Comparison of Screening Dilution and Automated Reading for Antinuclear Antibody Detection on HEP2 Cells in the Monitoring of Connective Tissue Diseases

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Background: Indirect immunofluorescence plays a major role in the detection of antinuclear antibodies (ANAs) and follow-up of their titers in the context of connective tissue diseases. Given the numerous unfavorable features of the conventional manual reading of HEP2 slides (need of time and expert morphologists for the reading, lack of standardization, subjectivity of the interpretation), the biomedical industry has developed automated techniques of slide preparation and microscope reading. **Methods:** We collected 49 sera beforehand analyzed by the conventional reading of slides. They were prepared again by QUANTA-Lyser[®] and re-analyzed in four different conditions: two dilutions of screening (1/40 and 1/80), two different systems of analysis, NOVA View[®] automated reading (INOVA Diagnostics), then confirmation by the operator, and conventional manual reading by two different qualified operators. The analysis was realized in blind of the first interpretation and clinical diagnosis. The sera were classified in four groups, on the basis of the results of the first analysis: negative sera (titer < 1/160; 11 patients), low positives (titer at 1/160; 18 patients), moderated positives (titers between 1/320 and 1/640; 10 patients), and strong positives (titers between 1/1,280 and 1/2,560; 10 patients). **Results:** Among the 49 patients, 13 presented a connective tissue disease includ-

ing 4 systemic scleroderma (SS), 3 rheumatoid arthritis (RA), 2 Goujerot-Sjogren (GS), 2 systemic lupus erythematosus (SLE), 1 polymyositis (PM), 1 Raynaud's syndrome (RS), and 1 CREST syndrome. One patient presented both an SLE and an SS. Regarding the screening dilution, the 1/40 dilution is less specific than the 1/80 dilution for both the systems of analysis (5.6% vs. 16.7% for the manual reading, and 27.8% vs. 50% for the automated reading). It also generates statistically more false positives ($P = 0.037$ for the conventional analysis and $P = 0.003$ for the automated system). The automated NOVA View[®] reading of slides allows a gain in specificity for both dilutions, and also statistically less false positives ($P = 0.002$ at the 1/40 and $P = 0.0006$ at the 1/80), and detriment of the sensitivity at the highest dilution (84.6% vs. 92.3% with manual reading). Thus, according to our analysis of 49 sera, the automated NOVA View[®] system of reading of slides at the dilution 1/80 seems to be a successful condition for the detection of ANAs on HEP2 cells, close to the significance ($P = 0.067$). **Conclusion:** The automated NOVA View[®] reading of slides allows saving time, and an improvement in the standardization. Nevertheless, it requires a confirmation by a qualified operator, to interpret mixed patterns in particular. J. Clin. Lab. Anal. 30:471–478, 2016. © 2016 Wiley Periodicals, Inc.

Key words: Connective tissue disease; ANA; IIF; HEP2 cells; NOVA View[®] automated reading of slides; conventional manual reading

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INTRODUCTION

The detection of antinuclear antibodies (ANAs) is an important tool for diagnosing many connective tissue diseases, especially systemic lupus erythematosus (SLE), Goujerot-Sjogren (GSJ), systemic sclerosis (SS), polymyositis, and mixed connective tissue disease (MCTD) (1). The reference method for screening nonorgan-specific autoantibodies is the indirect immunofluorescence (IIF) on HEP2 cells. It detects a wide range of autoantibodies to nuclear and cytoplasmic antigens and requires a manual interpretation by a qualified technician. After a screening of ANAs, the titer is obtained by serial dilutions of the serum. In fact, it is represented by the inverse of the last positive dilution. Nevertheless, the lack of standardization of this method, linked to the type of microscope and to interpretation by different operators, still remains a concern. Moreover, it is very time consuming. Therefore, the introduction of automation can eliminate these sources of variability, provides an objective output, and facilitates the exchange of results between labs and clinicians. The purpose of this study was to determine the best threshold of serum dilution (40 vs. 80) for the screening of ANAs, with the conventional manual reading and the automated method (NOVA View®) on HEP2 cells. The second objective was to compare the diagnostic performances of manual and automated interpretations with both the dilutions (40 and 80), according to the clinical diagnosis. Finally, this study focused on the conflicts in patterns analyzed between the two methods.

PATIENTS AND METHODS

Between November and December 2013, 49 sera were prepared and analyzed by the conventional method (Immunofluorescent Assay, QUANTA-Lyser®, INOVA Diagnostics) at the screening dilution 1/80 in our laboratory, consisting of 11 negatives (titer < 1/160), 18 low positives (titer at 1/160), 10 moderated positives (titers between 1/320 and 1/640), and 10 strong positives (titers between 1/1,280 and 1/2,560). According to our laboratory's practices, all the sera were analyzed by two different qualified operators and when ANAs were detected with a titer upper to 160 for adults and 80 for children, anti-ENA and anti-DNA antibodies were looked for by an Enzyme-Labeled Anti-Isotype Assay (ELiA, Phadia/Thermo Fisher Scientific) to precisely identify the target of antibody. Moreover, the specificity of the antibody can be sometimes detected by an immunodot blot technique. All sera were addressed by physicians within the framework of suspicion of inflammatory disorders or follow-up of known autoimmune disease. Each patient was followed in our teaching hospital of Saint-Etienne,

France. Patient's clinical data were retrospectively collected and anonymously computed with respect to the latest version of the Helsinki Declaration of Human Research Ethics.

For the study, we collected 49 sera, stored them at -20°C, and after defrosting, they were reanalyzed in 5 not consecutive days, at the rate of one series well-balanced by day. To verify the validity of the system, two levels of controls (negative and positive) were used at the beginning of each series. All the samples (diluted in 1/40 and 1/80) were prepared with the Quanta-Lyser® analyzer, and the fluorescence pattern and titers were interpreted visually by two qualified operators and by the automated system NOVA View® with a user's validation, in blind of the clinical diagnosis and first interpretation.

The NOVA View® automated system contains an Olympus microscope with an automated stage, a CCD digital camera, an LED light source, and a software that controls the motorized stage, and takes and archives digital images after categorization of the samples as positive or negative.

Statistical Analysis

Statistical analysis was performed by calculating the value of sensibility and specificity of each of the four conditions. The Wilcoxon signed-rank test was used to compare the false positives between the four different conditions and Student's test was used to determine the degree of agreement between the methods of interpretation and between the dilutions.

The statistical analysis of the data obtained in this study was performed using Prism, Version 5.02 (GraphPad, La Jolla, CA). A *P* value less than 0.05 was considered to indicate statistical significance.

RESULTS

Patient's Characteristics (Table 1)

The median age was 59 years (12–94 years). The group of patients who presented initially a moderated positive titer (from 1/320 to 1/640) of ANA presented the highest median age (64 years), whereas the minimal (39 years) was in the group of initially negative patients. The sex ratio was 1.88 (W/M). The most represented connective tissue disease was systemic scleroderma (Table 1).

From the 49 patients, 13 were considered as “real positives” concerning the ANA detection: 4 patients with SS (with 1 suffering from SLE); 1 SLE; 3 RA; 2 GS; 3 different other patients with PM, RS, and CREST syndrome, respectively.

TABLE 1. Characteristics of the Patients of the Study

General characteristics	Median age	59 years
	Age range	12–94 years
	Sex ratio (W/M)	1.88
Connective tissue diseases (number of patients) <i>N</i> = 13 patients “real positives”	SS	4
	RA	3
	GS	2
	SLE	2
	PM	1
	RS	1
	CREST syndrome	1
Other autoimmune diseases (number of patients) <i>N</i> = 17 patients	Glomerulonephritis	4
	Psoriasis	3
	IBD	3
	Horton disease	2
	Spondyloarthritis	2
	Autoimmune thrombocytopenic purpura	1
	Type I diabetes	1
	Biermer’s disease	1
	Juvenile idiopathic arthritis	1

SS, systemic scleroderma; RA, rheumatoid arthritis; GS, Goujerot-Sjogren; SLE, systemic lupus erythematosus; PM, polymyositis; RS, Raynaud’s syndrome; IBD, inflammatory bowel disease.

The main characteristics of the patients included in the study are indicated, in particular their autoimmune diseases.

SLE was associated with SS in one patient and spondyloarthritis was associated with IBD in one other patient.

Comparison of Dilutions 1/40 and 1/80 for the Screening of ANAs in HEP2 Cells

Among the 11 sera (initially negatives), the manual interpretation was positive in the dilution 1/40 and negative in the dilution 1/80 for 5 sera. The patterns of fluorescence were four speckled and one homogeneous. None of these patients presented a connective tissue disease. On the contrary, there was no conflict between the two dilutions with the automated NOVA View® interpretation, the 11 sera remained negatives. Among the 18 sera that were initially low positives (titer 1/160), the second analysis with the automated system revealed a conflict for 9 sera, which were positives only in the dilution 1/40. The patterns were speckled (four patients), homogeneous (three patients), and nucleolar (one patient). The pattern was unrecognized for the last patient, who was the only one to present a connective tissue disease—myopathic dermatomyositis. The manual viewing of this slide revealed a positivity of ANA, with a speckled pattern, at the two dilutions. Globally, the manual interpretation was positive at the two dilutions for the 18 sera. As expected, there were no differences between the two screening dilutions for the 20 other sera (titers from 1/320 to 1/2560).

Fig. 1. Differences of false positives between the two systems of reading and the two screening dilutions are significant.

The rates of false positives and false negatives are presented for each condition, even if false negatives are not comparable because of the few number of events.

P value less than 0.05 is considered to indicate statistical significance.

Fig. 2. ROC “curves” for different systems of reading and dilutions. The assimilated ROC “curves” are presented for each condition. Only the P value closest to the significance is indicated in this figure ($P = 0.067$); others are indicated in Table 3.

Comparison of Automated and Manual Methods for the Screening of ANAs in HEP2 Cells

At the screening dilution 1/40, ANAs were detected only by the manual interpretation for eight sera initially classified as negatives, with patterns speckled (six sera) and homogeneous (two sera). None of these patients presented a connective tissue disease. At the screening dilution 1/80, ANAs were detected only by the manual interpretation for 13 sera (among whom 4 initially classified as negatives), with patterns speckled (7 sera), homogeneous

TABLE 2. Performances of Manual and Automated readings of HEP2 Cells for the Detection of ANAs at the Two Dilutions of Screening

	Manual 1/40	Manual 1/80	Automated 1/40	Automated 1/80
Sensitivity (%)	92.3	92.3	92.3	84.6
Specificity (%)	5.6	16.7	27.8	50
Positive predictive value (%)	26.1	28.6	31.6	37.9
Negative predictive value (%)	66.7	85.7	90.9	90
Rate of false positives (%)	69.3	61.2	53.1	36.7
Rate of false negatives (%)	2	2	2	4
Rate of disease in the population (%)	26.5	26.5	26.5	26.5
Rate of positivity in the population (%)	93.9	85.7	77.6	59.2
Positive likelihood ratio test	0.98	1.11	1.28	1.85
Negative likelihood ratio test	1.38	0.46	0.28	0.15

(7 sera), mixed homogeneous and speckled (1 serum), and nucleolar (1 serum). Only one of these patients presented a connective tissue disease—the patient with the myopathic dermatomyositis. Moreover, the specificity of ANA for this patient was unknown (absence of identification by ELiA and immunodot).

Comparison of the Four Conditions for the Screening of ANAs in HEP2 Cells (Table 2 and Fig. 1)

The sensitivity is correct for the manual interpretation—same at the two dilutions, whereas it is slightly decreased in the 1/80 dilution with the automated system (more false negatives) even if there are very few events to be able to compare this criterion between the four conditions. The specificity of the test is better with the automated system than with the conventional reading, and for the highest dilution of the serum. In fact, the rate of false positives is highest with the conventional reading at the dilution of 1/40. This is concordant with the low positive predictive value (PPV), which represents the probability of having a connective tissue disease if the screening test is positive. On the contrary, this PPV is best if the detection is realized with an automated NOVA View[®] reading of slides, at the highest dilution of 1/80 (37.9%). The negative predictive value (NPV), which represents the probability of not having a connective tissue disease if the screening test is negative, is strongly improved by the dilution of the serum in the manual analysis (from 66.7% to 85.7%) but not in the automated reading where the NPV is slightly upper at the lower dilution of 1/40. Globally, the automated reading of slides allows an improvement in the predictive values. The positives and negatives likelihood ratios are the most informative with the automated interpretation at the highest screening dilution.

The degree of agreement between NOVA View[®] interpretation and manual viewing of slides is better at the

dilution 1/40 (83.7%, $P = 0.16$) than at the dilution 1/80 (73.5%) for which there are significantly more positive samples with the manual viewing ($P = 0.02$). The degree of agreement is satisfactory between the two dilutions for each system—87.8% for the manual analysis ($P = 0.49$) versus 81.6% for the automated analysis ($P = 0.11$). To summarize, the only statistically significant difference was between automated and conventional readings at the dilution 1/80.

ROC Curve Analysis for the Two Methods of Interpretation and for the Two Screening Dilutions (Fig. 2 and Table 3)

The performance of the automated system seems to be better than the manual viewing of slides, for example, the AUC (area under the assimilated ROC “curve”) passes from 0.54 to 0.69 at the dilution 1/80. In the same way, the dilution 1/80 seems to be more adapted to the test than the dilution 1/40, because the area passes from 0.6 to 0.69 in the automated reading. The area concerning the manual reading at 1/40 cannot be determined, because it is too low (very close of 0.5), thus this condition is not suitable for the ANA detection. On the contrary, the automated reading of slides, at the dilution 1/80, is the most discriminator among the four conditions. To summarize, an automated interpretation, after a dilution screening of 1/80, seems to be a successful test for the detection of ANA in HEP2 cells, because, in spite of a light decrease in sensitivity, it allows an important gain in specificity, with a P value close to the significance ($P = 0.067$).

Conflicts With Clinical Diagnosis of Autoimmune Disease and Between Patterns of ANAs (Table 4)

There is a conflict between clinical diagnosis of connective tissue disease and negativity of the automated NOVA View[®] interpretation at the dilution of 1/80 for two patients.

TABLE 3. Conflicts With Clinical Diagnosis of Autoimmune Disease and Between Patterns of ANA

Patient	Manual interpretation			Automated interpretation			Autoimmune pathology
	1/40	1/80		1/40	1/80		
1	Speckled	Speckled	Speckled	Speckled		Homogeneous	Behcet
2	Homogeneous + cytoplasmic	Homogeneous + cytoplasmic	Homogeneous + cytoplasmic	Homogeneous + cytoplasmic		Speckled + cytoplasmic	None identified
3	Speckled	Speckled	Centriole	Speckled		Negative	None identified
4	Homogeneous + speckled	Homogeneous + speckled	Homogeneous + cytoplasmic	Homogeneous + cytoplasmic		Homogeneous + speckled + cytoplasmic	None identified
5	Homogeneous + cytoplasmic	Homogeneous	Homogeneous	Homogeneous		Homogeneous	Psoriatic arthritis
6	Perinuclear + cytoplasmic	Speckled	Speckled + cytoplasmic	Speckled + cytoplasmic		Speckled + cytoplasmic	Rheumatoid arthritis
7	Speckled						Scleroderma + SLE

The first patient was an 18-year-old girl affected by amyopathic dermatomyositis. ANAs with a speckled pattern were initially detected during the first interpretation with an initial titer of 1/160. For the study, the serum was reanalyzed with the two methods. ANAs with speckled pattern were detected again by the manual interpretation at the two dilutions of screening. On the contrary, they were detected only at the dilution 1/40 for the automated interpretation. The specificity of these ANAs cannot be determined by our screening tests (anti-DNA antibodies, anti-ENA antibody testing, and immunodot targeting the ANAs in myositis).

The second patient was a 61-year-old woman affected by secondary GSI syndrome linked to spondyloarthritis. ANAs were never identified, neither in the first interpretation, nor in the second one (manual and automated).

Moreover, a conflict in the interpretation of the pattern was observed for seven sera (Table 4). We can note a conflict between speckled and homogeneous patterns for two sera, of which one patient was affected by Behcet's disease.

For three patients without any connective tissue identified, the difference of pattern is probably unpredictable and not really interesting to take into account. Among these cases, a centriole pattern was curiously observed only by the automated interpretation, at the dilution of 1/40. The homogeneous pattern displayed by the manual interpretation was not found by the manual analysis for one serum. On the contrary, a speckled pattern was observed only by the automated interpretation. Concerning the patients with an identified autoimmune disease, an additional speckled pattern was identified by the automated analysis at 1/80 for a patient with psoriatic arthritis. Moreover, a perinuclear and cytoplasmic pattern was surprisingly observed by the manual interpretation at the dilution 1/40 for one patient, affected by a rheumatoid arthritis, whereas it was a homogeneous pattern at the dilution 1/80 observed by the automated interpretation. An additional cytoplasmic pattern was visualized only by the automated interpretation. Patterns between the two methods demonstrated a very good agreement of 85.7%.

DISCUSSION

Detection and quantification of ANA by immunofluorescence assay represents a serological hallmark of connective tissue diseases, in association with clinical criteria. The question of the cut-off of dilution for screening is very important and hence discussed. INOVA[®] recommends a screening dilution 1/40, but in our daily laboratory's practice, we noticed that low positive titers of ANAs were frequently not specific of autoimmune disease, that

TABLE 4. The Automated System at the Dilution Screening of 1/80 Allows the Most Significant Detection of ANA on HEP2 Cells

	Manual 1/40 ^a	Manual 1/80	Automated 1/40	Automated 1/80
Area	Uninterpretable	0.54	0.6	0.69
Standard error	Uninterpretable	0.09	0.09	0.08
95% Confidence interval	Uninterpretable	(0.37–0.72)	(0.43–0.77)	(0.53–0.85)
<i>P</i> value	Uninterpretable	0.63	0.29	0.067

Areas under the assimilated ROC “curves” and their standard error, 95% confidence interval, as well as the *P* value for each condition are indicated.

^aArea under curve for the manual analysis of condition at the dilution 1/40 cannot be determined because it was too low (close to 0.5).

is to say, false positives. In fact, ANAs are not specific of connective tissue diseases and can be detected in many nonpathological situations (high age, pregnancy, taking of medicine) or non-autoimmune diseases (infections, cancers, hemopathies) (1). In a Brazilian population, ANAs were present in 12.9% of healthy individuals, at the 1/80 dilution (2). In fact, the reference screening dilution provided by the manufacturer is based on ethnic populations maybe different than ours. It becomes mandatory for each laboratory to determine its own screening dilutions for the local population to distinguish healthy and diseased states. In the study, we wanted, first, to compare the performances of the two screening dilutions of sera: 1/40 and 1/80 (this latter dilution was usually used for the detection of ANAs in our laboratory). Second, we tested the performance of the NOVA View[®] automated system that allows a completely automated reading of IIF images and the subsequent interpretation of the fluorescence pattern by software algorithms, and compared it to manual viewing of slides by two different experienced and certified medical technologists or biologists. This automated system saves time, reduces variability, and provides an interesting approach to standardize ANA interpretation.

For the first point, we can see that the screening dilution 1/80 is the most successful and more specific than the 1/40 dilution that generates too false positives. Assimilated ROC “curves” analysis showed the best area at the dilution 1/80, for the automated method (*P* = 0.067). In this way, the study of Ghosh et al., realized on 213 sera, showed that it was the best dilution for discrimination between healthy individuals and SLE (3), and it is also recommended by other authors (4).

For the second point, the automated interpretation allows reducing the rate of false positives in a consequent way, hence improving the specificity of the test (from 5.6% to 27.8 % at the dilution 1/40 and 16.7% to 50% at the dilution 1/80). The overall specificity of six automated systems of reading was superior with NOVA View[®] as shown in the study of Bizzaro et al. (Italy), about 89.2%. This could be explained by differences in the sampling: the precedent study was conducted on 92 ANA-positive sera and 34 ANA-negative sera, whereas the more

reduced size of our population could be a limiting factor for the interpretation of the results. The overall sensitivity in the Italian study was closer to ours, about 96.7% (5). The higher rate of positivity with the conventional reading could be explained by the unconscious fear of the technician or biologist to miss a diagnosis. In this way, the automated interpretation is probably more objective but presents, however, the limit of a decrease in sensibility, which is most important for a screening test.

In a study of Krieger, the correlation of the automated reading system to the conventional method was 88% (311 samples out of 353), and was improved to 93% by the reinterpretation run (328 out of 353). This correlation was lower in our study of limited size (of 83.7% at 1/40 and 73.5% at 1/80) (6).

Obviously, the final result of the determination in IIF depends also on the quality of the cellular substrate and quality of the slide's preparation, which is in all cases realized by an automated system in our laboratory (QUANTA-Lyser[®], INOVA Diagnostics).

Regarding the pattern identification, NOVA View[®] seems to have difficulty in producing the same pattern on all dilutions of the samples, for 5 sera of the 49 analyzed (10%), and there were differences concerning the pattern of the fluorescence for 3 sera of patients without any autoimmune disease (homogeneous, speckled, and centriole), according to the type of system. In a study of Mariz et al., the nuclear dense fine speckled pattern occurred more frequently in healthy individuals, whereas the homogeneous pattern was observed only in patients with autoimmune rheumatic diseases (2). This more specific pattern “homogeneous,” was not found by NOVA View[®] at the 1/80 dilution for two of the three patients. The centriole pattern observed only by the automated system at 1/40 is more surprising in a patient without any autoimmune disease (in particular, no CREST syndrome).

In Behcet's disease, there is classically no systematically ANA described (7) but in a study of Chun et al., concerning 554 cases of Behcet's disease, the authors showed that patients with this disease had significantly more ANA

than healthy control group ($P = 0.0003$); the patterns were mainly speckled (37% of the cases) and homogeneous (10.9% of the cases) (8). In this study, the patient with Behcet's disease presented ANAs with a homogeneous pattern only with the automated analysis, at the highest dilution. Concerning psoriasis, the arthritic form can be associated with ANA in some patients (9, 10), with a predominance of homogeneous nuclear pattern (11). An additional speckled pattern was found by the automated interpretation at 1/80 dilution only. Rheumatoid arthritis, scleroderma, and SLE are generally associated with ANAs. The pattern coarse speckled (12) is the most frequent during the rheumatoid arthritis, whereas nucleolar and homogeneous are found during scleroderma and SLE, respectively. Curiously, these patterns were not observed for the two patients suffering from these pathologies.

The use of the automated NOVA View[®] system of reading fluorescence on HEP2 cells offers a friendly software and the ability to interface with bar-coded slides and at the dilution 1/80, it seems to be more successful, as it can considerably improve the specificity and allows saving time and a possible establishment of ANA standardization. The main difficulties of this automated reading are the risk of generating false negatives, as it was the case for one of our patients (the patient with a myopathic dermatomyositis), for whom ANAs were not detected at the highest dilution by the NOVA View[®] system. Even if the serum presented a low antibody titer (1/160), this decline of sensitivity could be harmful for a screening test, whose aim is to detect all positive patients. Second, if the majority of automated systems are able to classify positive samples with the classic patterns, it seems to be more difficult for cytoplasmic (Golgi) or rare nuclear patterns (as PCNA, nuclear dots, centriole, etc.) (13). Nevertheless, the main objective of all the automated systems is, for the moment, to discriminate between positive and negative samples, and as declared by the producers, the identification of pattern is a secondary aspect, which must be validated visually by the qualified operator. The other problem is the reading of mixed patterns, which is often difficult with the automated reading, and can be solved by the dilution of the serum so that the masked pattern becomes visible.

In conclusion, the automated NOVA View[®] reading system is very promising, as a cost-effective method, improving the analytic precision, but it continued to be used as a screening method that must be followed by a human visualization and a second-level test that is able to detect the presence of several antibodies. Another possible advantage of this method, not tested in this study, is the measuring of fluorescence intensity to evaluate the antibody concentration, avoiding the step of titration. Moreover, this study confirms the interest of the 1/80 dilution screening, versus the 1/40, which generates too false

positives. It could be considered as the dilution of reference for the screening of ANAs in HEP2 cells.

CONFLICT OF INTEREST

Mr. Boutin is employed at INOVA Diagnostics, the society that manufactures and markets the QUANTA-Lyser[®] and NOVA View[®].

TAKE-HOME MESSAGES

- The best serum dilution for the screening of ANA on HEP2 cells is 1/80 and can be considered as the reference dilution.
- The automated detection of ANA on HEP2 cells with NOVA View[®] is very useful for the diagnosis of connective tissue disease.
- It reduces variability of the interpretation, providing a tool for the standardization, compatible with the current constraints of the biological medical labs.
- The manual viewing of images stays essential, after the automated reading, to verify the patterns or to take into account the clinical context for the samples close to the cut-off, hence limiting the risk of false negatives.

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