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Development of a Blocking ELISA Method for Detection of Getah Virus Antibodies in Horse Sera

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Recent climate change and global warming are likely to increase mosquito-borne diseases. Getah virus (GETV) is a mosquito-borne virus that infects horses and other mammals, including humans. Currently, GETV infection in horse sera is confirmed by the virus neutralization (VN) test. Therefore, there is a need for a new enzyme-linked immunosorbent assay (ELISA) to detect GETV-antibody in a large number of horse serum samples. We aimed to develop a blocking ELISA (B-ELISA) method for the specific detection of GETV antibodies in horse serum samples. Antibodies against GETV in the sera of 175 horses were measured using the VN test. The purified QIAG9301-100P virus was used as an antigen for the B-ELISA. A monoclonal antibody (1E1) was conjugated with horseradish peroxidase and used as a detection antibody. For the establishment of the B-ELISA, antigen concentration, serum dilution factor, and conjugate dilution concentration were determined as 5 μg/mL, 20 times, and 3.6 μg/mL, respectively. We evaluated the sensitivity, specificity, and accuracy of the B-ELISA using sera from 175 horses. The B-ELISA had a diagnostic sensitivity of 91.4%, a specificity of 94.0%, and an accuracy of 93.1% compared with that of the VN test. The B-ELISA was significantly correlated with the VN test (r = 0.83). The new B-ELISA could replace the VN test and be useful for the sero-surveillance of GETV in horse sera. It is also expected to be able to detect GETV antibodies in the serum of various animals including pigs.

Key Words: Arboviruses, ELISA, Serology, Horse disease

INTRODUCTION

Getah virus (GETV) is a mosquito-borne virus belonging to the *Alphavirus* genus of the family *Togaviridae* (1). Since its isolation from *Culex gelidus* mosquitoes in Malaysia in 1955 (2), many GETV isolates have been reported from several countries, including Japan, China, Korea, Mongolia, and Australia (3-6). The GETV genome encodes five structural proteins: C, E3, E2, 6K, and E1. Among the structural proteins, glycoproteins E1 and E2 play key roles in cell surface binding and are related to the induction of anti-infection immunity (1). GETV strains can be divided into four groups (I - IV) based on E2 gene homology, and the most dominant group has been identified as group III (7).

Although clinical signs of GETV infection in horses serving as amplification hosts in the natural transmission of GETV are generally mild, GETV causes several symptoms, such as fever, rashes, edema of the hind legs, lymph node enlargement, and urticaria in multiple areas of the body (8). GETV is considered a transboundary and re-emerging disease in Asia. GETV infections have also been reported in racehorses in Japan, China, and India (8-10). The number of GETV outbreaks has increased from horses to pigs, cattle, blue foxes, and red pandas (11). The seropositivity rates of GETV in horses vary by country and season, ranging between 17 and 39% (12). The incidence and risk of GETV infection in horse populations are increasing; however, serological studies of GETV in horses are still lacking in South Korea.

Several methods can be used to measure GETV-specific antibodies in serum samples. The virus-neutralization (VN) test is the classical and general method for measuring GETV-specific antibodies in animals (13). Although the VN test is accurate and useful for measuring anti-GETV antibodies, it requires skilled technicians, cell culture equipment and reagents (12). Therefore, it is necessary to develop a relatively simple assay, such as an enzyme-linked immunosorbent assay (ELISA) to measure GETV-specific antibodies in the sera of several animals. ELISA is the preferred replacement for the VN test and has several advantages. It is easy to handle, and requires only small amounts of serum. Therefore, an indirect ELISA (I-ELISA) was developed using recombinant E2 protein expressed in *E. coli* for swine and horse sera (12, 14). An ELISA using a 20-mer synthetic peptide for the E2 glycoprotein has been reported to detect GETV infection in horses (15).

Previously, we purified GETV using the sucrose gradient method and reported an I-ELISA with sensitivity (86.3%) and high specificity (94.5%) compared with that of the VN test (16). In this study, we developed a blocking ELISA (B-ELISA) with high sensitivity and specificity that can be applied to several animal species because GETV transmitted by mosquitoes can cause disease in several animals.

MATERIALS AND METHODS

Cells, viruses, and serum samples

Vero cells (African green monkey kidney cell line, ATCC, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 IU/mL of penicillin and 10 μg/mL of streptomycin, 0.25 μg/mL of amphotericin B (Gibco BRL, Grand Island, NY, USA), and 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL). Vero cells were used to propagate and titrate the QIAG9301-100P strain (KTCT no: 19077P), which contains seven amino acid mutations in the genome of the QIAG9301 strain (NCBI accession number: KR-081238). The cells were used in the VN test. To optimize the B-ELISA, five positive and three negative GETV serum samples with virus-neutralizing antibody (VNA) titers of 256-4 and <2, respectively, were prepared from horses. These sera obtained from horses raised in South Korea were selected as a result of VN test against GETV. In total, 175 horse sera were randomly selected from those sera collected for regular equine serum testing from horses residing in South Korea nationwide and subjected to the VN test and B-ELISA.

Growth kinetics

The growth kinetics of the QIAG9301-100P strain were examined to determine the optimal harvest date. To assess the growth kinetics of the QIAG9301-100P strain, Vero cells grown in 25 cm^2 cell culture flasks were inoculated with a virus containing 100 $TCID_{50}$/mL and harvested at 12 h intervals for 4 days. After three consecutive freeze-thaw cycles, 10-fold serial dilutions of each virus were titrated in 96-well microplates, and cytopathic effects (CPEs) were observed under a microscope daily for 4 days post inoculation (DPI). The viral titers of the QIAG9301-100P strain were determined in accordance with the standard method of Reed and Muench (17) and expressed as 50% tissue culture infectious dose ($TCID_{50}$/mL).

Propagation and purification of the QIAG9301-100P strain

Vero cells planted in a 175 cm^2 cell culture flask were infected with the QIAG9301-100P strain at a multiplicity of infection of 0.1. After incubation at 37°C for 4 days, the infected cells were frozen and thawed three times. Viral titers for each 12-hour period were determined in 96-well microplates. GETV was purified by discontinuous sucrose gradient centrifugation as described previously (18). The concentration of the purified GETV antigen was determined using a NanoDrop 1000 UV/Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and GETV particles were identified by an electron microscope.

Production and purification of monoclonal antibody against GETV

One monoclonal antibody (mAb) specific to GETV was prepared as a detection antibody. Purification of mAb were carried out as described previously (19). Briefly, the ratio of GETV antigen and Cabopol 974 PNF (Lubirzol, Wickliffe, OH, USA) adjuvant was mixed at 9:1 and the prepared antigen was inoculated intramuscularly into BALB/c mice two times to confirm immunogenicity (IACUC approval No. 2021-542). One hybridoma cell-secreting antibody against GETV was selected and designated 1E1 (KTCT no:19078P). The 1E1 hybridoma cells were intraperitoneally inoculated into mice to generate ascites. The collected ascitic fluid was purified using Protein G affinity chromatography (Bio-Rad, Hercules, CA, USA). Purified ascitic fluid was conjugated with horseradish peroxidase (HRP), as described previously (20). The 1E1-HRP conjugate was used as the detection antibody for B-ELISA.

VN test

The VN test was performed in 96-well cell culture plates, as described previously (21). The VNA titers of GETV were expressed as the reciprocal of the highest serum dilution that completely inhibited viral CPE. Serum was diluted from 1:2 to 1:256, and the measured VNA titers were used for optimization and comparison with the B-ELISA. A VNA titer ≥ 1:2 was determined to be positive.

Optimization and application of B-ELISA

Chessboard titration was performed to determine the optimal viral antigen concentration, serum dilution factor, and 1E1-HRP conjugate concentration. The purified GETV antigen at various concentrations of 10 to 0.005 μg/mL, serum panel samples at dilutions of 1:10 to 1:1,280 and 1E1-HRP conjugate at concentrations of 0.45 to 3.6 μg/mL were used to determine the optimal conditions for B-ELISA. A 96-well microplate (MaxiSorp; NUNC, Roskilde, Denmark) was coated with 100 μL of purified GETV antigen in carbonate buffer (pH 9.6) and incubated at 4°C for 8 h. After incubation, the solution was discarded, and the microplate coated with GETV antigens was blocked with 5% skim milk.

The plates were washed with phosphate buffered saline (PBS) with pH 7.2 containing 0.05% Tween 20 (PBST). After washing, 100 mL of 2,2'-azino-bis-(3-ethylbenzothiazoline) substrate (ABTS) solution was added to each well and incubated for 10 min at room temperature. The reaction was stopped by the addition of 50 μL of 0.5 M sulfuric acid. The absorbance was measured at 405 nm using a spectrophotometer (Sunrise ELISA reader, Tecan, Switzerland). Under optimized B-ELISA conditions, 175 horse serum samples were analyzed. The absorbance value of the negative sample was set to 1.0 or more, and the percentage inhibition (PI) was calculated by substituting the absorbance value of each sample into the PI arithmetic equation. The PI was calculated as follows: PI = (1 - absorbance value of sample/absorbance value of negative sample) × 100. Serum samples were evaluated as positive if the PI was greater than 0, because the 1E1 mAb did not have neutralizing activity against GETV (19). Specificity, sensitivity, and accuracy were calculated according to previously reported formulas: sensitivity (%) = (number of positives in B-ELISA/total number of positives in the VN test) ×

100; specificity (%) = (number of negatives in B-ELISA/total number of negatives in the VN test) × 100; and accuracy (%) = (number of positives and negatives in both tests/total number of samples) × 100.

Statistical analysis

Linear regression analysis (least squares method) was used to determine the correlation coefficient (r-value) between the absorbance of the B-ELISA and the VNA titer. The r-value was automatically calculated by Microsoft Excel 2010 software (Microsoft Corp., Redmond, WA, USA).

RESULTS

Identification of purified QIAG9301-100P strain

Vero cells infected with QIAG9301-100P were frozen up to 96 h at 12 intervals to determine the optimal harvesting time. As shown in Fig. 1A, the QIAG9301-100P strain propagated in Vero cells had the highest viral titer ($10^{7.0}$ $TCID_{50}$/mL) at 36 h. Therefore, the optimum harvest time for QIAG9301-100P was determined to be 36 h after inoculation. The purified antigen revealed spherical viral particles of 40-50 nm in diameter (Fig. 1B).

Establishment of the B-ELISA

The purified mAb was applied to Vero cells infected with the QIAG9301-100P strain, and as a result of confirming by an immunofluorescence assay, fluorescence was shown in the cytoplasm (Fig. 1C). The GETV antigens were tested to

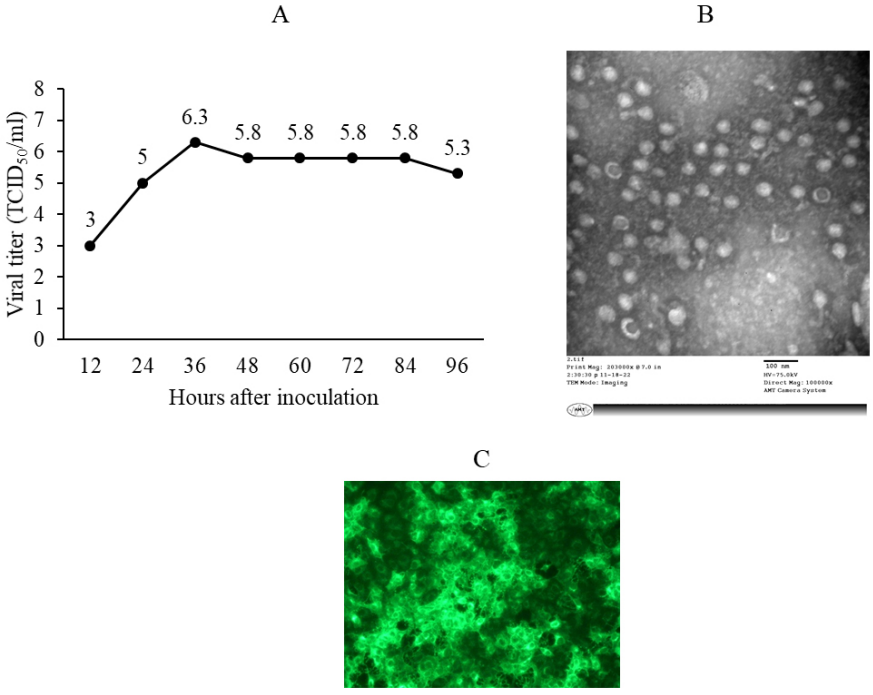

Fig. 1. The optimal harvest time is determined by measuring growth kinetics of the QIAG9301-100P strain (A). Virus particles of QIAG9301-100P strain identified by electron microscopy (B). The activity of 1E1 monoclonal antibody against Getah virus identified by FA test (C).

determine the optimal coating concentration. The GETV antigen at 5 μg/mL had approximately 14.6 times the value of the highest negative/positive (N/P) value (Fig. 2A). A positive serum sample diluted from 1:10 to 1:1280 was tested to determine the optimal dilution rate. A serum dilution rate of 1:20 had the highest N/P value (6.2) and was selected as the serum dilution rate (Fig. 2B). A detection antibody was used to determine the optimal conjugate concentration. The conjugate at 3.6 μg/mL showed the greatest difference in negative serum samples (Fig. 2C).

Application of the B-ELISA to animal serum samples

The absorbance value of the B-ELISA was compared with the VNA titers using 175 horse serum samples. B-ELISA results were considered positive if the PI of the test serum was ≥ 0% and negative if it was < -0.01%. The sensitivity, specificity, and accuracy of the B-ELISA were calculated using serum samples measured using the VN test. The sensitivity, specificity, and accuracy of the B-ELISA with 175 horse serum samples were 91.4, 94.0, and 93.1%, respectively (Table 1). The correlation between the absorbances of the B-ELISA and VNA titers is indicated by the regression line, and the r value was 0.86 (Fig. 3). As shown in Fig. 4, 90.6% of 64 horse serum samples showing an VN titer of 1:2 or higher had PI values ≥0.

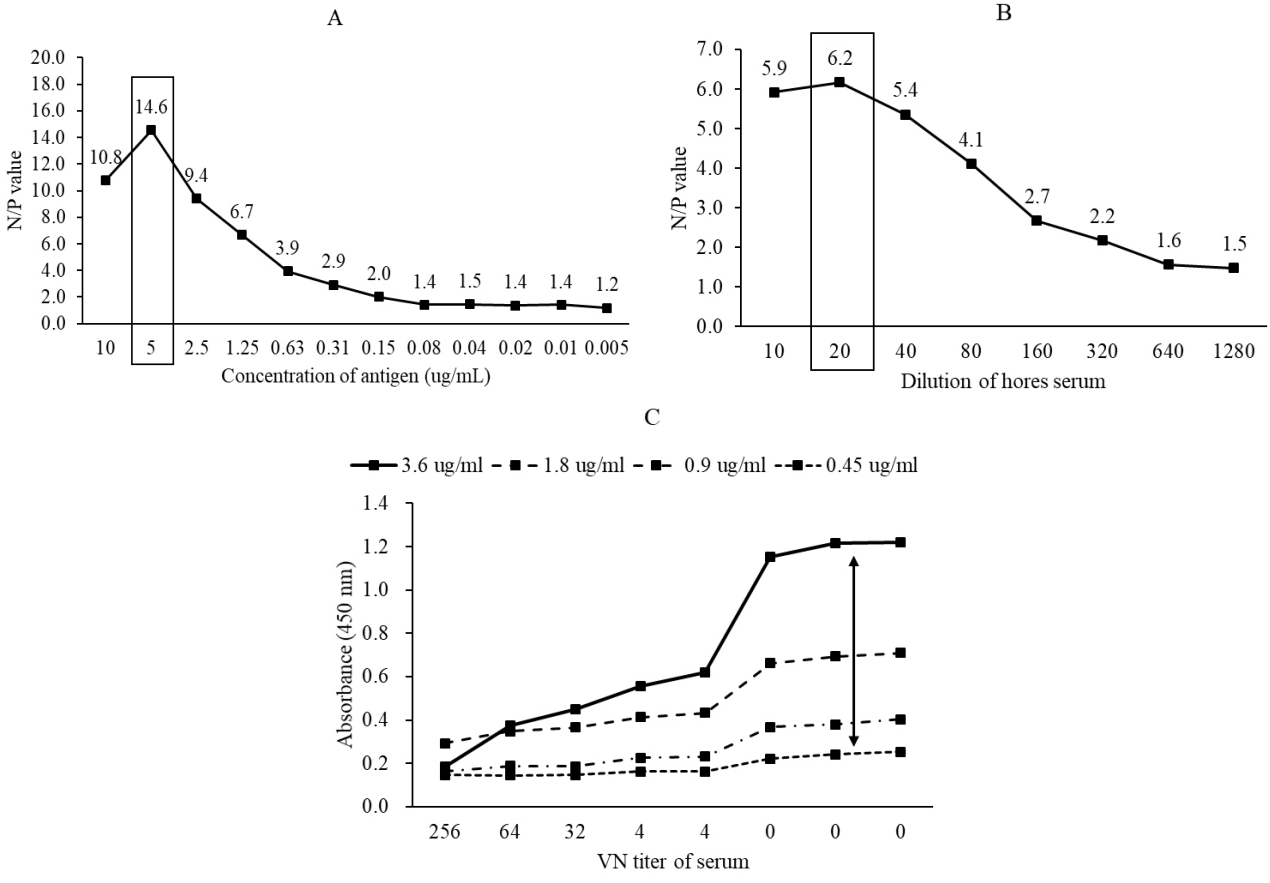

Fig. 2. Determination of the concentration of the purified GETV antigen (A), serum dilution factor (B) for the establishment of blocking ELISA based on the negative /positive serum absorbance value. Determination of the concentration of 1E1 HRP conjugate (C) based on the difference in absorbance. Double arrow in C indicates the difference in absorbance between 3.6 and 0.45 μg/mL of 1E1 HRP conjugate.

Table 1. The sensitivity, specificity, and accuracy of the B-ELISA to detect anti-Getah virus antibodies in comparison with the VN test

Serological test		B- ELISA		
		Positive	Negative	sum
VN	Positive	53	5	58
	Negative	7	110	117
	Sum	60	115	175
Sensitivity*		(53/58) 91.4%		
Specificity#		(110/117) 94.0%		
Accuracy†		[(53+110)/175]93.1%		

*Sensitivity = [(number of positives in B-ELISA)/ (number of positives in the FA test)] × 100,
#Specificity = [(number of negatives in B-ELISA)/ (number of negatives in the VN test)] × 100,
†Accuracy = [(number of positives and negatives in both tests)/ (total number of samples)] × 100.

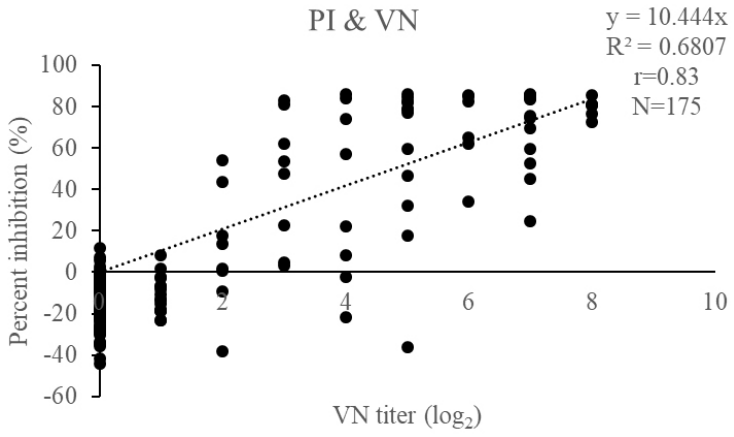

Fig. 3. Correlation between percent inhibition and the virus neutralizing antibody titer for detecting GETV antibodies in 175 horse serum samples. The correlation is indicated by the linear regression line and r value (0.83).

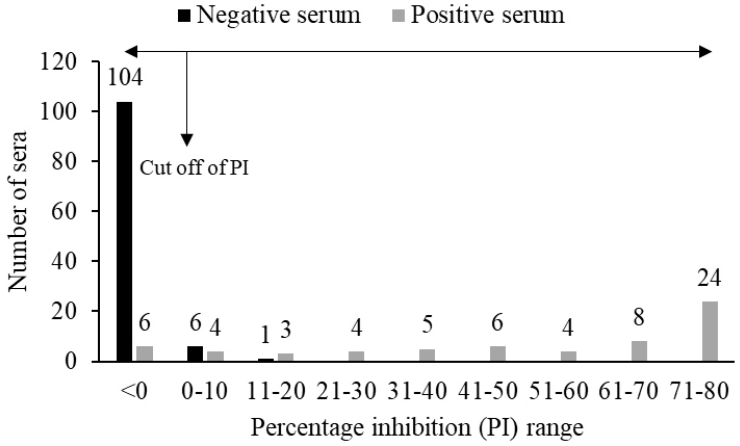

Fig. 4. Distribution of percentage inhibition (PI) values obtained by blocking enzyme- linked immunosorbent assay (ELISA) for horse sera (n=175). The arrows indicate cutoff values of 0 for horse sera. Of the horse serum samples, 90.6% (58/64) of positive serum samples for Getah virus had a PI value of >0.

DISCUSSION

GETV antigens and antibodies have been detected in pigs, cattle, horses, sheep, and mosquitoes, and their regional coverage is expanding (13). Outbreaks of GETV in horses have been reported in Japan and India, indicating that GETV infections can cause economic damage to the equine industry. Although there have been no outbreaks of GETV in South Korea, 12.4% of horses have been serologically reported to be exposed to GETV in the field (21). To date, no GETV vaccines or effective diagnostic kits have been commercialized in South Korea. Serological monitoring tools have also not been prepared or improved for the crisis of a pandemic. The development of a B-ELISA to measure GETV-specific antibodies in horses is timely.

The VN test for measuring anti-GETV antibodies requires skilled technicians and cell culture facilities, including microscopes and CO_2 incubators. Although the VN test is the standard method for serological monitoring of GETV, many laboratories prefer ELISAs, which are easy to perform without specialized facilities. Therefore, we developed a new B-ELISA using a purified antigen and mAb to measure GETV-specific antibodies in horse sera. Several I-ELISAs have been developed to measure anti-GETV antibodies in animals (10, 12, 14). An I-ELISA developed using a purified recombinant GETV E2 domain protein expressed in *E. coli* cannot be applied to several animals bitten by mosquitoes carrying GETV. This problem can be overcome by using B-ELISA, which allows serum samples to react with viruses coated on a solid phase. In B-ELISA, 1E1 mAb conjugated with peroxidase reacts with a coated virus that is not blocked by GETV antibodies in the serum. When the ABTS substrate was added to the B-ELISA plate, a decrease in color intensity indicated the presence of anti-GETV antibodies in the tested serum. Considering that antibodies against GETV have been confirmed in pigs and horses (21, 22), it is worthwhile to develop a tool for the sero-surveillance of several animals.

The sensitivity, specificity, and accuracy of B-ELISA were compared with those of the VN test using 175 horse serum samples. The sensitivity (91.4%) of the B-ELISA for horse serum compared with the VN test was slightly lower than those of the I-ELISA using the recombinant E2 domain protein or a synthetic peptide (93.3%-98.2%) (10, 12). The specificity (94.0%) of the B-ELISA was similar to that of the I-ELISA using recombinant or synthetic proteins (92.6-95.0%) (10, 12). The sensitivity of an ELISA can be improved using an mAb that blocks the epitope (23). In our study, we used mAb 1E1, which recognizes the GETV E2 protein, but does not have neutralizing activity, as the detecting antibody. The features of mAb 1E1 affected the sensitivity and specificity of B-ELISA. Moreover, B-ELISA showed high accuracy (93.1%) in horse sera, suggesting that it is suitable for the sero-surveillance of GETV in horses. Data on reproducibility, precision and stability will be described in the documents required for the industrialization process. Further studies on B-ELISA are warranted to perform sero-surveillance of GETV using sera from pigs and cattle.

In conclusion, this study described the development of a B-ELISA for the rapid measurement of GETV-specific antibodies in horse sera. The performance of B-ELISA was evaluated and compared with that of the VN test using 175 horse serum samples. The high sensitivity and specificity of B-ELISA suggested its potential for screening horses suspected of being infected with GETV or for measuring the immune response after vaccination. In addition, this new B-ELISA can be used to measure GETV antibodies in serum samples from various animals, including pigs.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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