Field evaluation of commercial Immunoglobulin M antibody capture ELISA diagnostic tests for the detection of Japanese encephalitis virus infection among encephalitis patients in Nepal

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1. Introduction

Japanese encephalitis (JE) virus is a mosquito-borne flavivirus and is the leading viral cause of childhood neurologic infection in countries of the Asia-Pacific region, including the Indian subcontinent. In tropical regions where JE is endemic, transmission generally occurs year-round, often with a peak during the rainy season, but in temperate climates there may be seasonal patterns of transmission and massive epidemics can occur. Besides the high mortality due to JE, life-long disability from neurologic sequelae among 50% of survivors makes it an even more devastating public health problem.

Although up to 50,000 cases of JE and 10,000 deaths are reported each year to the World Health Organization (WHO), these figures are widely acknowledged to be underestimates of the true global burden of disease. Approximately one-third of the world’s population lives in areas of Asia that are endemic for JE. Although some of these countries, including China, Japan, South Korea, Sri Lanka, and Thailand have introduced JE immunization programs resulting in a decline in JE incidence in these settings over the past 20 years, the burden of disease is still considerable in countries without access to vaccination. Applying a representative incidence rate of 2.5 per 10,000, as estimated from investigations using active case-finding with laboratory confirmation in a defined population, to the

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SUMMARY

Objectives: Japanese encephalitis (JE) is a devastating disease with high rates of death and disability that occurs particularly in resource-limited, rural regions of Asia. Simple, accurate and inexpensive diagnostics tests are vital for quantifying the burden of illness. This field study evaluated two commercial JE immunoglobulin M antibody capture (MAC) ELISA kits using samples from routine JE surveillance.

Methods: Positive (n = 132) and negative (n = 218) sera were randomly selected from patient samples collected as part of JE surveillance in Nepal in 2005. Samples were tested in a national public health laboratory with commercial kits produced by XCyton and Inverness (Panbio). Results were compared with those of the research lab-based reference standard, the Armed Forces Research Institute of Medical Sciences JE MAC ELISA.

Results: Positive and negative predictive values and 95% confidence intervals were 90% (82–95%) and 85% (79–89%) for Panbio1, 94% (88–98%) and 89% (87–93%) for Panbio2, and 84% (77–90%) and 96% (92–98%) for XCyton kits, respectively. Sensitivities of Panbio1, Panbio2, and XCyton kits were 71% (63–79%), 80% (72–87%), and 93% (88–97%); specificities were 95% (91–98%), 97% (94–99%), and 89% (85–93%), respectively. Overall percent agreement was 86% for Panbio1 and 91% for both Panbio2 and XCyton.

Conclusions: Both commercial kits had good predictive values when single serum samples from encephalitis cases were tested in a national laboratory. Either kit can be used in similar JE-endemic settings where co-transmission of dengue virus, a flavivirus which has strong cross-reactivity with JE, is limited. These results can inform decisions by countries and the World Health Organization laboratory networks on national-level use of these kits for JE surveillance.

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under-15-year-old population at risk in the absence of any intervention, there would be approximately 175,000 cases per year. A major reason for incomplete data on the incidence of JE is the lack of adequate laboratory- and population-based surveillance in many endemic countries. Even in countries that have functioning surveillance systems for acute encephalitis syndrome (AES), there is often limited availability of diagnostics and capacity to conduct laboratory testing to confirm the presence of JE virus (JEV) infection. Diagnosis on clinical criteria alone is insufficient to distinguish JE from other etiologies of central nervous system infection because JE symptoms are non-specific. A variety of laboratory techniques are available for confirmation of JEV infection, but the WHO-recommended methodology for JE surveillance purposes is demonstration of JE immunoglobulin M (IgM) antibodies in blood and/or cerebrospinal fluid (CSF).

Several research institutions around the world have ‘in-house’ IgM antibody capture enzyme-linked immunosorbent assays (MAC ELISAs), but some of these have shown variable performance when used outside of the research laboratory and in field laboratory settings. Fortunately, in the past few years, commercial JE MAC ELISA kits have become available. Commercial ELISA kits allow convenient, standardized, and simplified testing at public health laboratories and will thus improve and expand laboratory-based surveillance for JE in endemic countries. Such quality-controlled commercial assays for JE are a vital component of the WHO-coordinated regional JE laboratory network.

An evaluation of three newly available JE ELISA test kits was undertaken in 2005 using a panel of well-characterized specimens. About 30% of the panel comprised dengue-positive samples to evaluate the issue of cross-reactivity between dengue and JEV infections. Dengue virus infection can cause neurological disease, and there is also the possibility that a patient presenting with AES caused by a different pathogen could have concurrent asymptomatic dengue virus infection with IgM in serum. However, the composition of the sample panel was artificially high for dengue in that it is unlikely that dengue IgM would be present in the serum of 30% of patients tested during routine AES surveillance. Therefore, further evaluation of these kits was needed, in particular to determine the positive predictive value, when testing samples collected from patients presenting with symptoms compatible with encephalitis (i.e., in a real-world clinical setting).

The objective of the current study was to evaluate the diagnostic accuracy, specifically the predictive values, of two of the commercial JE MAC ELISA kits evaluated in the 2005 study – the JEV-Chex kit (manufactured by XCyton Diagnostics Ltd, India) and the Panbio JE-dengue IgM combo ELISA kit (manufactured by Inverness Medical Innovations formerly Panbio Ltd, Australia) – using samples collected during routine encephalitis surveillance in Nepal. Nepal has been tracking cases of encephalitis since 1978, and in 2004 a laboratory-based surveillance system for JE was established using the extensive acute flaccid paralysis/polio surveillance network. For JE surveillance, medical officers identify cases on the basis of a standard WHO clinical case definition for AES and collect at least one serum sample for diagnostic testing. An AES case is defined as a person of any age, at any time of year, with an acute onset of fever, a deterioration in mental status (e.g., confusion, disorientation, coma, or inability to talk), and/or new onset of seizures excluding simple febrile seizures. An assay produced by the Department of Virology, US Army Medical Component–Armed Forces Research Institute of Medical Sciences (AFRIMS), was used as the reference standard. AFRIMS is a recognized regional reference laboratory for Asia and is a designated WHO Collaborating Center. The AFRIMS, kit was the reference standard used in the previous 2005 study.

2. Methods

2.1. Study population

Samples for this retrospective study were randomly selected from among AES cases in 2005 for whom serum specimens were available in the sample archives, stored at −20°C after initial testing. The Nepal AES surveillance samples were transported under cold-chain conditions for testing with the AFRIMS ELISA at one of two designated laboratories: the National Public Health Laboratory (NPHL) of the Department of Health Services and the B. P. Koirala Institute of Health Sciences in Dharan. All surveillance samples collected and tested during 2005 (based on the result of AFRIMS JE IgM ELISA testing performed at NPHL) were randomized by technicians at the Walter Reed/AFRIMS Research Unit Nepal (WARUN). A randomized list was generated of all archived serum samples of AES cases from 2005 JE surveillance (both JE IgM-positives and JE IgM-negatives). The first 105 JE IgM-positive specimens in the randomized list and all the JE IgM-negative specimens listed consecutively between those positive specimens were selected for a total of 350 study specimens for testing with the commercial kits. Specimens (positive as well as negative) with inadequate volume, that were missing from the storage site, and/or those with indistinct NPHL ID numbers were skipped and replaced by the subsequent positive or negative specimens in the randomized list. The total study population selected should therefore be representative of the normal encephalitis patient population in Nepal in 2005.

2.2. Commercial (index) kits and reference standard

The two commercially available ELISA kits evaluated in this study, XCyton and Panbio, are interpreted based on a qualitative result indicating presence or absence of JE antibodies in the sample. Both index test kits use a cell culture-derived recombinant particulate JE antigen and, additionally, the Panbio assay uses recombinant dengue-1 through –4 antigens. The Panbio kit has dual antigens (JE and dengue) and the XCyton kit contains only a JE antigen.

The reference standard for this study was the AFRIMS in-house JE MAC ELISA performed at the AFRIMS laboratory. Trained and experienced technicians tested each study sample, either initially in 2005 as part of the routine quality control system that is in place for Nepal’s JE surveillance system (10% of negatives and all positives are retested with JE and dengue antigen for confirmation at AFRIMS) or if this result was not available, the sample was retested as part of this study.

2.3. Laboratory and data management procedures

Preparation and testing of surveillance specimens are described elsewhere. After receiving the selected 350 study subject specimens from NPHL, WARUN prepared two aliquots of each specimen. Laboratory personnel were blinded to each sample’s reference standard result when conducting the testing with the commercial kits. A single, trained laboratory technician at NPHL, who was masked to the reference standard assay results, tested each specimen with both index kits. The technician was trained in basic ELISA methodology and followed kit instructions based on the product insert, and laboratory supervisors were available to answer questions and monitor the testing. The test kits were run singly on each sample, with retesting conducted when
indicated, according to the index product information (e.g., an initial equivocal result). The initial testing and subsequent retesting were each completed within a one-week period. The technician completed a qualitative questionnaire on the usability of the two assays after completing the laboratory testing.

Final results for each sample with the index and reference assays were classified as 'JE-positive', 'JE-negative' (including those that were determined to be dengue-positive by Panbio), or 'equivocal' (including other suspected flavivirus infections determined by XCyton), according to the manufacturers' instructions. All results from the two commercial assays were entered into an Excel (Microsoft Corp., Seattle, WA, USA) database, rechecked against source documents, such as ELISA reader printouts, to confirm accurate data entry, and double-checked by personnel at WARUN. The samples were then unmasked (i.e., all reference results were matched to each sample) and a master database containing AFRIMS, Panbio, and XCyton results for each sample was created for analysis.

2.4. Data analysis

The study results were analyzed to determine the concordance or agreement, positive and negative predictive values, and sensitivity and specificity (definitions given below) of each of the two kits in relation to the AFRIMS JE IgM ELISA. For all quantitative measures, 95% exact binomial confidence intervals (CIs) were calculated. For the purpose of this evaluation and as per the protocol, equivocal results or flavivirus unspecified (defined according to the kit instructions) from the two test kits were coded as 'JE-negative' in the final analysis. Data were analyzed using Stata version 9.2 (StataCorp, College Station, TX, USA). Qualitative results from the technician's responses to the test kit usability questionnaire were recorded.

The positive predictive value (PPV) and negative predictive value (NPV) are defined as the probability of the subject having or not having JE disease following a positive or a negative test result, respectively. Predictive values are calculated using the likelihood ratios and assume the given prevalence is correct. Sensitivity is defined as the proportion of pathogen-positive subjects correctly identified, and specificity is the proportion of pathogen-negative subjects correctly identified, by the test. A measure of concordance or agreement between the index test results and the reference test results is calculated along with the kappa statistic, which provides a measure of the reliability of a categorical variable beyond the role of chance alone.16

### 2.5. Retesting of Panbio

The laboratory results from Panbio and XCyton testing at NPHL were analyzed for PPV and NPV, sensitivity, and specificity. The sensitivity of the Panbio kit was found to be low and inconsistent with that found in previous studies and evaluation settings. In order to investigate the possible reasons for the low sensitivity of this kit under the existing field conditions, a retesting of all the 350 samples with a new batch of Panbio kits was undertaken at NPHL and also at AFRIMS, using identical laboratory protocols. The first test results at NPHL are referred to hereafter as Panbio1, the second retest results as Panbio2, and the AFRIMS retest results as Panbio3. The results were compared to get a better understanding of whether there was a batch or transport/storage issue with the Panbio kit, or whether there were problems with laboratory testing processes in Nepal.

### 2.6. Sample size

A sample size calculation for a binomial proportion was used where the sample size (N) depends on: the Z score (1.645 for a 90% confidence limit); expected sensitivity or specificity of the test (p); and the desired precision of this expected proportion (L), set at 0.05. The expected sensitivity and specificity of these test kits was assumed to be at least 90%, and not less than 85% compared with the AFRIMS reference standard (with a precision of 5%). The formula,

\[ N = \left( \frac{Z^2 \times p \times (1 - p)}{L^2} \right), \]

with an estimated disease prevalence of 35%, yielded a sample size requirement of 98 JE-positives and 98 JE-negatives. To account for error in specimen handling and processing that occurs with any laboratory-based activity, at least 105 JE-positive and 105 JE-negative specimens were estimated.

### 3. Results

#### 3.1. Study population demographics

The demographic characteristics of the study population of 350 randomly selected AES samples from the 2005 surveillance population are shown in Table 1. The initial sample selection procedure used the NPHL-AFRIMSJE IgM result, which yielded 105 JE-positive samples. However, 132 of the 350 samples were JE-seropositive according to the testing done at AFRIMS (the reference standard for this study), giving a prevalence of 38%. None of the study samples were positive for dengue by the AFRIMS dengue test conducted at AFRIMS. The median age of subjects in the study was 12 years (mean 17 years), and 45% were female. The AES cases in this study had onset dates between 20 May and 17 December 2005, in effect capturing one JE transmission season spanning the months of June through October. With regard to district of residence, 59% of subjects in this study came from the four hyperendemic western districts and the city of Kathmandu. The average number of days from symptom onset to collection of the sample was 7 days (median 5 days), which is at the lower end of the 7 to 10 days recommended by the WHO.17

#### 3.2. Diagnostic accuracy and kit performance characteristics

##### 3.2.1. Initial analysis

The first round of testing was done at NPHL with both XCyton and Panbio kits run simultaneously on the 350 samples. For the Panbio kit (Panbio1), PPV and NPV were 90% (95% CI 82–95%) and 85% (95% CI 79–89%), respectively; the sensitivity was 71% (95% CI 63–79%) and the specificity was 95% (95% CI 91–98%). For the XCyton kit, the PPV and NPV were 84% (95% CI 77–90%) and 96% (95% CI 92–98%), respectively; the sensitivity was 93% (95% CI 88–97%) and the specificity was 89% (95% CI 85–93%).
3.2.2. Retest results

In the retest of the Panbio kit (Panbio2), undertaken because the sensitivity of the Panbio kit in the initial analysis was inconsistent with previous results, the sensitivity of the Panbio kit conducted at NPHL was 80% (95% CI 72–87%) and the specificity was 97% (95% CI 94–99%). PPV and NPV for Panbio were 94% (95% CI 88–98%) and 89% (95% CI 87–93%), respectively. The results of the NPHL initial (Panbio1) and retesting (Panbio2) with the Panbio kit, the Panbio testing by AFRIMS (Panbio3), and the XCyton kit were compared (Figure 1).

The cross-tabulation of the positive and negative results of the two commercial kits, including the two sets of Panbio testing, are shown against the AFRIMS MAC ELISA reference standard result in Table 2. Results of the analysis of key parameters of diagnostic performance of the two commercial kits compared to the AFRIMS reference standard are shown in Figure 1. Overall, the concordance or measure of agreement of the kits with the AFRIMS reference standard was very good and the same (91%) for both XCyton and Panbio2, but was slightly lower (86%) for Panbio1. The agreement values corresponded to kappa statistics of 0.69 (95% CI 0.61–0.77; \( p < 0.0001 \)) for Panbio1; 0.79 (95% CI 0.73–0.86; \( p < 0.0001 \)) for Panbio2; and 0.81 (0.75–0.87; \( p < 0.0001 \)) for XCyton. These statistically significant results indicate that the agreement between the index kits and the reference standard for Panbio and XCyton, respectively, are not likely due to chance alone.\(^{16}\)

3.3. Kit usability

In general, the technician’s responses to usability for each of the two kits were very similar, particularly with regard to preparatory steps such as additional equipment required, handling and processing of specimens, and clarity of instructions. According to the technician report, processing with the XCyton kit took about 4 h, with 2 h of hands-on time with 88 samples per plate (single well for each sample). Testing with the Panbio kit was marginally faster, taking approximately 3.25 h, with approximately 1.5 h of hands-on time, however testing only 43 samples (two wells for each sample for the JE and dengue antigens). No major difficulties were encountered with either kit.

4. Discussion

In many countries where JE is endemic there is almost no systematic, population-based routine surveillance for the disease.\(^{4}\) Where surveillance does exist, the quality of the system is variable, depending on multiple factors, including timely care-seeking behavior, recognition of symptoms, case finding and reporting, sample collection at the optimal time, and perhaps most importantly, the collection and laboratory testing of appropriate clinical samples because of lack of test reagents. It is absolutely essential, therefore, that easy-to-use, standardized, affordable test kits are available in order to strengthen JE diagnostic and surveillance capacity in resource-poor, endemic settings. This study was the first to do a field evaluation of the diagnostic performance characteristics of two relatively new commercial diagnostic assays for JE IgM detection when used with samples collected in a routine JE surveillance system and with testing conducted in a national-level laboratory. In particular, this study was able to determine the predictive values, positive and negative,
because the set of samples used for this study were randomly selected from a population of AES patients reported to Nepal’s national surveillance system. Because many JE-endemic countries have limited capacity for collection of CSF and thus serum is the primary sample available for JE diagnostic testing, we considered it important to evaluate both the commercial kits using serum samples collected during routine surveillance, even though CSF is the preferred diagnostic sample.

The predictive values are indicators of the usefulness of these kits in distinguishing patients who truly have JE from those who do not, when the tests are utilized in applied field settings for a given population prevalence. The Panbio kit demonstrated very good predictive values with Panbio1 (PPV 90% and NPV 84%) and slightly higher predictive values with Panbio2 (PPV 94% and NPV 90%). The XCyton kit had similarly good results with PPV at 84% and NPV at 96% for this population, with a prevalence of 38%. Both the Panbio and XCyton kits demonstrated excellent specificity (Panbio1: 95%; Panbio2: 97%; XCyton: 89%). These high specificity results are encouraging, because with the progressively more widespread implementation of JE immunization programs, the incidence of JE will decrease, and high specificity of assays will become increasingly important. The Panbio kit showed good sensitivity with the second run (Panbio2: 80%), but lower sensitivity with Panbio1 (71%), and the XCyton kit showed very good results for sensitivity (95%). Because predictive value is affected mainly by prevalence of disease in the population and the specificity of the test when used in populations where the disease is uncommon, in these evaluations it remained largely unaffected by the lower sensitivity observed for Panbio. These performance characteristics, in particular the high predictive values, indicate that both commercial assays are useful for JE surveillance in similar population settings.

In 2005, an evaluation of commercial JE ELISA kits was conducted at AFRIMS with a set of pre-selected serum specimens that were well-characterized as JE, dengue, or JE-negative specimens. The specificity of the Panbio kit was very similar, 99% (95% CI 97–100%) for the 2005 study compared to 95% (95% CI 91–98%) for Panbio1 and 97% (95% CI 94–99%) for Panbio2 in the current study. However, the sensitivity was much lower in the current study at 71% (95% CI 63–79%) for Panbio1 and 80% (95% CI 72–87%) for Panbio2 compared to the 2005 study at 89% (95% CI 82–94%). The fact that the confidence intervals of each of the parameter estimates overlap indicates non-statistically significant differences, except for the lower sensitivity for Panbio1 in the current study compared to the same parameter in the 2005 study, which is significantly different (Chi-square, p = 0.0001).

With regard to the XCyton kit, sensitivity was similar in both studies at 97% (95% CI 91–99%) and 93% (88–97%), but specificity was vastly improved from 65% (95% CI 59–71%) in the 2005 study to 89% (95% CI 85–93%) in the current study. This was a statistically significant difference (Chi-square, p < 0.0001). The XCyton assay does not include a dengue antigen and testing algorithm like the Panbio assay. Thus, the lower specificity in 2005 was likely a result of substantial dengue IgM cross-reactivity with the JE antigen in the serum samples. In the current study, however, the marked improvement in specificity was presumably because there were no detectable dengue antibodies in the population tested by the AFRIMS reference assay.

The retesting of all the samples with the Panbio kit showed that the performance characteristics of the three sets of testing (Panbio1 and Panbio2 at NPHL and Panbio3 at AFRIMS) were similar and the differences were not statistically significant. This suggests that a national laboratory such as NPHL is capable of producing similar caliber work to a reference laboratory using a commercial kit for JE, such as the Panbio kit. Although not statistically significant, differences in sensitivity were observed. These differences could be related to laboratory and transportation factors that were not measured in this study, including temperature control in kit transportation and storage. However, these differences could also reflect lot-to-lot variability in the commercial kit. These observations emphasize the importance of the WHO JE laboratory network with lab accreditation, quality control and proficiency testing to help account for such issues. In addition to using validated kits, the network includes standardized testing protocols, procedures for calibration of equipment and instruments, and a strong laboratory quality assurance program.

Overall, the study population was comparable to the broader AES surveillance population. In a review by Wierzb and colleagues of JE surveillance in Nepal between 2004 and 2006, 32% of AES cases with diagnostic specimens collected (3198/4652 or 69%) were IgM-positive for JE. This compares to a slightly higher figure of 38% in this study population. Just over half (52%) of the AES cases in this study came from the four western hyperendemic Terai districts, whereas 44% of the 2004 to 2006 AES surveillance cases with specimens were from these four districts. Of those cases, the mean age was 18 years, and 43% were female. The population in this study was comparable, with a mean age of 17 years and 45% females. It should however be noted that approximately twice as many JE cases were detected during the 2005 season (n = 664) than the one in 2004 (n = 371), which could reflect increased case detection or normal seasonal variability in JE cases.

A strength of this study is that it used a large number of randomly selected samples from clinically-diagnosed encephalitis cases within a national AES surveillance system. These samples were from Nepal’s fully-functional population- and hospital-based surveillance system for AES, which has covered more than 24 endemic districts since 2004. This study also evaluated the capabilities of a national laboratory (NPHL) compared to a reference laboratory (AFRIMS) in the use of commercial kits for JE (Panbio2 compared to Panbio3). In addition, the reference standard – the AFRIMS JE ELISA kit – was selected because it had been used in previous evaluations of the same commercial kits and could therefore provide a measure of comparability to prior evaluations.

The JE surveillance system in Nepal is very strong and one of the best models of JE surveillance in a developing, JE-endemic country. The system does have some limitations for this study which should be noted. WHO surveillance standards support the diagnosis of JE in AES from a single serum sample, and in Nepal mostly single serum samples are collected for diagnosis. However, one-third of AES cases do not have a sample collected. Frequently, the sample is collected early in the course of illness, without a second convalescent sample collected. Samples, including those that are JE-negative, are not routinely tested for other etiologies. WHO standards recommend that if an initial serum specimen is negative, a further sample should be taken 7 to 10 days following onset to allow antibodies to rise to a detectable level.

Dengue virus circulation has not been reported extensively in Nepal, and no dengue IgM was detected in the samples from 2005 tested in our study, whereas many countries endemic for JE have co-circulating dengue virus. Thus a limitation of our study is that the AES population is not necessarily representative of populations in other countries in the Asia-Pacific region. Another limitation is that the AFRIMS reference testing in many cases was conducted earlier than the study evaluation of the commercial kits, so there is a possibility that antibody could have degraded during sample storage. However, this is unlikely as good storage conditions were maintained in the interval between AFRIMS testing and the study testing.

The performance characteristics of the Panbio and XCyton JE kits in this evaluation suggest that these kits could be considered acceptable in routine JE diagnostic testing in a similar patient population. To better understand the impact of dengue virus...
circulation and potential for cross-reactivity with JE IgM in these commercial kits, it would be useful to conduct additional studies in a country in Asia where both JE and dengue are endemic. Further evaluation of the usability of the kits from additional laboratory technicians would be helpful in establishing functionality of these commercial kits. In settings with significant dengue co-circulation, false-positive results could be obtained. This is programmatically of low significance in routine surveillance before JE vaccination, but as JE vaccination becomes standard practice, false-positive results could give an erroneous indication of vaccine failure. After JE vaccine introduction, samples collected for case confirmation in JE surveillance should be CSF, which, unlike serum, does not have antibody after immunization. XCyton recommends the use of their kit on CSF, especially where dengue is co-endemic, and both kits recommend confirmatory testing with plaque-reduction neutralizing testing.

In summary, both commercial kits performed well in this field evaluation in a JE-endemic area with limited dengue virus circulation. PPV, NPV, sensitivity, and specificity values demonstrated generally good performance characteristics for diagnostic accuracy, indicating that the two kits would perform reasonably well within national surveillance systems for detection of JE among encephalitis cases. Additional evaluation in other JE-endemic areas where there are high rates of dengue virus transmission would be valuable. The results of this study provide important information that will assist with designing laboratory-based surveillance systems for JE and help facilitate ascertainment of JE disease incidence in endemic populations. Population-based surveillance for JE that enables understanding of disease burden and epidemiological patterns is an important contributing factor in national-level decision-making on the introduction of JE vaccine.

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