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Trop Anim Health Prod
DOI 10.1007/s11250-017-1332-6

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REGULAR ARTICLES

Epidemiology behavior of leptospirosis in Ciénaga de Oro, Córdoba (Colombia)

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Received: 15 March 2017 / Accepted: 31 May 2017
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Abstract The purpose of this study was to determine the epidemiology of leptospirosis in rural areas of Ciénaga de Oro, Córdoba, Colombia, a convenience sampling was carried out on 13 farms. The sample size was 325 reproductive age cows, 11 canine samples, and 20 humans. The samples were subjected to MAT analysis with 11 serogroups of *Leptospira interrogans* sensu *lato*. Once the MAT results were received, urine samples were collected from 78 cows, along with 39 water samples, for bacteriological cultures and PCR for the 16S rRNA gene in *L. interrogans* sensu *lato*. Positive PCR samples were sequenced to determine the possible genome species. The leptospirosis seroprevalence was 74.5% in the cattle, 70.0% in the dogs, and 45.5% in the humans. Although isolation was not achieved, *L. interrogans* sensu *lato* was detected by PCR in three urine samples and in a sample of wastewater. The sequencing confirmed the circulation of pathogenic species. The high prevalence of antibodies for *L. interrogans* sensu *lato* and the molecular evidence led to the inference that the rural areas of Ciénaga de Oro are endemic and that cattle can act as renal carriers and contaminate water sources, which increases the risk of contracting leptospirosis.

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Keywords *Leptospira interrogans* · Serovar · Public health · Zoonoses

Introduction

Leptospirosis is a zoonotic disease that infects humans and domestic and wild mammals around the world. This disease has global distribution and potentially lethal effects on humans (Ayral et al. 2014). The infection is caused by leptospires within the pathogenic complex *Leptospira interrogans* sensu *lato* (Adler and Peña 2010).

Humans can become infected with any pathogenic serovar of *Leptospira* spp. through contact with fluids from infected animal or the environment (Goris et al. 2013). Soil (Saito et al. 2013), and surface waters (Hochedez et al. 2013) contaminated with chronically infected water remain an important source of human leptospirosis transmission worldwide.

Infected cattle are generally asymptomatic and can disseminate the bacterium through urine (leptospiruria) for long periods (Salgado et al. 2014). Economic losses are represented by reproductive failures, abortions, births of weak calves, and decreased milk production. Some cattle with chronic infections act as reservoirs for other cattle and other species, such as infections caused by Hardjo serovar resulting in acute disease in humans or dogs, producing acute disease (Ellis 1984; Adler and Peña 2010; Yoo 2010; Fonzar and Langoni 2012; Tilahun et al. 2013; Mwachui et al. 2015).

The municipality of Ciénaga de Oro, Córdoba (Colombia), has characteristics and conditions that favor the presence, multiplication, and infection by bacteria from the genus *Leptospira* in water sources, animals, and man, such as temperature (27 °C), a predominantly tropical humidity, and presence of mammals that can serve as a source of infection. The objective of this study was to determine the epidemiological

Published online: 28 June 2017

behavior of leptospirosis in the rural areas of Ciénaga de Oro, Córdoba (Colombia).

Materials and methods

Study zone This study was carried out on livestock farms with a dual-purpose system, located in the municipality of Ciénaga de Oro, department of Córdoba (Colombia), located at 8° 52′ 44″ N and 75° 42′ 8″ 0, in the Colombian Caribbean zone.

Type of study and sampling A cross-sectional descriptive study was performed. The sample size was 325 cows (Fedegan 2014). To determine the number of cattle studied per farm, the British Cattle Veterinary Association (BCVA) (1992) was followed, with 25 animals per farm. Twenty workers participated in the zootechnical management of the cows and 11 dogs belonging to these farms. On each of the evaluated farms, three samples of water were taken: untreated water, drinking water, and wastewater.

Blood sampling and serological tests In the cattle, 10 mL of whole blood was taken by puncturing the jugular or coccygeal vein. In the dogs, sedation and immobilization (acetyl promazine) were done, and asepsis was performed with venipuncture of the jugular vein, extracting 10 mL of blood. In humans, 10 mL of blood was extracted, after disinfecting the radial, ulnar, and/or median vein. All samples were transported under refrigeration.

The blood samples obtained were centrifuged at 3000 rpm for 5 min to obtain the blood serum, which was deposited in labeled cryovials and stored at −70 °C until the serological tests were performed. Antibodies against *L. interrogans* sensu *lato* were detected using the microagglutination test (MAT), according to the specifications of the International Office of Epizootics (WHO 2003). Eleven (11) serogroups of *L. interrogans* sensu *lato* were used: Icterohaemorrhagiae (serovar Icterohaemorrhagiae and Ccopenhageni), Autumnalis (serovar Autumnalis), Grippotyphosa (serovar Grippotyphosa), Sejroe (serovars Hardjo, Sejroe and Saxkoebing), Pomona (serovar Pomona), Australis (Serovar Bratislava and Australis), Tarassovi (serovar Tarassovi), Canicola (serovar Canicola), Pyrogens (serovar Zanoni), Batavie (serovar Batavia e), Celledoni (serovar Celledoni), and Louisiana (serovar Louisiana). A sample was considered positive when the serum agglutinated at least 50% of the concentration of the leptospires used as antigens at a dilution equal to or greater than 1: 100.

Collection of urine samples in the cattle for bacteriological culture and PCR Once the MAT results were obtained, six cows per farm were selected: one that was negative; two with titers between 100 and 400; and three with titers over 400. Furosemide was administered intravenously and the urine samples were collected after disinfecting the perianal area, waiting until the cows urinated to collect 30 mL of the sample.

Water sampling for bacteriological culture and PCR Three samples of water were taken on each cattle farm. The first sample corresponded to unused water such as water sources, ravines, wells, and storage tanks; the second sample came from animal drinkers; and the third sample was taken from wastewater. The sample volume was 15 mL. The urine and water samples were transported at room temperature and processed for cultures within two hours.

Bacteriological cultures Water and urine samples were seeded in liquid and semisolid EMJH media, enriched with 1% rabbit serum, and supplemented with or without nalidixic acid and 5-fluorouracil. A portion of the sample (0.5 mL) was seeded directly into the liquid and semisolid media, and another fraction (2 mL) was membrane-filtered with 0.45-μm pores and 0.5 mL was inoculated into the liquid and semisolid media. All of the cultures were incubated between 29 and 30 °C and monitored biweekly for 4 months.

Extraction of DNA The urine and water samples were centrifuged at 14000 rpm for 5 min, and the sediment obtained from each sample was washed with PBS. The extraction of the genetic material was done with a commercial kit (ID: 51306). The DNA was stored at −20 °C. The detection of pathogenic leptospires by PCR was performed using PFA and PRA primers (Fearnley et al. 2008) that delineated a 357-bp fragment of the 16S ribosomal RNA (rRNA) gene from the pathogenic leptospires. The final microliter concentration of each of the components of the PCR reaction mixture was 1× PCR buffer, 1.25 mM dNTPs, 2 mM $MgCl_2$, 1 μM primers, Taq DNA Pol 0.4 U, and 1 μl DNA, in a final reaction volume of 25 μl. The thermal profile was 94 °C for 5 min, 34 cycles consisting of 94 °C for 3 min, 64 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min.

Confirmation of PCR products with sequencing The fragments obtained from the amplification of the ribosomal gene, encoding the 16S rRNA subunit (357pb), were sequenced in both directions using fluorescence-tagged chain terminators in a 3730xl capillary sequencer, Applied Biosystems 3730xl DNA Analyzer. The sequences were compared with sequences deposited in GenBank using the local alignment search tool (BLAST). The phylogenetic reconstructions were done using methods based on distances (maximum probability). These analyses were performed with the MEGA program, version 6 (Tamura et al. 2013). As an external group, the sequence of *Turneriella parva*, Parva DB61 strain (accession no. JQ988843), was used. In addition, based on the evolution model, the genetic distances were calculated between

the individuals of a species and between the species to obtain reference values for confirming the phylogenetic relationships observed between the taxa.

Ethical aspects The study was considered low risk by the Ethics Committee of the Instituto de Investigaciones Biológicas del Trópico (IIBT) and the Facultad de Medicina Veterinaria Zootecnia of the Universidad de Córdoba. The procedures for sample collection, management, and conservation were based on the standards of good laboratory practice and ethical, technical, and scientific standards, following Law 84 (Congreso de Colombia 1989). Volunteers were invited to participate, and, after reading and signing the written informed consent, blood samples were taken by a bacteriologist. Resolution 00 8430/93 (Ministerio de Salud, República de Colombia) was followed. Throughout the study, confidentiality of all information was maintained and the results were only reported to the people involved in the study.

Results

Of the cows, 74.5% (242/325) were seropositive. Seroreactivity was found in the 11 serogroups of the evaluated panel. Table 1 shows the distribution in the different evaluated species by serogroups.

Of the cows, 25.5% (83/325) did not present any titers for the 16 evaluated serovars; 10.8% (35/325) had titers up to 100, 37.3% (121/325) between 200 and 400, 13.8% between 400 and 800 titers, and 12.6% (41/325) had titers ≥800 titers. None of the cows presented clinical indications; they were apparently healthy on the day of sampling. In the dogs, 54.5% (6/11) did not present any titers for the 16 evaluated serovars, 18.2% (2/11) had titers between 200 and 400 and 27.3% (3/11) had titers ≥1: 800 titers. There were no clinical indications of the disease. In the evaluated humans, no serovars were seen in 30% (6/20), 5% (1/20) had titers of 100, 40% (8/20) between 200 and 400 titers, 15% (3/20) had between 400 and 800, and 10% (2/20) had titers ≥800. The correlation between molecular and serological evidence of *L. interrogans* sensu *lato,* is show in Table 2.

In the 78 cultured urine samples and 39 evaluated water samples, there was no growth of pathogenic leptospires during the 4 months of follow-up. However, *L. interrogans* sensu *lato* was seen in three urine samples and one wastewater from farm 2 (labeled A76), using PCR.

Four 356-base sequences were obtained, which were deposited in GenBank using codes BiCCC0H2-33, BiCCC0H4-76, BiCCC0H4-76, and EWCCC0H2-W76 (Fig. 1). The BLAST analysis showed that the sequences of 356bp obtained in this study were homologous to the sequences of the gene coding for the 16s RNA subunit, located between positions 2.672,328 and 2.797,1972 of chromosome 1 of *L. borgpetersenii* serovar Hardjo strain NVSL S818, with a coverage value of 100%, 99% identity, and an E-value of 2e −180. In the alignment of the compared species, 149 variable sites were observed from the 341 analyzed sites, including 117 parsimoniously informative sites (data not shown). It was determined that the evolution model that best explained the polymorphism observed in the alignment was the Kimura 2-

Table 1 Percentage distribution of serogroups of *L. interrogans* sensu *lato* among the different species in Ciénaga de Oro, Córdoba (Colombia)

Serogroup/species	Cows (%)	Dogs (%)	Humans (%)
Hardjo	58.2	0.0	0.0
Saxkoebing	38.4	0.0	0.0
Tarassovi	36.8	0.0	0.0
Grippotyphosa	30.2	80	71
Bratislava	18.6	40	57
Pomona	14.9	40	57
Serjoe	7.6	0.0	0.0
Celledoni	7.4	0.0	0.0
Zanoni	6.2	0.0	14
Batavie	4.9	0.0	42
Luisiana	4.1	0.0	0.0
Autumnalis	4.1	80	7.0
Cynoptery	1.7	0.0	0.0
Canicola	1.7	20	0.0
Icterohaemorrhagiae	0.4	20	0.0

Table 2 Correlation between molecular and serological evidence of *L. interrogans* sensu *lato* in cattle herds in Ciénaga de Oro (Córdoba)

Farm	Specie	Serovars	Titers
2	Bovine (#33)[a]	Saxkoebing	3200
		Hardjo	1600
		Grippotyphosa	200
		Celledoni	200
		Cynopteri	100
	Human (woman)	Grippotyphosa	800
		Bratislava	400
		Pomona	100
	Dog	Pomona	3200
		Grippothyphosa	800
	Water (A76-wasterwater)[a]	Autumnalis	800
4	Bovine (#76)[a]	Pomona	100
	Human	Negative	
	Dogs	Autumnalis	100
		Grippothyphosa	200
7	Bovine (#162)[a]	Negative	
	Human	Negative	
	Dog	Negative	

[a] Molecular evidence

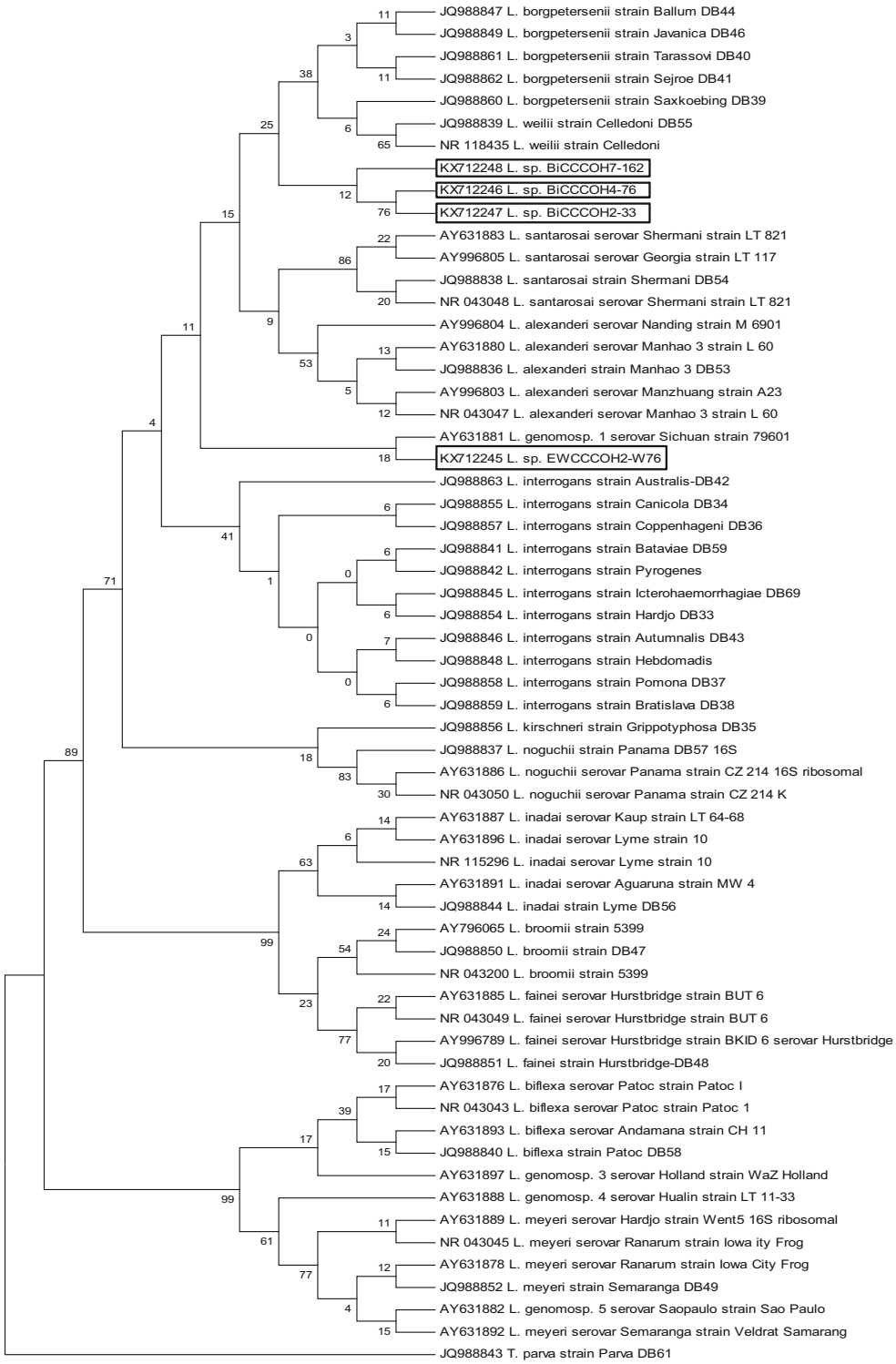

Fig. 1 Phylogenetic analysis of the bacteria of the genus *Leptospira* based on the gene sequence 16s RNA by the method of maximum probability

parameter model, and the phylogenetic reconstructions were visualized using methods based on distances (maximum probability) (Fig. 1).

It is worth noting that, although this analysis did not determine the genome of the *Leptospira* species because the low support of the branch in the clades that grouped the individuals of each species and individuals of some species grouped in different clades. However, it was found that the sequences BiCCC0H2-33, BiCCC0H4-76, and BiCCC0H4-162 are closely related to each other and to the clade that grouped *L. borgpetersenii*/*L. weilii*; on the other hand, sample EWCCC0H2-W76 grouped with *Leptospira* genomosp., one

serovar Sichuan strain 79601 16S (AY631881), and, although the support was low, the strains obtained in the urine and water samples from Ciénaga de Oro were pathogenic species of the genus *Leptospira*. Further studies are needed to establish the identity of the genome for the species found in this study.

Discussion

The prevalence of antibodies against *Leptospira* spp. was determined to be 74.5% in the cows in Ciénaga de Oro (Cordoba). This seroprevalence is high compared to that determined in recent studies: 41% in Córdoba (Betancur et al. 2013), 60.9% in Don Matias, Antioquia (Ochoa et al. 2000), and 16.4% in Pereira (Zuluaga 2009). The higher seroprevalence in the current study may be due to the higher number of serogroups/serovars included in the antigen battery used in the MAT, geo-climatic conditions of the area such as high precipitation and relative humidity, preventive sanitary deficiencies, exposure to contaminated water sources, or the habit of sharing cattle and pork grazing areas, a very common situation in the Colombian Caribbean zone. This variable was not considered in the current study. In Córdoba, strains of *L. interrogans* sensu *lato* have been isolated from water sources (Calderón et al. 2014). Based on the information provided in the epidemiological survey, cows from these farms were not vaccinated against leptospirosis.

In the cows, the most prevalent serovar was Hardjo (58.7%), followed by Saxkoebing (38.4%), both belonging to the serogroup Sejroe: Tarassovi (36.8%) and Grippotyphosa (30.1%). In the coffee zone, it has been reported that the predominant serovar was Hardjo with 45.7% (Zuluaga 2009). In Colombia, it has been proposed that the most frequent serovars in cattle are Hardjo, Pomona, Canicola, and Grippotyphosa (Rodríguez 2000). These results are based on previous research that indicated the presence of Hardjo as the main serovar involved in bovine leptospirosis in Brazil (Pimenta et al. 2014), Chile (Salgado et al. 2014), Venezuela (Alfaro et al. 2004), and Mexico (Moles et al. 2002).

Only one cow was positive for the serovar Icterohaemorrhagiae, which is considered of great importance for public health and whose main reservoir is rodents (Faine et al. 1999). The seroreactivity for 15 of the 16 MAT serovars suggests the presence of cross-reactions or shared infectivity in the different serovars of leptospires because of the presence of common antigens.

The 20 sampled humans were permanent workers on the farms and performed different tasks in the bovine production systems, activities carried out mainly by men, leaving women to the domestic tasks. These workers were always in permanent contact with the cows and dogs; the risk of infection was increased by their possible direct and indirect contacts with the etiological agent and the lack of use of clothing and bioprotection, as evidenced in the current study.

Thirty percent (6/20) of the workers did not present titers against any of the evaluated serovars; 70% (14/20) had titers of 400 or more for one or more serovars, of which 6 had titers of 800 or more. The serovar Grippothyphosa was the highest: two individuals had titers of 1600, and one had a titer of 3200. The most frequent associations were Grippothyphosa/Bratislava. The epidemiological survey carried out on the workers did not show data of clinical symptomatology, leading to the conclusion that the disease is not easily diagnosed either clinically or microbiologically and that this is an endemic area.

In Cordoba, 75.8% seroprevalence was reported in workers on pig farms (Calderón et al. 2014) and 67.9% in displaced persons (Rodríguez et al. 2009); these seroprevalences were higher than in other areas of the country, for example, Valle del Cauca with 22.7% (Ferro et al. 2006), Antioquia with 13.3% (Agudelo-Flórez and Restrepo-Isaza 2007), 14.1% in a study on the epidemiological characterization of non-malarial febrile syndrome in three municipalities of Urabá Antioquia (Arroyave et al. 2013), 6% in the north of Tolima (Romero et al. 2010), and 25% in zoo workers in Pereira (Romero et al. 2011).

The presence of *L. interrogans sensu lato* was demonstrated using PCR in three cows, which were considered renal carriers. The finding of *L. interrogans sensu lato* in the urine samples suggested a chronic carrier status for these cows and that the transmission dynamics of *Leptospira* on the farms active. A study in Rio de Janeiro (Brazil) that tested urine using PCR showed that 33.6% were positive (Hamond et al. 2015); in Sri Lanka, the carrier status of pathogenic leptospires was determined by *flaB*-PCR to be 12.2% in bovines (Gamage et al. 2014).

L. interrogans sensu *lato* was observed in a water sample, wastewater from farm 2 (Table 2), where molecular evidence was observed in a cow and in humans, suggesting possible infection between the different species. In the Sinú medium (Córdoba), *L. interrogans* sensu *lato* was detected in two water samples using PCR. The first strain came from drinking water, and the second one came from wastewater (Calderón et al. 2014). Water sources have been implicated in outbreaks of leptospirosis (Hochedez et al. 2013), along with the presence of *Leptospira* spp. in water sources (Wynwood et al. 2014) and *Leptospira* spp. pathogens in freshwater sources (Andre-Fontaine et al. 2015).

The epidemiological survey revealed that the management of the water sources on the farms is mostly inadequate because the water deposits are generally in the open, exposing them to the urine of domestic, peridomestic, and wild animals and facilitating transmission to other animals and humans since these water sources are used for different domestic tasks.

The PCR results for the *L. interrogans* sensu *lato* in the three urine samples suggested the carrier status of these cows and also that the transmission dynamics of leptospirosis on these farms was active. It has been suggested that the highest incidence of bovine excretion of leptospires in urine occurs in calves and that most cows older than 3 years are not leptospiruric (Ellis 1983). This phenomenon could be due to the development of some immunity found in these animals for the different leptospira serovars to which they are exposed, especially those for which they are considered maintenance hosts.

For the *Leptospira* genus, although the definitive species could not be determined based on the 16S rRNA sequences available, it can be stated with certainty that the sequences BiCCC0H2-33, BiCCC0H4-76, BiCCC0H4-76, BiCCC0H4-162, and EWCCC0H2-W76 are closely related to the pathogenic species of the *Leptospira* genus, which demonstrates the importance of considering cattle and water sources as reservoirs of pathogenic leptospires in the study area.

Conclusions

The high presence of antibody titers against serovars of *Leptospira interrogans* sensu *lato* and the molecular evidence in four urine samples and a water sample demonstrated the endemicity of the disease and the transmissibility that exists between the different animal species, the humans, and the environment. Although the sequencing of the PCR products revealed a high similarity with the clade that grouped *L. borgpetersenii*/*L. weilii* and *Leptospira* genomosp. 1, further studies are needed to better determine the species genome and serovars.

Acknowledgments We thank Cristopher Stacey for the translation and Ben Adler, for the reading, correction, and suggestions.

Compliance with ethical standards

Conflicts of interest The authors declare they have no conflicts of interest.

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