Serological Studies and Isolations of Serotype *hardjo* and *Leptospira biflexa* Strains from Horses of Argentina

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Received for publication 6 January 1976

Three pathogenic leptospires and 12 saprophytic *Leptospira biflexa* strains were isolated from 72 apparently normal horse kidneys collected at an abattoir in Argentina. Cross-agglutination reaction patterns of the pathogens showed that they were antigenically homologous with members of the Hebdomadis group. When one of the strains was compared to Hebdomadis serotypes in reciprocal agglutination-absorption tests, it was found to be serologically homologous to serotype *hardjo*. This is the first known report of an isolation of this serotype from horses. Serological tests were also carried out on randomly collected abattoir sera from 245 horses to determine the prevalence of equine leptospirosis. Significant antibody titers (1:100 or greater) were found in 74.6% of the sera. Predominant reactions occurred with the antigens *pomona*, *hebdomadis* group, *pyrogenes*, *tarassovi*, and *canicola*. Agglutination tests performed with antigen prepared with one of the saprophytic *biflexa* isolates showed seropositive reactions in 99.1% of the equine sera, with agglutination titers ranging from 1:100 to 1:3,200. Absorption of selected horse sera with the saprophytic strain removed the agglutinins to *Leptospira interrogans* serotypes. This suggests the possibility that *L. biflexa* strains may act as an antigenic stimulus and account for some of the persistent multiple cross-reaction patterns of equine sera with pathogenic serotypes.

Equine leptospirosis is a disease of considerable importance to countries where horses are still used as a major source of labor and transportation, especially in the less mechanized nations where animal husbandry and agriculture are widely practiced. The breeding and raising of horses is also increasing in economic importance because of their greater demand for pleasure use and in racing.

Comparatively little has been reported in the literature concerning leptospiral isolations and their role in the etiology of equine disease. Available world-wide data show that only 11 *Leptospira* isolations from natural equine infections have been reported (4, 20). Evidence of infection in most other cases has generally depended upon serological tests and the demonstration of clinical signs of ocular involvement (6, 7, 12, 17).

The results of numerous serological studies suggest a diversity of *Leptospira interrogans* serotype agglutinins occurring in horses. The actual significance of the positive serological findings has not been clearly understood because concurrent attempts at isolation of leptospires were for the most part unsuccessful. The predominant serotypes in horses, based upon

serological surveys, are *pomona*, *canicola*, *gripotyphosa*, and *icterohaemorrhagiae* (1).

Horses may acquire disease from infectious urine of rodents, dogs, pigs, cattle, or other farm animals. Existing evidence indicates that the horse can excrete viable leptospires in the urine for 14 weeks or more (17). This prolonged leptospiuria may permit this animal species to serve as an important reservoir for transmitting infection to either animals or man.

The purpose of the present study was to reexamine serologically the prevalence of leptospirosis in a randomly selected equine population of Argentina and to report on the isolation of 15 strains of leptospires from equine renal tissues collected at an abattoir.

MATERIALS AND METHODS

The horses used in this study were apparently normal animals, randomly selected, 13 years of age or older, and slaughtered at the Frigorifico Lamar, Mercedes, Province of Buenos Aires, which is engaged in horse meat export. The kidneys and blood samples were obtained through the collaboration of the abattoir. The animals were received at the abattoir from sources throughout Argentina.

One entire kidney was collected from each of 72 horses in groups of 10 or 11 during seven visits to the

abattoir between October 1974 and April 1975. Simultaneously, 245 random blood samples were collected in groups of 25 or more at the time of each visit for serological testing.

Serological testing. Serological examinations of the sera were done by using the microscopic-agglutination (MA) test method as described by Galton et al. (5), using the following living *L. interrogans* serotypes grown in Stuart (Difco) liquid medium (19) containing 10% pooled normal rabbit serum as antigens: *pomona* strain Pomona, *australis* strain Ballico, *ballum* strain Castellon 3, *grippotyphosa* strain Moskva V, *tarassovi* strain Perepelicin, *hebdomadis* strain Hebdomadis, *bataviae* strain Van Tienen, *canicola* strain Hond Utrecht IV, *hardjo* strain Hardjoprajitno, *wolffi* strain 3705, *pyrogenes* strain Salinem, *sejroe* strain M84, *icterohaemorrhagiae* strain RGA, and *autumnalis* strain Akiyami A. The sera were diluted with Sorensen phosphate-buffered saline (pH 7.2) to give final dilutions of 1:50 and 1:100. Those sera showing 50% agglutination or more in the 1:100 dilution were further titrated in serial twofold dilutions to determine their end points. For all agglutination tests, equal volumes of 0.2 ml of standardized antigen suspension (5) and diluted serum were used. The highest twofold serum dilution showing 50% agglutination or more after 2 h of incubation at room temperature was considered the end-point titer.

Isolation procedures. Each kidney was removed immediately after slaughter, transported to the laboratory in individual sealed plastic bags, and processed for *Leptospira* isolation within 4 h. In the laboratory the kidneys were cleaned to remove the covering renal fat, immersed in a disinfectant solution (Hyamine, 1:1,000, Rohm & Hass Co., Philadelphia, Pa.) for 10 min to reduce surface contamination and dried with a sterile towel. A suspension of renal tissue was prepared from the inner surfaces by cutting each kidney in half longitudinally with a sterile knife and scraping the exposed cortex, medulla, and renal hilus with a sterile metal bottle cap which had previously been punched with holes to act as a scraper (15). Tenfold dilutions of the tissue suspensions were made in sterile phosphate-buffered saline and each 10^{-2} to 10^{-4} dilution (0.5 ml) was used to inoculate triplicate tubes containing 5.0 ml of Fletcher (Difco) semisolid medium (3) (with 10% pooled rabbit serum). The same dilutions of renal tissue were also used to inoculate triplicate tubes containing 5.0 ml of modified bovine albumin-polysorbate 80 (BA-P80) medium containing bovine fraction V albumin (Pentex) and prepared as described by Johnson et al. (8, 10).

To control contamination, one set of cultures contained the pyrimidine analogue 5-fluorouracil (Hoffman-LaRoche, Inc., Nutley, N.J.) at a final concentration of 200 μ g/ml (9), one set of culture tubes of Fletcher and BA-P80 media was implanted with a 5- or 30- μ g disk of neomycin (Difco), respectively (16), and the other set of culture tubes was used without antimicrobials. In addition, a 10^{-2} dilution of renal tissue from each kidney in the buffered saline was exposed to the combined action of both neomycin and furazolidone at a final concen-

tration of 25 μ g/ml each for 1 h before culture (13), after which the 10^{-3} and 10^{-4} dilutions were made in antimicrobial-free saline. Each dilution (0.5 ml) was then used to inoculate one tube each of Fletcher and BA-P80 semisolid media.

Cultures were incubated at 30 C and examined weekly for 8 weeks and once again at 12 weeks by dark-field microscopy to detect leptospiras. When growth was detected, successive transfers were made in the semisolid media until growth was sufficiently abundant for adaptation to the liquid media. Further subcultures into Stuart (19) or BA-P80 liquid medium were made until sufficient organisms were obtained for use as antigen. These antigens were then tested against a battery of hyperimmune antileptospiral rabbit sera for identification (5) or used in cross-agglutination-absorption tests and in serum absorption studies (11).

The relative resistance of the isolated leptospiras to the growth-inhibiting action of copper sulfate and 8-azaquinine (ICN Pharmaceuticals, Inc., Cleveland, Ohio) (21) was also determined.

To determine if the equine *Leptospira* isolates would produce infection in laboratory animals, groups of four 21-day-old hamsters were inoculated intraperitoneally with 0.5 ml of cultures of each of the 15 isolates grown in BA-P80 semisolid medium. Blood was obtained 1, 3, 5, and 8 days later by cardiac puncture and one drop of blood was cultured in one tube each of Fletcher and BA-P80 semisolid media. Three weeks postinoculation all of the hamsters were sacrificed and the kidneys were removed aseptically. Kidneys were triturated and suspended in 9 parts of buffered saline, and then decimal dilutions were cultured in the same manner indicated above for the horse kidneys.

RESULTS

Significant antibody titers (1:100 or greater) were found in 183 (74.6%) of the sera from the 245 horses examined by serological tests. With relatively few exceptions, positive sera contained agglutinins against 2 to as many as 10 of the 14 screening antigens. Particularly noteworthy was the presence of cross-agglutinins for antigenically unrelated serotypes (Fig. 1). The distribution of the reactions according to the predominant leptospiral agglutinins obtained with the various antigens is shown in Table 1. The data show that serotypes *pomona*, *hebdomadis* group, *pyrogenes*, *tarassovi*, and *canicola* were the prevalent leptospiral agglutinins and accounted for 55% of the reactors.

Fifteen (20.8%) *Leptospira* isolations were made from the 72 randomly collected equine kidneys. All of the isolations were achieved by using the BA-P80 medium and no growth occurred in renal cultures made in Fletcher medium (Table 2). On the basis of cross-agglutination reactions against selected hyperimmune antisera representative of the 17 serogroups of pathogenic leptospiras and serogroups Semar-

FIG. 1. Rate of seropositive microscopic agglutination titers (1:100 or greater) to one or more *Leptospira* serotypes in 183 reactors among 245 equine sera of Argentina.

TABLE 1. Prevalence of predominant leptospiral agglutinins in 245 sera from apparently normal horses of Argentina

Antigen	Distribution of titers				Total positive	
	1:100 to 1:200	1:400 to 1:800	1:1,600 to 1:3,200	1:6,400 and higher	No.	%
<i>pomona</i> alone	2					
<i>pomona</i> plus 1 to 9 other antigens	1	14	12	15	44	17.9
<i>hebdomadis</i> group ^a alone	12	5	2			
<i>hebdomadis</i> group plus 1 to 8 other antigens	3	14	1	1	38	15.5
<i>pyrogenes</i> alone	4	2				
<i>pyrogenes</i> plus 1 to 6 other antigens		15	1		22	8.9
<i>tarassovi</i> alone	5	5				
<i>tarassovi</i> plus 1 to 7 other antigens	4	2		2	18	7.3
<i>canicola</i> alone	7	2	1			
<i>canicola</i> plus 1 to 5 other antigens		4			14	5.7
Other ^b	10	2	2		14	5.7
Miscellaneous ^c	22	10	1		33	13.4
Total	70	75	20	18	183	74.6
Percentage positive	28.5	30.6	8.1	7.3		

^a Hebdomadis group antigens: *hardjo*, *wolffi*, *sejroe*, *hebdomadis*.

^b Less than 3% reactions to any of the following: *ballum*, *autumnalis*, *australis*.

^c Multiple reactions to two or more antigens to the same titer.

anga and Andamana, it was established that three of the horse isolates (laboratory numbers 28, 31, and 38) were members of the *Hebdomadis* group. These three strains were slow growing, requiring 8 weeks of incubation before leptospire could be detected by dark-field examinations, and repeated transfers of the cultures into Fletcher semisolid media containing rabbit serum failed to grow. All three strains were easily recovered from experimentally infected hamsters either from blood cultures obtained 8 days postinoculation or from kidney cultures performed 3 weeks later. Again, growth occurred only in the albumin-base medium. Clinical signs of infection were not demonstrated in any of the animals.

Antigens and specific antisera for each of the three isolates were used in cross-agglutination tests against the 28 recognized serotypes of the *Hebdomadis* group. The cross-agglutination reaction patterns of horse isolate no. 28 with individual members of the group are shown in Table 3. Similar cross-reaction patterns were obtained with the other two isolates (no. 31 and 38). All three strains were found to be serologically identical on the basis of reciprocal agglutination studies conducted with strain no. 28 and the other two equine isolates. When the isolate no. 28 was compared to *Hebdomadis* serotypes in reciprocal agglutination-absorption tests, it was found to be serologically homologous to serotype *hardjo* on the basis of current taxonomic criteria (Table 4).

The other 12 *Leptospira* horse isolates shown in Table 5 demonstrated biological characteristics comparable with saprophytic strains of the species *L. biflexa*. These isolates readily grew on subculture in both Fletcher and Stuart media containing 10% rabbit serum and showed a greater resistance to copper sulfate and 8-aza-

TABLE 3. Results of cross-agglutination tests with *Leptospira* horse isolate no. 28 showing the antigenic relationship to serotypes of the *Hebdomadis* group

Antiserum	Agglutinin titer ^a with antigen		Antiserum isolate no. 28 against the reference strains
	Homologous	Isolate no. 28	
<i>hebdomadis</i> , <i>Hebdomadis</i>	102.4	0.2	0.2
<i>nona</i> , <i>Nona</i>	204.8	0.1	— ^b
<i>kambale</i> , <i>Kambale</i>	102.4	0.4	1.6
<i>kremastos</i> , <i>Kremastos</i>	25.6	3.2	0.8
<i>worsfoldi</i> , <i>Worsfold</i>	25.6	1.6	0.2
<i>jules</i> , <i>Jules</i>	51.2	0.8	0.2
<i>maru</i> , CZ 285B	25.6	0.4	0.2
<i>borincana</i> , HS-622	12.8	3.2	1.6
<i>kabura</i> , <i>Kabura</i>	102.4	3.2	3.2
<i>mini</i> , <i>Sari</i>	12.8	3.2	0.1
<i>szawjizak</i> , <i>Szawjizak</i>	25.6	3.2	1.6
<i>georgia</i> , LT 117	51.2	0.8	0.1
<i>perameles</i> , <i>Bandicoot</i> 343	102.4	3.2	1.6
<i>hardjo</i> , <i>Hardjopr</i> - <i>jitno</i>	51.2	25.6	25.6
<i>medanensis</i> , <i>Hond HC</i>	6.4	6.4	6.4
<i>wolffi</i> , 3705	25.6	51.2	3.2
<i>sejroe</i> , M 84	25.6	6.4	6.4
<i>balcanica</i> , 1627 <i>Bur-</i> <i>gas</i>	25.6	51.2	6.4
<i>polonica</i> , 493 <i>Poland</i>	102.4	25.6	6.4
<i>saxkoebing</i> , <i>Mus 24</i>	6.4	0.8	0.2
<i>nero</i> , <i>Gamsulin</i>	51.2	0.8	3.2
<i>haemolytica</i> , <i>Marsh</i>	6.4	12.8	3.2
<i>ricardi</i> , <i>Richardson</i>	204.8	1.6	3.2
<i>recreo</i> , LT 957	102.4	12.8	6.4
<i>trinidad</i> , LT 1098	51.2	12.8	3.2
<i>gorgas</i> , LT 829	25.6	12.8	3.2
<i>beye</i> , LT 844	25.6	0.4	0.2
<i>tabaquiteo</i> , TRVL 34056	51.2	0.4	0.4

^a Reciprocal of the highest dilution in thousands showing 50% or more agglutination.

^b —, Less than 50% agglutination at a 1:100 serum dilution.

TABLE 2. Comparative value of Fletcher and bovine albumin-polysorbate 80 media and antimicrobial agents in the isolation of 15 *Leptospira* strains from 72 horse kidneys

Medium	No. of isolations		
	No anti-biotic	5-Fluorouracil (200) ^a	Neomycin or combined neomycin/furazolidone ^b
Fletcher medium	0	0	0
BA-P80 medium	4	10	12

^a Final antibiotic concentration (micrograms per milliliter).

^b Neomycin disks contained 5 and 30 µg/disk, or renal tissue exposed to 25 µg/ml each of neomycin-furazolidone combined in saline before culture.

quanine. After inoculation of each of the 12 isolates into weanling hamsters, none of the strains was recoverable by culturing either blood or kidney.

Agglutination tests carried out against a battery of 22 hyperimmune antisera of pathogenic and saprophytic serotypes revealed a broad reactivity with seven of the isolates with titers to 19 of the various antisera ranging from 1:100 to 1:1,600. The antigens of the other five cultures did not react with any of the antisera. Specific antiserum prepared in rabbits with one isolate representative of each of the two groups (no. 48 and 55, respectively) showed that both isolates were antigenically distinct when used in cross-agglutination tests. When culture no. 48 was used as antigen in the MA test against the 245 randomly selected horse sera mentioned

rototypes and their possible relationship to the *L. biflexa* isolates. For these tests, 10 of the random equine sera were absorbed with the horse kidney isolate no. 48 culture. When the 10 absorbed sera were retested by the MA test against the absorbing culture and the battery of pathogenic serotypes, most of the agglutinins were removed (Table 6). All seven of the sera reacting positively to serotype *pomona* antigen became negative after absorption with isolate no. 48. In contrast, three of the sera after absorption reacted to the same titer against serotype *canicola*. When the equine serum no. 91 was again absorbed using serotype *canicola* organisms and retested, all specific agglutinins to this serotype and cross-reactivity to *ballum* and *pyrogenes* were removed.

One equine serum (no. 82) was treated with 2-mercaptoethanol (Eastman Kodak Co., Rochester, N.Y.) at final concentrations of 0.1 M and 0.05 M for 2 h at 37 C. When the treated

serum was examined in comparative MA tests against the positively reacting pathogenic serotypes and to the horse isolate no. 48 culture, all agglutinin activity to the various pathogenic strains became negative. However, the agglutinating activity of the treated serum to the isolate no. 48 antigen was the same as that of the untreated sample. These results show that the serological activity of this horse serum to the pathogenic serotypes was apparently restricted to the immunoglobulin M proteins, and the serological reactivity to the isolate no. 48 antigen involved the immunoglobulin G fraction of the serum. These findings also suggest that the 2-mercaptoethanol-sensitive immunoglobulin M immunoglobulins may give non-specific reactions and account for a portion of the multiple cross-reactivity to pathogenic serotypes seen in equine sera by agglutination tests. To further support this hypothesis, 70 (28.5%) of the 245 horse sera that were positive

TABLE 6. Results of agglutination tests with 10 horse sera absorbed with horse isolate no. 48 against the absorbing culture and 14 pathogenic *Leptospira* serotypes

Antigen	Unabsorbed serum									
	91	15	12	76	82	81	32	63	8	83
<i>pomona</i>	400	800	— ^a	—	400	—	100	400	100	200
<i>australis</i>	—	100	—	100	—	—	—	100	—	—
<i>ballum</i>	400	100	200	—	400	100	—	200	200	—
<i>grippotyphosa</i>	—	—	—	—	—	—	—	—	—	—
<i>tarassovi</i>	—	—	—	—	—	—	400	—	—	—
<i>hebdomadis</i>	—	100	—	100	400	400	—	—	—	100
<i>bataviae</i>	—	200	—	—	—	—	100	—	—	—
<i>canicola</i>	400	100	100	100	200	100	—	100	—	—
<i>hardjo</i>	—	100	200	400	100	200	—	—	100	—
<i>wolffi</i>	—	—	100	200	—	200	—	—	—	—
<i>pyrogenes</i>	200	200	200	200	1,600	100	100	100	200	100
<i>ictero-</i>	—	100	—	—	100	—	—	—	—	—
<i>sejroe</i>	—	100	400	200	—	800	—	—	100	—
<i>autumnalis</i>	—	100	—	—	100	—	—	—	—	—
Isolate 48	1,600	1,600	1,600	400	1,600	800	800	800	800	1,600
Serum absorbed with isolate 48										
<i>pomona</i>	—	—	—	—	—	—	—	—	—	—
<i>australis</i>	—	—	—	—	—	—	—	—	—	—
<i>ballum</i>	100	—	—	—	—	—	—	—	—	—
<i>grippotyphosa</i>	—	—	—	—	—	—	—	—	—	—
<i>tarassovi</i>	—	—	—	—	—	—	—	—	—	—
<i>hebdomadis</i>	—	—	—	—	—	—	—	—	—	—
<i>bataviae</i>	—	—	—	—	—	—	—	—	—	—
<i>canicola</i>	400 ^b	—	—	—	200	—	—	100	—	—
<i>hardjo</i>	—	—	—	—	—	—	—	—	—	—
<i>wolffi</i>	—	—	—	—	—	—	—	—	—	—
<i>pyrogenes</i>	200	—	—	—	100	—	—	—	—	—
<i>ictero-</i>	—	—	—	—	—	—	—	—	—	—
<i>sejroe</i>	—	—	—	—	—	—	—	—	—	—
<i>autumnalis</i>	—	—	—	—	—	—	—	—	—	—
Isolate 48	100	—	1,600 ^c	—	—	—	400	100	800	100

^a —, Less than 50% agglutination at a 1:100 serum dilution.

^b Serum absorbed with *canicola* cells removed all agglutinins to serotype *canicola*.

^c Repeat absorption with isolate 48 cells showed agglutinin titer as 1:200.

in MA tests to serotype *pomona* (Fig. 1) were examined after treatment with 0.05 M (final concentration) 2-mercaptoethanol for 2 h at 37 C. Repeated MA tests with the treated sera against *pomona* antigen showed that reactivity of 44 (62.8%) of the 70 sera was completely destroyed by 2-mercaptoethanol. These sera did not show any antibody activity in MA tests at a 1:100 serum dilution.

DISCUSSION

In the present study serological evidence of naturally occurring leptospiral agglutinins in horses of Argentina was found in 74.6% of the animals sampled. These findings were based upon results obtained with the MA test procedure using 14 screening serotypes. The majority of the positive reactions were to *pomona*, *Hebdomadis* group (*hardjo*, *wolffi*, *sejroe*, *hebdomadis*), *pyrogenes*, *tarassovi*, and *canicola* serotypes.

Previous studies reported in Argentina have also shown high reactor rates in horses against diverse and multiple antigens. Savino and Rennella (18) reported an incidence of 61% positive reactions, predominantly against the leptospiral antigens *pomona* and *canicola*. Subsequently, Cacchione (2) reported 80% or greater seropositive horses in the city of Buenos Aires. The predominant reactions were to antigens *pyrogenes* and *icterohaemorrhagiae*. In the same report a positive rate of 31% was given for the Province of Buenos Aires, with predominant reactions occurring with *pomona* and *tarassovi* antigens. The etiological significance of the serological findings of multiple leptospiral agglutinins in a high percentage of horses has not been established.

In this study, three serotype *hardjo* strains were isolated in studies on 72 kidneys of apparently normal horses collected at an abattoir in Buenos Aires. This is the first known isolation of this serotype from horses and may implicate this animal, because of its close ecological relationship to cattle and man, as playing an important role in the epidemiology of leptospirosis, by serving as a previously unrecognized maintenance host for infection of cattle by this serotype (14).

Twelve other *Leptospira* isolations were also made from the equine kidneys. All of these cultures shared biological characteristics that were comparable to saprophytic strains of *L. biflexa*. One of the isolates (no. 48) used as antigen in MA tests was agglutinated by serum dilutions (1:100 or greater) of 243 (99.1%) of 245 equine sera examined. Absorption of selected

horse sera with this saprophyte removed most of the leptospiral agglutinins to the parasitic serotypes. This suggests that certain *L. biflexa* strains may be responsible for some of the diverse cross-reactivity, frequently encountered with horse sera in agglutination tests with pathogenic serotypes. The very limited number of leptospiral isolations of the less fastidious pathogenic serotypes, viz., *pomona* or *canicola*, that have been reported in horses, and the few pathogenic strains recovered using albumin-based media considered excellent for growing all leptospires and using improved techniques for preparing renal tissue suspensions for isolation of leptospires, support the hypothesis that most of the positive reactivity seen in agglutination tests with horse sera is elicited by saprophytic strains.

The question may be raised concerning the possibility of outside contamination of the horse kidneys with the saprophytic strains during collection, but the collection method used in this study makes this unlikely. It was further established that the benzylammonium chloride disinfectant solution used to disinfect the exterior of each kidney was leptospiricidal to the horse kidney isolate (no. 48) culture with an exposure period of 1 min. This same methodology has been used for a number of years in this laboratory for *Leptospira* isolations from bovine and swine kidneys obtained from abattoirs. No *L. biflexa* strains have ever been isolated from cultures of renal tissue in the albumin-base medium. Leptospiral contamination of the bacteriological media is obviated by the fact that the basal medium is prepared in distilled water and then autoclaved. The dry-powdered, fraction V-filtered, albumin enrichment portion which completes the medium is also dissolved in distilled water. No uninoculated culture tubes of the completed medium have ever been found to be contaminated with a *biflexa* strain.

The findings obtained in this study with *biflexa* isolates may indicate that persistence of leptospires in the kidney provides a continuing antigenic stimulus. Such a stimulus might explain the persistent leptospiral serum titers observed in horses. However, further immunological studies are required to clarify this point and are in progress.

ACKNOWLEDGMENTS

Appreciation is expressed to the Frigorífico Lamar, Mercedes, Buenos Aires, and to Jorge Lasta, Facultad de Ciencias Veterinarias, La Plata, Buenos Aires, who contributed to this study by providing the equine kidneys and samples of blood, and to Alicia A. Siniuk for excellent technical assistance.

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