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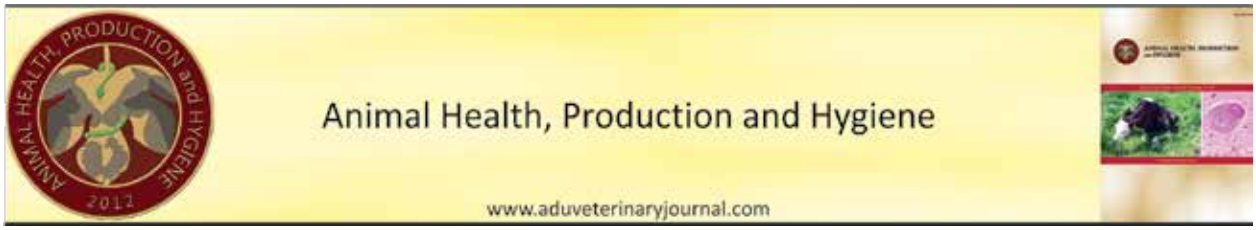


Research Article

# The Detection of *Brucella sp.* and *Leptospira sp.* in Cattle by Multiplex Polymerase Chain Reaction (mPCR)

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A B S T R A C T

In this study, blood samples obtained from 200 cattle in Aydın Region were used as material. Following DNA extractions, identification of the specific genes related with *Brucella* and *Leptospira* was carried out by using multiplex PCR method. Results obtained from this study revealed that *Brucella sp*. and/or *Leptospira sp.* were detected from 77 (38.5%) samples investigated. Bands related with both *Brucella sp*. and *Leptospira sp* were detected 24 (31.2%) out of 77 mPCR positive samples. From the remaining 53 (68.8%) samples, 33 samples (% 62.3) showed positive bands only related with *Brucella sp.* and 20 samples (37.7%) showed positive bands only related with *Leptospira sp*. The main conclusion of this study was the use of multiplex PCR as a reliable, sensitive and fast technique could prevent the hazards originated from contamination and false results caused by conventional and serum analysis methods in detection of Brucella and Leptospira.

*Keywords: Brucella sp., Leptospira sp., Multiplex PCR*

# Sığırlarda Brucella ve Leptospira Türlerinin Multiplex Polimeraz Zincir Reaksiyonu (mPCR) ile Tanımlanması

Ö Z E T

Bu çalışmada Aydın ilindeki sığır sürülerinden 200 adet kan örneği materyal olarak toplandı. Bu kan örneklerinden DNA ekstraksiyonları yapıldıktan sonra Brucella ve Leptospira bakterilerine ait spesifik genlerin multipleks PCR yöntemi ile identifikasyonu yapıldı. Yapılan multipleks PCR sonucunda 200 örneğin 77 (%38.5)'sinde *Brucella sp*. ve *Leptospira sp.* tespit edilmiştir. mPCR pozitif toplam 77 örneğin 24 (%31.2)'ünde hem *Brucella sp.*'ne hem de *Leptospira sp.*'ne ait bantlar ortak olarak görülmüştür. Kalan 53 (%68.8) örneğin 33 (%62.3)'ü sadece *Brucella sp.* ve 20 (% 37.7)'si ise sadece *Leptospira sp*. için pozitif bulunmuştur. Sonuç olarak çalışmada konvansiyonel mikrobiyolojik kültür yada serum analiz teknikleri ile *Brucella* ve *Leptospira* türlerinin tayini konusunda meydana gelen hatalı sonuçların ve kontaminasyon tehlikesinin hassas ve hızlı bir teknik olan multiplex PCR yöntemi ile elimine edilmiştir. Ayrıca her iki bakteri türünün identifikasyonunda da zaman kaybetmeden güvenilir sonuç alınabilecektir.

*Anahtar Kelimeler: Brucella sp., Leptospira sp., Multipleks PCR*

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## Introduction

Brucellosis and Leptospirosis infections are worldwide infections, seen frequently, in cattle. These causative infectious agents do not only cause economical losses and forfeit animal health, indeed these infections are also zoonotic infections which threat human health.

Brucella and Leptospira infections cause abortion, decrease of milking capacity, and decrement of brood property, and by this reason, they bring along serious losses for breeder and country economy. By determining these negations, Brucellosis and Leptospirosis are accepted as most important diseases of domestic animals (Sangari et al., 1994; Savio et al., 1994). The importance of these diseases are increasing by several reasons such as fast spreading, difficulty of control and prevention, time consuming and expensive therapy. These diseases cause critical consequences like prevention of trading animals and animal products, also these diseases inhibit social and economical development of breeders mostly found on pastoral regions (Dieffenbach and Dveksler, 1995).

Brucellosis is transmitted generally by direct contact with the infected animals or contaminated environment with discharges of infected animals. Aborted infants, infant membranes and fluids, vaginal discharges of infected animals which had abortion contain the infectious agent excessively. Animals are infected by ingesting these materials or by ingesting water of food contaminated with Brucella agents. Milk, urine, feces and synovial fluids are also sources for Brucella agents. Beyond this, transmission of the disease is seen in pregnancy from cow to calf, or with consumption of infected colostrum in postpartum period (Arda, 1997). Most cows with Brucellosis spread the agent by their milk for weeks, even for months after abortion. Excessive numbers of microorganisms were found in the fluxes of animals after abortion or giving birth. However, in spite of the decrease in the numbers of the agent in 1-2 weeks, the agent continues to remain in the fluxes for 6 months or more (Nicoletti, 1980).

The infectious agent is found mostly in uterus content, fetus and fetal membranes of pregnant animals. Transmission is formed primarily with digestive system, proof skin or skin with portantre, conjunctiva, coitus and contamination of the teats by milking (Verger et al., 1985).

Infected pregnant animals cause spread of disease by abortion or giving birth, especially by deployment of fetus and fetal membranes, amnion and placenta also cause environmental contamination. Infected uterus discharges and tail contaminated with urine, may transmit the agent to skin and conjunctiva of other animals (Nicoletti, 1980).

Infected bulls with testis inflammation may cause the spread of the disease. Bulls transmit the disease mechanically, and they also cause the infection in healthy cows with their infected semen (Verger et al., 1985).

Beyond this, the arthropods like flies, mosquitoes, bedbugs, ticks, fleas; and the rodents like wild rabbits, rats, and mice take role in the transmission of the disease. It is reported that avian like sparrows, crows, and vultures may become porter for the disease and sometimes the infection is seen in deers, mountain goats and gazelles, therefore these animals may also take role in the transmission of the disease (Smith and Ficht, 1990).

Brucellosis, the most important zoonosis, transmited from animals to human, is especially spread by direct contact of hands to eyes and mouth (Sangari et al., 1994). The disease is easily transmitted to individuals who digest butter, cream, fresh cheese and sour milk made by raw milk containing infectious agent. During abortion, rough births and the fall of afterbirth, the veterinarians may also be infected; therefore the Brucellosis carries another importance by being an occupational disease (Smith and Ficht, 1990).

The susceptibility of the body against the disease increases with increasing age of the animal. The disease may remain for long years in animals which have reached puberty. Second abortion cases are rarely seen in the animals which had first abortion (Verger et al., 1985).

The importance of gregariousness of too many animals is excess for the epidemiology of Leptospirosis. For example, it is reported that the infection is seen in the season when mouse population increases for individuals working at rice production. Rats, cats, dogs, goats, cattle, pork, deer, rabbits and even birds may carry the agent. These animals are the last reservoirs of *Leptospira sp.* and infection proceeds subclinically, therefore the clinical signs are generally underestimated. *Leptospira sp.* especially locates in the kidneys of rodents and may plunge for years with urination. The bacteria burst to the outer environment may be transmitted to human by different routes primarily the crack lesion on the skin. There are reports about development of the disease in the result of direct contamination (Levett, 2005).

For both of the diseases considered above, the diagnosis could be carried out by detection of the specific antibodies in the sera. However, generally false positive or false negative results may be obtained with this method. Beside this, the diagnosis obtained by detection of serum antibodies is hard to adapt routine because of the false positive and false negative results, plus, this situation may be hazardous for the laboratory staff. By the development of Polymerase Chain Reaction, this method has been started to apply for the detection of *Brucella sp.* (Fekete et al., 1990, Herman and Ridder, 1992; Romero et al., 1995a, b). PCR has also been started to be used for the detection of Leptospirosis afterwards (Van Eys et al., 1989; Woodward et al., 1991; Me´rien et al., 1992; Woodward and Redstone, 1993; Savio et al., 1994; Heinemann et al., 2000). Multiplex PCR method which is a novel procedure may be advantageous for detecting more than one target DNA sequences in single reaction. This direct method is generally applied for the isolation of bacteria from the hosts (Dieffenbach and

Dveksler, 1995).

In this study, it is aimed to diagnose *Brucella sp.* and L*eptospira spp.* in cattle as the abortion causative agents on molecular basis by using mPCR method. Thus with the epidemiological point, rapid diagnosing of these two bacterial agents which cause abortions by the effective and safe method, and prevention of the losses because of these zoonotic diseases will fasten the process of protection and control effectiveness.

## Materials and Methods

*Samples*

In this study, 200 full blood samples were collected with random sampling method from the cattle herds located in Aydın province and then the samples were brought to Adnan Menderes University Faculty of Veterinary Medicine Department of Microbiology Routine Diagnosis Laboratory in cold chain. The blood samples were kept in -20 °C deep freeze until the experimental stage.

*DNA Extration Kit and Other Reagents*

Genomic DNA Extraction Kit of MBI Fermentas® firm, 500 U Taq DNA polymerase enzyme of MBI Fermentas® firm, dNTP mix set consists of 200 µm each dNTP of MBI Fermentas® firm were used for DNA extraction.

*Primers*

In the study oligonucleotide primer sequences were applied, used by Bailey et al. (1992) and Mérien et al (1992) previously. These oligonucleotide primer sequences were formed as;

B4 =5'-TGGCTCGGTTGCCAATATCAA-3' and

B5 = 3'-CGCGCTTGCCTTTCAAGGTCTG-5' (Bailey et al. 1992)

Lep1 = 5'-GGCGGCGCGTCTTAAACATG-3' and

Lep2= 3'-TTAGAACGAAGTTACCCCCCTT-5' (Mérien et al. 1992).

*Standard Strains*

In the study *B. abortus* S 19 vaccine strain and *L. icterohemorrhagiaee* (obtained from Prof. Dr. Nihat Toplu, associate of Adnan Menderes University Faculty of Veterinary Medicine) were used as standard strains.

*DNA Amplification*

The multiplex Polymerase Chain Reaction Method (mPCR) was used for amplification of extracted DNA's. The solutions and components used for mPCR were prepared as consisting of 15 µl Milli-Q water, 5 µl taq reaction buffer 10x (500 mM KCl, 100 mM Tris HCl, pH 9.0), 8 µl dNTPmix, 1.5 µl $MgCl_2$ (50 mM), 2.5 µl B4, B5, Lep1 and Lep2 10 pm/ml from each primer, 0.5 µl Taq DNA polymerase (5 U/ml) and 10 µl extracted DNA, a total volume of 50 µl microtubes (Richtzenhain et al., 2002).

The mastermixes prepared for mPCR were applied to PCR process as 1 cycle 3 min pre-denaturation at 94ºC, 35 cycles 1 min DNA denaturation at 94ºC, primer annealing for 1 min at 60ºC, DNA extension for 1.5 min at 72ºC and 1 cycle 10 min final extension at 72ºC (Richtzenhain et al., 2002).

*Screening of PCR Products*

The products obtained at the end of mPCR were run by 2% gel electrophoresis and stained with ethidium bromide. Then, the products were screened in UV screening systems. 100 bp molecular weight marker (100 bp ladder MBI Fermentas®) was used as standard measure. Amplicons were observed at fragment space between 223 bp for *Brucella sp*. and 331 bp for *Leptospira sp*. bacteria (Richtzenhain et al., 2002).

## Results

In this study, DNA extractions from the collected samples were carried out and mPCR were applied for *Brucella sp*. and *Leptospira sp*. then mPCR products were screened. As a result of this screening, it was detected that *Brucella sp*. positive products were observed in the fragments of 223 base pair and *Leptospira sp.* positive products were observed in the fragments of 331 base pair (Figure 1).

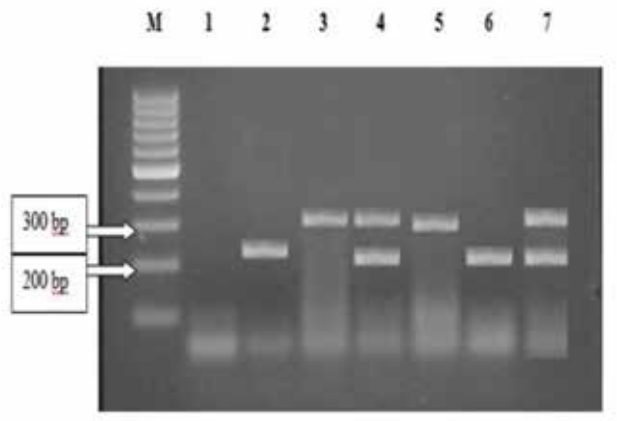


**Figure 1.** mPCR for *Brucella sp.* and *Leptospira sp.* (spesific amplificon is approximately 223 bp for Brucella species and spesific amplificon is approximately 331 bp for Leptospira species). Column M: 100 bp molecular weight marker (100 bp ladder), Column 1: negative control, Column 2: *Brucella sp.* positive control (*B. abortus* S19 vaccine), Column 3: *L. icterohemorrhagiaee* positive control, Column 4: *Brucella sp.* and *Leptospira sp.* positive sample, Column 5: *Leptospira sp.* positive sample, Column 6: *Brucella sp.* positive sample and Column 7: *Brucella sp.* and *Leptospira sp.* positive sample.
**Şekil 1.** *Brucella sp.* ve *Leptospira sp.* için mPCR (Brucella türleri için spesifik amplifikon yaklaşık olarak 223 bp ve Leptospira türleri için spesifik amplifikon yaklaşık olarak 331 bp aralığındadır). Sütun M: 100 bp'lik Moleküler ağırlık işaretleyicisi (100 bp ladder), Sütun 1: negatif kontrol, Sütun 2: Brucella pozitif kontrol (*B. abortus* S19 aşı), Sütun 3: *L. icterohemorrhagiaee* pozitif kontrol, Sütun 4: Brucella ve Leptospira pozitif örnek, Sütun 5: Leptospira pozitif örnek, Sütun 6: Brucella pozitif örnek ve Sütun 7: Brucella ve Leptospira pozitif örnek.

According to the mPCR results obtained, *Brucella sp.* and *Leptospira sp*. were detected from 77 (38.5%) of 200

samples investigated. There were detected collaborate positive bands obtained from 24 (31.2%) of the 77 mPCR positive products samples associated with both *Brucella sp.* and *Leptospira sp.*. Thirty-three (62.3%) of the samples were detected positive only for *Brucella sp*., and 20 (37.7%) of the samples were detected positive only for *Leptospira sp*. in the remaining 53 (68.8 %) samples.

*Specifity and Sensitivity of mPCR Process*

Two serial dilutions were prepared in the ratio of $3x10^4$, $3x10^3$, $3x10^2$, $3x10^1$, 3 CFU/ml for the sensitivity of mPCR process with the strains used for positive control; *B. abortus* S 19 vaccine strain and *L. icterohemorrhagiaee.* DNA extractions were carried out by using these dilutions. The extracted DNAs were applied to mPCR process (Richtsenhain et al., 2002). As a result of mPCR, it was obtained that the sensitivity of this method was $3.10^1$ bacteria/ml, thus it was considered as a spesific and sensitive method for DNA detection of *Brucella sp*. and *Leptospira sp*. (Figure 2).

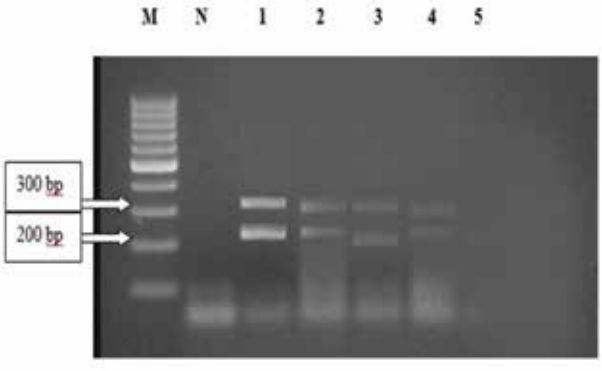


**Figure 2.** mPCR sensitivity for *Brucella sp.* and *Leptospira sp.* Column M: 100 bp molecular weight marker (100 bp ladder), Column N: negative control, Column 1: $3x10^4$ CFU/ml *Br. abortus* and *L. icterohemorrhagiaee* dilution, Column 2: $3x10^3$ CFU/ml *Br. abortus* and *L. icterohemorrhagiaee* dilution, Column 3: $3x10^2$ CFU/ml *Br. abortus* and *L. icterohemorrhagiaee* dilution, Column 4: $3x10^1$ CFU/ml *Br. abortus* and *L. icterohemorrhagiaee* dilution and Column 5: 3 cell CFU/ml *Br. abortus* and *L. icterohemorrhagiaee* dilution.

**Şekil 2.** Brucella sp. ve Leptospira sp. için mPCR sensitivitesi. Sütun M: 100 bp'lik Moleküler ağırlık işaretleyicisi (100 bp ladder), Sütun N: negatif kontrol, Sütun 1: $3x10^4$ CFU/ml *Br. abortus* ve *L. icterohemorrhagiaee* dilusyonu, Sütun 2: $3x10^3$ CFU/ml *Br. abortus* ve *L. icterohemorrhagiaee* dilusyonu, Sütun 3: $3x10^2$ CFU/ml *Br. abortus* ve *L. icterohemorrhagiaee* dilusyonu, Sütun 4: $3x10^1$ CFU/ml *Br. abortus* ve *L. icterohemorrhagiaee* dilusyonu ve Sütun 5: 3 cell CFU/ml *Br. abortus* ve *L. icterohemorrhagiaee* dilusyonu.

**Discussion**

Different methods were used in past researches to apply by using pure cultures for identification of *Brucella sp.* from various specimens by PCR (Fekete et al., 1990; Herman and Ridder, 1992; Romero et al., 1995a). Furthermore, the blood and milk samples obtained from cattle, cheese samples, human blood samples and the samples obtained from naturally infected cows were also used in the researches (Ficht et al., 1996). There are also studies available evaluating the infection agents by PCR in cattle and in sheep with Brucellosis cases ended with abortion (Fekete et al., 1992; Cetinkaya et al., 1999).

The urine samples obtained from cattle (Van Eys et al., 1989), experimentally infected blood, urine and cerebrospinal fluid samples (Me´rien et al., 1992), purely isolated microbiological cultures (Woodward et al., 1991), blood samples collected from gerbils as experimental animals, experimentally contaminated pork kidneys (Savio et al., 1994), semen, blood and urine samples of brood bulls and cerebrospinal fluid samples collected from human were also used in some studies (Romero et al., 1995a).

There are some studies about the usage of microbiological culture for detection of *Brucella sp.* by PCR. Fekete et al. (1992) used microbiological culture in a study conducted by using primer sequences targeted to 43 kDa *Brucella abortus* outer membrane protein, presented 98% sensitivity and 96% specifity. Cetinkaya et al. (1999) detected 80% sensitivity and 91% specifity in their research conducted by using primer sequences targeted to 16S rRNA in which microbiological cultures obtained from gastric content of aborted lambs were used. Rijpens et al. (1996) reported that in the isolation of Brucella agents from milk, the agents showed great affinity to the fatty part of the milk, and reported $2.8x10^4$ sensitivity for their PCR study which was based on enzymatic extraction of milk components.

Ana et al. (1997) reported from their studies that all samples examined in the research gave presults based upon spesific amplification by laboring with 6 strains of *Brucella sp*.; *B. abortus* (biovar 1), *B. canis*, *B. ovis*, *B. melitensis* (biovar 1), *B. suis*, *B. neotoma*. 66ºC temperature was used for primer annealing, thus it was reported that Omp2 gene locci attached to the primers more specifically. However in their results they also reported that the strains indicated 4 different signal amplifications as 900 bp, 720 bp, 600 bp and 200 bp electrophoretic bands respectively. In spite of the different amplifications obtained, it was reported that Omp2 gene that encodes the porin outer membrane found in *Brucella sp.* occured in the sequence analysis (Ana et al., 1997).

Moriyo´n and Berman (1982) reported that the cell wall of *Brucella sp*. is more resistant to solvents like nonionic detergents, EDTA (ethilen diamin tetra asetic acid) and Tris rather than other gram negative bacteria. For this reason, they reported that appropriate lysis solutions should be used in the extraction step in order to lyse cell wall of *Brucella sp.*

Bricker et al. (2000) reported that $10^6$ inactive bacteria or 5 ng purified genomic DNA (50 µl in each sample) should be used for the amplification of DNA products obtained from *B. abortus* S19 vaccine strain. Furthermore, in the PCR study applied to genes obtained from *B. abortus* 544, S19, 2308 and RB51 vaccine strains for 35 cycles in conditions of 1.2 min at 94 ºC, 2 min at 55.5 ºC, 2 min at 72 ºC, it was reported that the DNA's obtained from *B.*

*abortus* 544 and S19 strains showed band fragment at 498 bp and the DNA's obtained from *B. abortus* 2308 and RB51 strains showed band fragment at 364 bp (Bricker et al., 2000).

Vaccine strains are generally used as control group in PCR studies, for this reason the strain type of the vaccine product must be specified precisely (Bricker et al., 2000). Especially, *B. abortus* S19 strains are incubated by microbiolgical culture method, however this modal is not fast and there are risks of contamination in this method. It was also reported that erythritol should be added to the solid culture media for growth of this strain (Jones et al. 1965), thus the locus named as *eri*, which catabolyses erythritol could also be detected (Sangari et al., 1994).

Saad et al. (1997) reported that PCR method is more specific and sensitive rather than FAT (Florescense Antibody Test) and MAT (Microscopic Agglutination Test) for detection of Leptospira agents in semen, urine and sera of bulls. FAT and MAT tests could not show any efficiency in distinction of naturally infected animals and vaccine applied animals. It was reported that Microscopic Agglutination Test detected a titer in the ratio of 1:50 in 50 µl sera sample of naturally infected bulls (Saad et al., 1997). It was reported that *Leptospira sp*. agents could be detected one week after experimental infection when 50 µl sera sample was used in PCR study, (Saad et al., 1997).

By evaluating the studies given above, it was noticed that *Brucella* and *Leptospira* spesices were studied separately, and there was not any consideration of investigating these two bacteria together until the multiplex PCR study was conducted by Richtzenhain et al. (2002). In that study, it was reported that that diagnose of *Brucella spp.* and *Leptospira spp.* could be made in one tube by using mPCR and the sensitivity and specifity of this process was reported as 100%, and 92 - 93%, respectively. It was also reported that the diagnosis of these two pathogens should be made more rapidly and more sensitive in routine laboratories.

By the guidance of these data given above, we decided to diagnose Brucella and Leptospira species in cattle for our research. In our study, separation of nucleotides was made by DNA purification kit and molecular typing was carried out by using multiplex PCR. According to the obtained mPCR results, *Brucella sp.* and *Leptospira sp.* was detected from 77 (38.5%) of 200 samples. Collaborate positive bands were also detected in 24 (31.2%) of the 77 mPCR positive products samples associated with both *Brucella sp.* and *Leptospira sp.* 33 (62.3%) of the samples were detected positive only for *Brucella sp.*, and 20 (37.7%) of the samples were detected positive only for *Leptospira sp.* out of the remaining 53 (68.8%) samples. It was also observed that mPCR method had sensitivity with 98% for detection of low level agents in blood samples.

The sensitivity was detected in the ratio of $3.10^1$ bacteria/ml at the end of the mPCR application, *Brucella sp.* and *Leptospira sp.* was detected from 77 (38.5%) of 200 samples. There were detected collaborate positive bands from 24 (31.2%) of the 77 mPCR positive products samples related for both *Brucella sp.* and *Leptospira sp.* 33 (62.3%) of the samples were detected positive only for *Brucella sp.*, and 20 (37.7%) of the samples were detected positive only for *Leptospira sp.* out of the remaining 53 (68.8%) samples. It was detected that no Brucellosis and Leptospirosis infections were present in 123 (61.5%) of 200 cattle.

It is indicated that molecular method presents faster and more sensitive results rather than classical culture methods while considering the conventional methods in microbiology.

It is also approved that multiplex PCR method is useful in optimal level rather than PCR method based upon single locus sequence principle in order to detection of two bacteria species at the same time.

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