 2016; Schares et al., 2011). PCR primers (Toxoplasma: Tox5/Tox-8 (Homan et al., 2000; Reischl et al., 2003); Neospora: Np6 + /Np21 + (Müller et al., 1996)) were used at a final concentration of 0.5 µM and dNTPs at 250 µM each (Amersham Biosciences, Piscataway, USA). DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland) was added at 1 U/25 µl with the provided buffer. The reaction mix was supplemented with bovine serum albumin at a concentration of 20 µg/ml. Water PCR Reagent (Sigma-Aldrich, Taufkirchen, Germany) served as a negative control. The reactions were performed in a thermal cycler (Eppendorf Mastercycler, Personal Thermal Cycler, Hannover, Germany). Amplification products were visualized after electrophoresis in 1.5% agarose gels stained with ethidium bromide. A 100 bp DNA ladder (Invitrogen GmbH, Karlsruhe, Germany) served as a size standard.

2.5. Immunoblot (IB) analysis and Indirect Fluorescent Antibody Tests (IFAT) analysis

The NC-1 strain of *N. caninum* (Dubey et al., 1988) and RH strain of *T. gondii* (Sabin, 1941) were maintained in cell cultures and purified as previously described (Schares et al., 1998, 1999). Cell-culture-derived tachyzoites were frozen as a pellet at −80 °C until used for immunoblot. Pellets of 8×10^7 tachyzoites of *N. caninum* or 2×10^8 *T. gondii* were incubated in non-reducing sample buffer (2% [w/v] sodium dodecyl sulfate [SDS], 10% [v/v] glycerol, 62 mM TrisHCl, pH 6.8) for 1 min (94 °C), separated in 12% [w/v] SDS polyacrylamide minigels of 60 × 70 × 1 mm size, and transferred to PVDF membranes (Immobilon-P, Merck-Millipore GmbH, Germany). After the transfer, membranes were blocked using PBS-TG (PBS with 0.05% [v/v] Tween 20 (Sigma, Germany) and 2% [v/v] liquid fish gelatine [Serva, Germany]) and cut into 50 stripes and examined as described below. To detect antibodies against *N. caninum* or *T. gondii* tachyzoite antigens, the incubation of stripes was performed as previously described (Schares et al., 1998), with a few modifications. Sera were diluted 1:100 in PBS-TG. Serum of an experimentally *N. caninum*-infected cow (Schares et al., 1999) was used as a *N. caninum* positive control. A serum of an experimentally *T. gondii*-infected pig (Azevedo et al., 2010) was used as a *T. gondii* positive control. As negative controls preinfection sera of cattle and pigs were applied. Stripes incubated with tapir-serum were examined using an anti-horse IgG H/L peroxidase conjugate (Dianova, Germany), 1:500 diluted in PBS-TG, while stripes with bovine or pig sera were analysed with an anti-bovine IgG H/L or anti-pig IgG H/L peroxidase conjugate (Dianova, Germany), both 1:500 diluted in PBS-TG.

For IFAT ten µl of a suspension of cell culture-derived *N. caninum* or *T. gondii* tachyzoites (5 × 106 ml − 1) in PBS were used to sensitize IFAT slide wells. Slides were air-dried and stored frozen at −20 °C until use. The slides were fixed with ice-cold acetone for 10 min and then incubated in PBS for 10 min. Moose serum was titrated in PBS in 2-fold steps starting at a dilution of 1:25. The test was performed as described (Schares et al., 1998) and rabbit anti-horse IgG (whole molecule) coupled to FITC (Dianova, Hamburg, Germany) diluted 1:50 in PBS with 0.2% Evans Blue was used to detect the primary antibodies. The slides were examined using a Nikon Eclipse Ti microscope (Nikon GmbH, Düsseldorf, Germany). Only complete peripheral fluorescence of the tachyzoite was considered specific. A titre of 1:100 was defined as the positive cut-off titre. The same sera as described for IB were used as positive or negative controls in IFAT and detected with rabbit anti-bovine or anti-pig IgG (whole molecule) coupled to FITC (Dianova, Hamburg, Germany) diluted 1:50 in PBS with 0.2% Evans Blue, respectively.

2.6. Microsatellite analysis

To further characterize the *N. caninum* DNA, positive samples were subjected to microsatellite analysis. For nested-PCR amplification of the *N. caninum* microsatellites (MS) MS1B, 2, 3, 5, 6A, 6B, 10, 12 and 21 and amplicon sequencing previously described methods were used (Basso et al., 2009a,b, 2010).

Fig. 1. Aborted Malayan tapir fetus in an early stage of mummification.

3. Results

3.1. Macroscopical, histological and immunohistological findings

The tapir fetus (CRL 19 cm) weighed 530 g, and revealed an early stage of mummification (Fig. 1). Parts of the small intestine protruded through an umbilical opening of the abdominal wall. No other macroscopic findings were found at necropsy.

Histological examination of the brain revealed multifocal mild encephalitis with perivascular cuffs, few glial nodules (Fig. 2A, B) and a mild lymphocytic meningitis. There was a severe multifocal mononuclear myocarditis (Fig. 2C). Despite severe autolysis a periportal mononuclear hepatitis was recognizable. Protozoal stages could neither be detected in H & E stained sections nor immunohistologically.

3.2. Bacteriological and virological findings

Bacteriological examination of stomach content, lung and liver revealed an unspecific bacterial flora. *Chlamydia* spp. and *Coxiella burnettii* were not detected by PCRs. Equine herpes virus 1 (EHV1) antigen could not be detected in frozen tissue sections of the fetus by immunofluorescence.

3.3. Neospora caninum and Toxoplasma gondii PCR

By PCR using primer Np6+ and Np21+ a band at 328 base pairs specific for *N. caninum* was detected in brain, liver, lung and heart. *T. gondii* PCR with primer Tox5 and Tox-8 failed to produce a specific nucleotide product.

3.4. IB and IFAT analysis

Sera of the tapir cow with abortion and the tapir bull showed a *N. caninum* specific banding pattern identical to that of experimentally infected cattle (Fig. 3). The *N. caninum* IFAT titer of the cow was 1:1600 and the bull showed an IFAT titer of 1:800. When tested with *T. gondii* antigen, no reactions were observed in IB and in IFAT only the bull showed a *T. gondii* titer of 1:50 while the tapir cow remained negativ.

3.5. Microsatellite typing

Out of 9 markers, only the MS1B, MS2 and the MS10 marker (Regidor et al., 2006) could be amplified from DNA extracted from the aborted tapir fetus and subsequently sequenced. The pattern in MS1 B

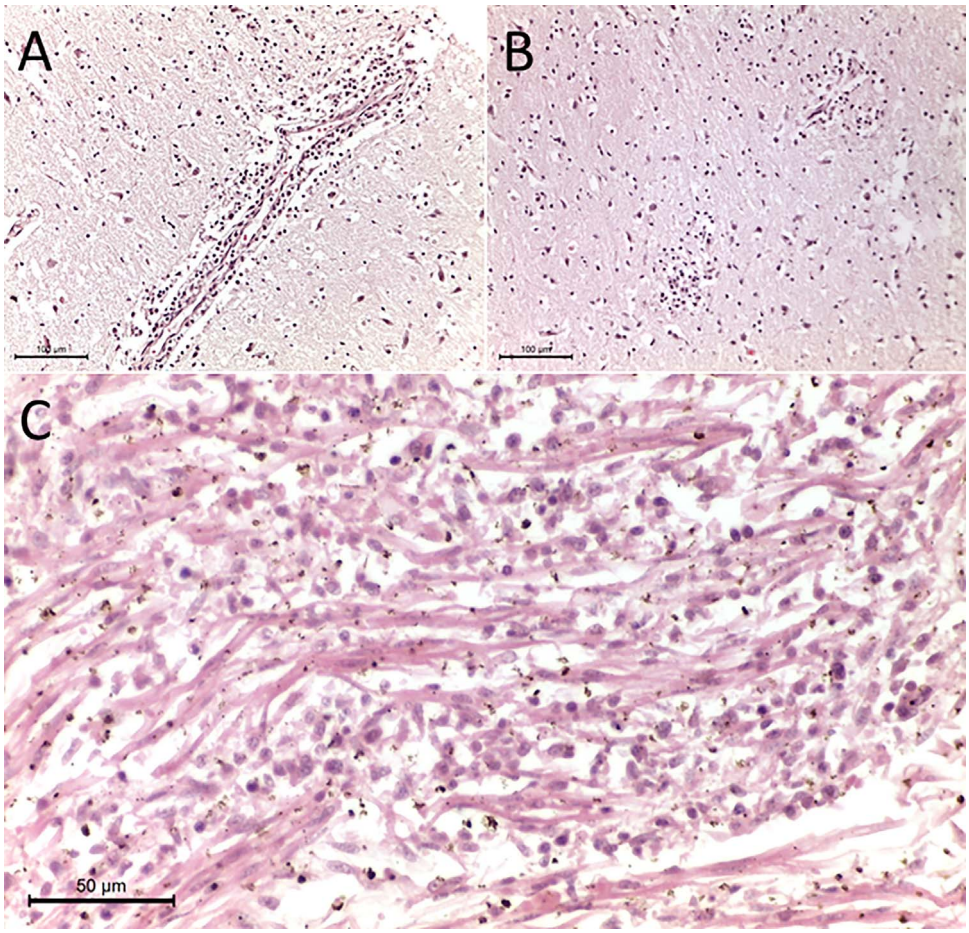

Fig. 2. Pathohistological findings. Lymphocytic perivascular infiltration (A) and multifocal glial nodules (B) in brain tissue. Severe non purulent myocarditis in the autolytic myocardium (C).

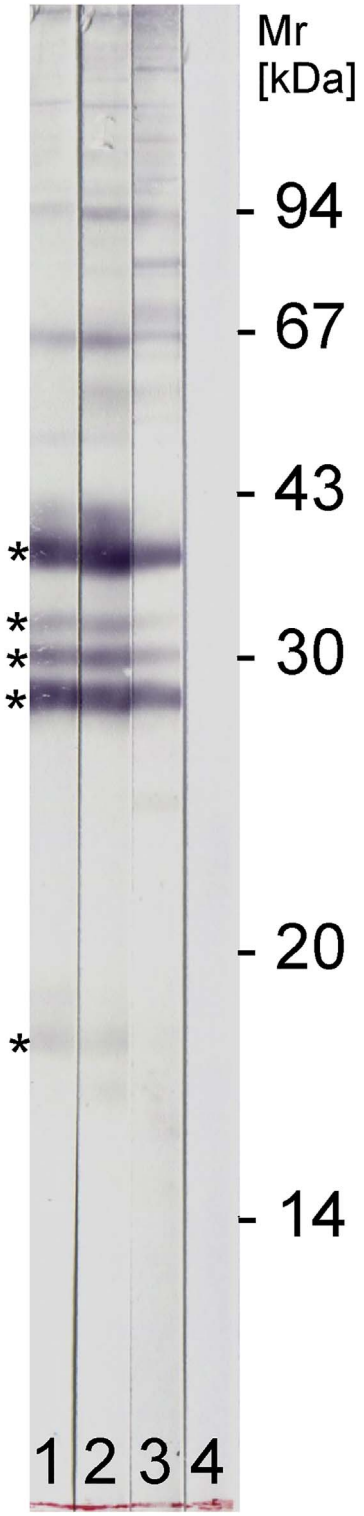

Fig. 3. *N. caninum* specific antibody response in the Malayan tapir cow with abortion and in a non-related tapir bull. (1) serum tapir cow, (2) serum tapir bull, (3) serum bovine positive control, (4) serum bovine negative control. *, Immunodominant antigens with relative molecular masses of 17–19, 29, 30, 33, 37 kDa are regarded as specific.

was AC_{13} and in MS2 $(AT)_6$-TTGTATC-$(AT)_{10}$-GT-$(AT)_2$. These patterns have been previously described for aborted fetuses in cattle herds and in *N. caninum* oocysts isolated from dogs in Germany (Basso et al., 2009a,b, 2010). The pattern of MS10 was $(ACT)_6$-$(AGA)_{14}$-$(TGA)_9$. This pattern is not described for Germany yet but it is very similar to those reported for *N. caninum* isolated from dog and cattle in Germany (Basso et al., 2010). Only small differences between the MS10 pattern of tapir *N. caninum* and others were observed, when it was compared to MS10 pattern of *N. caninum* observed in a dog (oocyst isolate Nc-GER6: $(ACT)_7$- $(AGA)_{17}$-$(TGA)_8$; Basso et al. (2010)) and in aborted bovine fetuses (in bovine Herd 5: $(ACT)_6$- $(AGA)_{15}$-$(TGA)_8$; Basso et al. (2010)).

4. Discussion

Here we report for the first time transplacental transmission of *N. caninum* in a Malayan tapir. In addition, our results strongly suggest that *N. caninum* was the most likely cause of abortion. Although the fetus was already in an early stage of mummification, characteristic histological lesions indicative for a protozoal abortion were still detectable. Immunohistochemical staining failed to detect protozoa, but *N. caninum* infection was confirmed by a positive PCR reaction in brain, heart, lung and liver. In contrast, *T. gondii* DNA could not be demonstrated by PCR. The failure to detect *N. caninum* by immunohistochemistry in fetal tissues was most likely due to the poor state of tissue conservation and/or low numbers of protozoa in the tissue. Infection of the fetus with *N. caninum* must have occurred transplacentally as is proven in other intermediate hosts of *N. caninum*. The tapir cow was seropositive for *N. caninum* with an immunoblot binding pattern resembling that of experimentally infected cattle and with a relative high IFAT titer of 1:1600 (Dubey and Schares, 2006). Unfortunately, fetal serum as well as placenta tissue was not available for further serological, respectively histological examination.

The gestational length of the Malayan tapir is 390–410 days (Fahey, 1999), after which a single offspring, weighing around 15 pounds (6.8 kg) is born. In the reported case the date of service was not known. The weight of premature tapir fetus was only 530 g and its CRL 19 cm. Ultrasonographic measurement of the fetal growth in a captive Malayan Tapir over three successive pregnancies revealed CRLs of approximately 13,5 cm at day 135 (week 19) of pregnancy (Hoyer and van Engeldorp Gastellaars, 2014). After day 135 the CRLs of the fetuses could not be measured accurately by ultrasound as the CLRs became too large for the field of view. In our case we estimate the age of the fetus before abortion to be approximately 5–6 months. Mummification of the fetus confirmed that it had died in the absence of bacteria some time before it was expelled. Otherwise a macerated fetus would have been expected. Potentially abortifacient bacteria and pathogens were not isolated from or detected in fetal tissues.

Not only the tapir cow, but also the unrelated tapir bull was serologically positive for *N. caninum* with an identical specific immunoblot pattern. It is unclear when and how the animals, which were kept in captivity all their lives, became infected. The animals were kept in an enclosure only together with Sumatran orangutans. Natural infections of humans and non-human primates with *N. caninum* have hitherto not been reported (Donahoe et al., 2015). In Dortmund zoo visitors are not allowed to take their dogs with them when visiting the zoo. Therefore, contact with feces of dogs as definitive hosts for *N. caninum* within the park is very unlikely. Other known definitive hosts for *N. caninum* as dingoes, coyotes and gray wolves are not kept by the zoo. Tapirs were fed with grass and hay harvested from a meadow in urban location frequently haunted by dogs. Infection via oocyst-contaminated food therefore seems the most likely route of infection. Transplacental infection of the tapir cow acquired from her own mother cannot be ruled out as well. Unfortunately, stored blood samples of the cow and her former offspring were not available for further serological examinations. Therefore, the time of infection of the cow and the bull as well remains unclear.

Microsatellite typing of the *N. caninum* DNA extracted form fetal tissues was used to further characterize the *N. caninum* strain in the aborted tapir. Only three of the 9 markers usually used, i.e. MS1B, MS2 and the MS10 were amplified from DNA extracted from the aborted tapir fetus and were subsequently sequenced. This might be due to the low DNA concentration in the tissue of the fetus and is a common

problem in microsatellite typing from clinical cases (Regidor-Cerrillo et al., 2006; Pedraza-Diaz et al., 2009; Basso et al., 2010). Nevertheless, the resulting MS10 pattern $(ACT)_6$ $(AGA)_{14}$ $(TGA)_9$ is not described for Germany yet, although it is similar to those reported for *N. caninum* isolated from a dog (oocyst isolate Nc-GER6) and in aborted bovine fetuses (bovine Herd 5) (Basso et al., 2010), both also isolated in the same German Federal State Dortmund zoo is located. This seems to support the hypothesis that animals became infected through oocyst contamination in grass or hay collected on local meadows. On the other hand, microsatellite typing using MS10 marker had also been used to characterize *N. caninum* in another aborted odd toed ungulate, a southern white rhinoceros (*Ceratotherium simum simum*) from an Australian zoo. The rhinoceros dam was wild caught in South Africa and had been transferred to Australia. Analysis revealed a different, but also similar MS10 pattern $(ATC)_6$ $(AGA)_{19}$ $(TGA)_8$ (Sangster et al., 2010). The MS2 pattern has been previously described for aborted fetuses in cattle herds and in *N. caninum* oocysts isolated from dogs in Germany (e.g. oocyst isolate Nc-GER6; Basso et al. (2010)) but also in other parts of the world, including e.g. Spain (Pereira Garcia-Melo et al., 2010), Portugal (Basso et al., 2009a), Korea, Brazil (Regidor-Cerrillo et al., 2006) and Argentina (Campero et al., 2015).

In conclusion, *N. caninum* is able to use Malayan tapirs as intermediate hosts and can be transplacentally transmitted to their offspring possibly resulting in abortion.

Conflict of interest

The authors report no conflict of interest.

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