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Testing of Susceptibility of *Mycobacterium tuberculosis* to Pyrazinamide with the Nonradiometric BACTEC MGIT 960 System

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The reliability of the novel BACTEC MGIT 960 pyrazinamide (PZA) kit (Becton Dickinson Microbiology Systems, Sparks, Md.) was assessed for testing of susceptibility of *Mycobacterium tuberculosis* to PZA. Results generated by the BACTEC MGIT 960 system (Becton Dickinson) were compared with those obtained with the BACTEC 460TB system. Extensive proficiency testing (phase I) and reproducibility testing (phase II) as well as susceptibility testing of blinded strains of *M. tuberculosis* from the Centers for Disease Control and Prevention (phase III) were performed prior to testing 58 strains isolated from clinical specimens (phase IV). After resolution of discrepant results obtained by the two BACTEC methods by two other laboratories which acted as independent arbiters (phase V), overall agreement of the BACTEC MGIT 960 system with the BACTEC 460TB system for PZA testing of phase IV strains was 96.6%. Between the two systems there was no statistically significant difference in time until results were obtained, i.e., 6.8 days (BACTEC MGIT 960) versus 5.4 days (BACTEC 460TB), the latter not counting the time required for a subculture with a growth index of 200, however. The new BACTEC MGIT PZA susceptibility testing procedure works equally well for inocula prepared from liquid (MGIT) and solid (Löwenstein-Jensen) cultures. PZA MGIT medium in plastic tubes yielded results equivalent to medium dispensed in glass tubes.

Since its introduction in 1952, pyrazinamide (PZA) has remained one of the most important components in an effective treatment regimen for tuberculosis, mainly affecting semidormant, intracellular *Mycobacterium tuberculosis* (7). The drug is, however, active at a lower pH value only, making drug susceptibility testing in the clinical mycobacteriology laboratory more demanding. Based on a modified 7H12 medium at pH 6.0, the radiometric BACTEC 460TB technique is, to date, the only culture-based method for PZA susceptibility testing (see the BACTEC 460TB system product and procedure manual [publication MA-0029 of Becton Dickinson and Company, Sparks, Md.] by S. H. Siddiqi) which is recommended by the National Committee for Clinical Laboratory Standards (6).

To eliminate the hazards associated with the use of ^{14}C-labeled substrates and the use of needles by laboratory personnel, nonradiometric culture systems have been established in the past few years which offer, in parallel, rapid, growth-based testing of susceptibility of *M. tuberculosis* to front-line drugs. Among those novel methods is the MGIT (Mycobacteria Growth Indicator Tube) technology, for which results on susceptibility testing are available for isoniazid (INH), rifampin (RMP), ethambutol (EMB), and streptomycin (STR) (1, 8), but not for PZA, due to the well-known drawbacks of measuring the drug's activity at a more acidic pH.

We report here the results of susceptibility testing of *M. tuberculosis* to PZA performed in the fully automated BACTEC MGIT 960 system (Becton Dickinson), utilizing the novel BACTEC MGIT PZA medium (Becton Dickinson) which contains a modified 7H9 broth with a pH value adjusted to 5.9. Our study consisted of five phases during which results were compared on a one-to-one basis with those generated by the standard PZA susceptibility testing by the BACTEC 460TB system.

MATERIALS AND METHODS

Study sites. PZA testing was done by the Swiss National Center for Mycobacteria (center 1) and the German Reference Center for Mycobacteria (center 2). Two additional laboratories (California Department of Health Services, Berkeley, Calif., and VA Medical Center, West Haven, Conn.), acted as independent arbiter sites to which strains were sent for retesting if BACTEC MGIT 960 results were discrepant from BACTEC 460TB results.

Reagents. The BACTEC MGIT 960 PZA medium has recently been developed by Becton Dickinson. The tube contains 7 ml of a modified 7H9 broth adjusted to pH 5.9 and 110 μl of a fluorescent indicator (Tris 4,7-diphenyl-1,10 phenanthroline ruthenium chloride pentahydrate) in a silicone rubber base. In the PZA kit there are two vials of lyophilized PZA and six vials of BACTEC MGIT PZA supplement containing bovine serum albumin, dextrose, catalase, polyoxyethylene stearate, and oleic acid.

Drug concentration. The PZA concentration was 100 μg/ml for both BACTEC MGIT 960 and BACTEC 460TB tests.

Identification of strains. All *M. tuberculosis* strains included in this study had been identified by classical biochemical criteria (5).

Preparation of inocula. Prior to inoculating the PZA set tubes, 0.8 ml of BACTEC MGIT 960 PZA supplement was added to both growth control and PZA tubes and 100 μl of PZA solution was added to the PZA tubes with an Eppendorf repeater pipette. Inocula were prepared following the instructions of the manufacturer. (i) MGIT cultures were used for PZA susceptibility testing no sooner than the day following instrument positivity (day 1) and no later than 5 days following the day of instrument positivity (≥1 day, ≤5 days). On days 1 and 2 following positivity, an undiluted inoculum was used, while on days 3 through 5 suspensions were diluted 1:5 with sterile saline. Half a milliliter was then inoculated into the MGIT PZA tubes by using a GILSON Pipetman with sterile tips and safety plugs. The growth control tube was inoculated with 0.5 ml of a 1:10 dilution of the *M. tuberculosis* suspension. (ii) Cultures grown on Löwenstein-Jensen (LJ) medium were used for PZA susceptibility testing no later than 14 days after the first appearance of colonies on the slant. Colonies were scraped from the medium with a sterile loop. A suspension adjusted to be equivalent to

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TABLE 1. Testing of proficiency (phase I) and reproducibility (phase II) of *M. tuberculosis* strains at the two reference centers

Phase	No. of results	No. of results agreeing with reference method[a]	Agreement (%)
I[b]			
BACTEC MGIT 960 system	20	20	100
BACTEC 460TB system	20	20	100
II[c] (BACTEC MGIT 960 system)			
PZA susceptible	72	71	98.6
PZA resistant	18	18	100
PZA susceptible and PZA resistant	90	89	98.9

[a] BACTEC 460TB system.
[b] In phase I, proficiency was assessed with five strains of *M. tuberculosis* from liquid and solid media, respectively, per center.
[c] In phase II, reproducibility was assessed with five strains of *M. tuberculosis* in triplicate from three separately prepared inocula (i.e., nine replicates per strain) per center.

a 0.5 McFarland standard was prepared by using glass beads to ensure homogeneity and then diluted 1:5 prior to inoculating 0.5 ml of the suspension into the MGIT PZA set. All inoculated PZA sets were loaded into the BACTEC MGIT 960 instrument within 8 h of inoculation.

Interpretation of results. (i) BACTEC MGIT 960 system. Using predefined algorithms, readings are automatically interpreted by the BACTEC MGIT 960 instrument and reported as either susceptible or resistant. The "unloaded PZA set report" listed growth units, time to result, and susceptible, resistant, or invalid results.

(ii) BACTEC 460TB system. Results of the BACTEC 460TB system were judged according to the established criteria for calculating susceptible, resistant, and borderline results (BACTEC 460TB manual).

Quality controls. *M. tuberculosis* (H37 Rv, ATCC strain 27294) was used for each lot of BACTEC MGIT 960 PZA medium and BACTEC MGIT 960 PZA drug used in this study. Batch quality control was done at least weekly, using the above American Type Culture Collection strains. Inclusion and exclusion criteria for patients and their clinical specimens as defined in the study protocol were strictly followed.

Purity checks. Purity checks on sheep blood agar and Middlebrook 7H10 agar plates were performed with the suspensions (grown in MGIT medium and on LJ medium) used in the PZA susceptibility test; 7H10 subcultures were performed with the growth controls of both BACTEC MGIT 960 PZA and BACTEC 460TB.

Study design. The study consisted of five phases. In the first three phases both centers received the same blinded panels of *M. tuberculosis* strains from the manufacturer of the BACTEC MGIT 960 PZA kit (Becton Dickinson Diagnostic Systems).

(i) Phase I. Proficiency of PZA susceptibility testing by BACTEC MGIT 960 and BACTEC 460TB (according to the product manual) from liquid (MGIT) and solid (LJ) media was done by testing five strains of *M. tuberculosis* (three susceptible to PZA; two resistant to PZA). No more than one individual drug error result per method was permitted per site. Failure to meet this criterion would have resulted in full repeat testing of the whole panel.

(ii) Phase II. Reproducibility of PZA susceptibility testing by the BACTEC MGIT 960 was done by testing another panel of *M. tuberculosis* strains ($n = 5$) in triplicate from three separately prepared inocula at three different separate points in time (cycles), i.e., nine replicates per strain. For PZA testing, inocula were prepared either from MGIT medium (center 1) or from LJ medium (center 2). Reproducibility was expressed as the ratio of the number of correct results to the number of expected results.

(iii) Phase III. PZA susceptibility testing of challenge strains of *M. tuberculosis* was done with the BACTEC MGIT 960 and BACTEC 460TB systems. These blinded strains were provided by the Centers for Disease Control and Prevention (CDC) (Atlanta, Ga.). Susceptibility to PZA, previously determined by the CDC by the agar proportion method (25 μg of PZA/ml [2]), with the BACTEC 460 system, and by the analysis of the *pncA* gene (11), remained, however, blinded until all results were finalized. The panel consisted of 10 strains of *M. tuberculosis* which were cultured in MGIT broth (center 1) and on LJ medium (center 2), respectively, prior to drug testing. Susceptibility results were analyzed by comparing the observed results with the expected results (as determined by the CDC).

(iv) Phase IV. PZA susceptibility testing was carried out with the BACTEC MGIT 960 and BACTEC 460TB systems for a total of 58 strains of *M. tuberculosis* isolated from clinical specimens at the two centers (Zurich, 32 strains; Borstel, 26 strains). Both centers tested all 58 strains. Of those strains, 50 (86%) were isolated from primary clinical specimens. The remaining strains originated from the strain collections of both centers and were multidrug-resistant *M. tuberculosis* strains. Mainly, they had been chosen to increase the proportion of PZA-resistant strains. Inocula for susceptibility testing were derived from both liquid (MGIT) and solid (LJ) media. Seventy-six of 116 tests (68%) were done in glass tubes, and 40 tests were done in plastic tubes.

(v) Phase V. Resolution of discrepant results (BACTEC MGIT 960 system results versus BACTEC 460TB system results) was performed by the independent arbiter sites. The two arbiter laboratories repeated testing with the BACTEC 460TB system. Their results were considered final and correct.

RESULTS

The present study first concentrated on proficiency (phase I) and reproducibility testing (phase II) in each of the two centers. As shown in Table 1, in phase I there was a 100% agreement between results generated with the BACTEC MGIT 960 system and those obtained with the BACTEC 460TB system, regardless of whether strains of *M. tuberculosis* were initially grown in liquid (MGIT) or on solid (LJ) medium. In phase II, tests run in triplicate from three separate inocula prepared at three different points in time (90 in total) yielded highly reproducible results. As illustrated in Table 1, there was one single discordant result out of 90 results (1.1%).

Based on the CDC's susceptibility testing on agar and with the BACTEC 460TB system, challenge strains 97-2144, 96-2373, and 96-2676 were resistant to PZA; strains 96-2203, BN 288, and 96-2124 were partially resistant; and strains 99-2620, 99-2569, 99-2619, and 99-2526 were susceptible (Table 2). Results of sus-

TABLE 2. Susceptibility of CDC challenge strains of *M. tuberculosis* to PZA as determined by the CDC, the study centers, and the independent arbiter sites (phase III)

CDC strain no.	*pncA* gene[f]	PZA susceptibility[a] according to method[b] at test site[c]					
		CDC		Center 1		Center 2	
		APM	460	960	460	960	460
97-2144	Mutant	R	R	R	S	R	R
96-2373	Mutant	R	R	R	R	R	R
96-2676	Mutant	R	R	R	R	R	R
96-2203[d,e]	Wild type	R	S	R	R	R	R
BN 288[d]	Wild type	R	S	S	S	S	S
96-2124[d]	Wild type	R	S	S	S	R	R
99-2620	Wild type	S	S	S	S	S	S
99-2569	Wild type	S	S	S	S	S	S
99-2619	Wild type	S	S	S	S	S	S
99-2526	Wild type	S	S	S	S	R	S

[a] Abbreviations: R, resistant; S, susceptible.
[b] Abbreviations: APM, agar proportion method; 460, BACTEC 460TB system; 960, BACTEC MGIT 960 system.
[c] Center 1, Zurich; center 2, Borstel.
[d] Borderline strain.
[e] PZA susceptibility unresolved (see Discussion). Both arbiter sites received a blinded subculture of this strain from four sites (center 1, center 2, Becton Dickinson Diagnostic Systems, Sparks, Md., and the CDC). Arbiter 1 obtained susceptible results throughout (four of four tests); arbiter 2 obtained resistant results throughout (four of four tests).
[f] The presence or absence of a point mutation in the *pncA* gene was determined in analyses done at the CDC.

TABLE 3. Susceptibility testing of clinical strains of *M. tuberculosis* (n = 112) to PZA utilizing glass and plastic tubes[a]

Results	Initial culture medium (a)	No. of tests	No. of tests with indicated results				Overall agreement[c] (%)	Agreement (%)	
			960-S, 460-S	960-R, 460-S	960-S, 460-R	960-R, 460-R		Glass tubes	Plastic tubes
Initial	MGIT	58	44	1		13	98.3	97.5	100
	LJ	58	45	3[d,e]	1	9	93.1	91.9	95.2
	MGIT + LJ	116	89	4	1	22	95.7	94.7	97.5
Corrected	MGIT	58	44	1		13	98.3	97.5	100
	LJ	58	45	2[d]	1	10[f]	94.8	94.6	95.2
	MGIT + LJ	116	89	3	1	23	96.6	96.1	97.5

[a] Strains were tested in glass tubes (n = 76) or in plastic tubes (n = 40).
[b] In the paired results shown "960" and "460" refer to results obtained with the BACTEC MGIT 960 and 460 TB systems, respectively; "S" and "R" indicate susceptible and resistant results, respectively.
[c] Between glass and plastic tubes.
[d] One strain was susceptible upon repeat testing.
[e] One strain was borderline according to the BACTEC 460TB system.
[f] One strain was borderline according to the BACTEC 460TB system at one arbiter site.

ceptibility testing of this set of strains with the BACTEC MGIT 960 and BACTEC 460TB systems agreed largely with the results generated by the CDC. Divergent results arose, however, with the partially resistant (borderline) strains. After retesting by the arbiters the PZA susceptibility profile of strain no. 96-2203 had to be considered unresolved. While one arbiter's results agreed with the results of both study centers, the other arbiter's results agreed with the CDC's results.

In phase IV, a total of 58 strains of *M. tuberculosis* isolated from clinical specimens were tested in both BACTEC systems (Table 3). All these strains were cultivated in MGIT medium (center 1) and, in parallel, on LJ slants (center 2) prior to inoculating the BACTEC MGIT PZA tubes. Combining the inocula from solid and liquid growth, 5 out of 116 (4.3%) results were initially discordant; 4 were resistant according to the BACTEC MGIT 960 system but susceptible according to the BACTEC 460TB system. Conversely, one strain was susceptible according to the former system but resistant according to the latter system. For one strain, arbiters unequivocally agreed that the MGIT system gave a resistant result and the BACTEC 460 system gave a susceptible one, while for the other strains, results generated by the study centers were judged to be erroneous. Although statistically not different, there was greater agreement when the inoculum was taken from liquid medium (liquid medium, 98.3%; solid medium, 93.1%). Similarly, there was no statistically significant difference between the values generated in plastic tubes versus those generated in glass tubes (although agreement of results was slightly higher for the former).

As far as time to complete susceptibility results is concerned, there was no statistically significant difference between the two methods: mean turnaround time for the BACTEC MGIT 960 system was 6.8 days, and that for the BACTEC 460TB system was 5.4 days, not including, however, the time required to obtain a radiometric subculture with a growth index (GI) of 200 (Table 4). Susceptible results were generated more rapidly by both techniques (6.3 days with the BACTEC MGIT 960 system versus 4.8 days with the BACTEC 460TB system, on average); resistant results took a few days longer (8.5 days [BACTEC MGIT 960 system] versus 8.0 days [BACTEC 460TB system]). PZA testing using inoculum from liquid growth was more than a day faster on the average (although statistically not significant).

DISCUSSION

Historically, initial efforts to develop a culture-based method for reliable testing of susceptibility of *M. tuberculosis* to PZA were not very successful. When utilizing a modified 7H10 agar

TABLE 4. Turnaround time to susceptibility results for PZA[a] (phase IV)

PZA profile	Inoculum source[b]	BACTEC MGIT 960 system			BACTEC 460TB-system[c]		
		No. of tests	Turnaround time (day)		No. of tests	Turnaround time (day)	
			Mean	Range		Mean	Range
Susceptible	Liquid	44	5.8	4.0–11.2	45	4.8	3.0–9.0
	Solid	46	6.8	4.7–11.3	47	4.7	3.0–16.0
	Liquid and solid	90	6.3	4.0–11.3	92	4.8	3.0–16.0
Resistant	Liquid	14	7.2	4.4–11.8	13	8.5	3.0–21.0
	Solid	12	10.0	4.9–16.8	11	7.3	3.0–21.0
	Liquid and solid	26	8.5	4.4–16.8	24	8.0	3.0–21.0
Susceptible and resistant	Liquid	58	6.1	4.0–11.8	58	5.7	3.0–21.0
	Solid	58	7.4	4.7–16.8	58	5.2	3.0–21.0
	Liquid and solid	116	6.8	4.0–16.8	116	5.4	3.0–21.0

[a] Glass and plastic tubes.
[b] Inoculum was derived from strains grown in liquid (MGIT) medium or on solid (LJ) medium.
[c] A GI of 200 was considered to be time zero. To reach this GI, four extra days were needed on average.

medium at pH 5.5, for instance, many strains either failed to grow or grew very poorly (2). Addition of egg yolk improved growth of mycobacteria; the procedure was, however, cumbersome and therefore not well suited for a clinical mycobacteriology laboratory (13). Eventually, combined efforts of different groups led to today's recommendation to use the radiometric BACTEC 12B medium at pH 5.9 to 6.0 (BACTEC PZA medium), with a concentration of PZA higher than that used in the conventional solid medium at pH 5.5 to compensate for the increase in pH (3, 9, 10).

The very recent development of the BACTEC MGIT 960 PZA susceptibility test is a significant breakthrough since it allows testing of the drug in a liquid, nonradiometric medium with a high concentration of PZA (100 μg/ml). Our study was a one-to-one comparison of two susceptibility testing procedures, which are both based on liquid medium and measure metabolic activity of *M. tuberculosis* organisms. While the classical BACTEC 460TB system detects $^{14}CO_2$ liberated during decarboxylation of ^{14}C-labeled palmitic acid present in the medium, the new MGIT technology is based on fluorescence which becomes stronger as oxygen is depleted from the medium due to actively metabolizing microorganisms.

Prior to testing the susceptibility of clinical strains of *M. tuberculosis* to PZA at the two study sites, it was important to establish performance parameters to guarantee an optimum baseline for objective results. Based on a rigid study protocol, the two laboratories first analyzed PZA susceptibility of blinded panels of *M. tuberculosis* strains whose susceptibility patterns were known to the manufacturer only (phases I and II) or to the CDC (phase III).

The CDC challenge strains had been thoroughly tested by that institution prior to inclusion in our study. In addition to being tested by radiometric susceptibility methods, those strains had also been tested by the agar proportion method and were analyzed for a mutation in their *pncA* gene (11). Point mutations had been found in the three PZA-resistant strains (97-2144, 96-2373, and 96-2676). Without exception, resistance in these strains was correctly detected with the BACTEC MGIT 960 system at both centers. Among the susceptible strains (99-2620, 99-2569, 99-2619, and 99-2526) there was one false-resistant result with the BACTEC MGIT 960 (center 2). As expected, variable results were obtained for the partially resistant strains (96-2203, BN 288, and 96-2124). None of them had a point mutation in the *pncA* gene, but they repeatedly yielded resistance to PZA upon susceptibility testing by the agar proportion method. The PZA susceptibility profile remained unresolved for strain 96-2203. While it was resistant according to both BACTEC techniques at both study centers and one arbiter site, it was susceptible by BACTEC 460 at the other arbiter site and at the CDC. In light of these contradictory results, DNA fingerprinting was performed and ensured that all centers tested the very same strain, 96-2203. Assuming that these three strains (96-2203, BN 288, and 96-2124) are resistant to PZA, a mutation on a gene other than *pncA* can be hypothesized. This is easily conceivable, since a significant number of resistant strains do not show mutations in *pncA* (4, 12). Provided the agar proportion method has yielded correct results at the CDC, these three strains may, thus, be considered "borderline" strains, similar to those reported by Gross et al. (W. Gross, J. Ridderhof, I. George, H. Lipman, B. Metchock, B. Robinson-Dunn, A. Sloutsky, G. Washabaugh, and B. Madison, Abstr. 101st Gen. Meet. Am. Soc. Microbiol. 2001, abstr. C-247, p. 208, 2001).

Focusing on the 58 clinical strains, the results for 5 out of a total of 116 tests were discordant. After arbiter resolution of discrepant results, the number of discordant results decreased to 4 (3.4%). Eventually, there were three major errors and one very major error. Overall, there were fewer discordant results when the inoculum came from liquid (MGIT) medium than when the inoculum came from solid medium. This could be due to excessive clumping of the growth on solid medium. However, the difference was statistically not significant. The tendency of the BACTEC MGIT 960 technology to generate major errors rather than very major errors has previously been observed in a comprehensive study concentrating on the testing of the susceptibility of 110 strains of *M. tuberculosis* to INH, RMP, EMB, and STR. In total, there were 34 discrepant results between the two BACTEC methods, none of them being very major errors (1).

The periods of time needed to establish susceptibility to PZA with the BACTEC MGIT 960 system (on average, 6.3 days for susceptible strains and 8.5 days for resistant strains) and with the BACTEC 460TB system (on average, 4.8 days for susceptible strains and 8.0 days for resistant strains) are in line with what has been reported for testing of susceptibility of *M. tuberculosis* to INH, RMP, EMB, and STR. Based on the results of more than 570 susceptibility tests, turnaround times ranged from 4.6 to 11.7 days for the BACTEC MGIT 960 system and from 4 to 10 days for the BACTEC 460TB system (1). At first sight, the radiometric technique appears to be slightly more rapid. In practice, this does not, however, hold true. While the BACTEC MGIT 960 system starts counting turnaround time at time zero, i.e., as soon as the inoculated MGIT PZA tube is placed into the instrument, the radiometric vial has to be subcultured first. Susceptibility testing can only be performed once the GI reaches 200. Thus, four additional days, on average, are necessary, which increases the time to completion of results by the BACTEC 460TB system to 9.4 days for all the tested cultures. Therefore, it is clear that the BACTEC MGIT 960 system is more efficient than radiometry and that time-to-results data cannot be compared on a one-to-one basis.

This study answers other important questions. First, from the results it is evident that susceptibility testing can be carried out using inocula from both liquid and solid culture. Even though there was no statistically significant difference ($P > 0.05$) in the overall agreement (98.3% [MGIT inoculum] versus 94.8% [LJ inoculum]), inoculating the MGIT PZA tube with a primary MGIT culture yielded slightly better results. This is advantageous since most of the time, PZA tubes are inoculated from liquid medium, which yields growth considerably earlier than does inoculation from solid medium. Second, plastic tubes were as suitable as glass tubes (agreement of 97.5% for plastic tubes versus 96.1% for glass tubes). For a clinical mycobacteriology laboratory, this finding is significant because the use of plastic tubes (currently marketed in certain countries outside the United States) increases safety for the personnel handling the tubes. Finally, compared to the radiometric technology, the new BACTEC MGIT 960 system automatically interprets results as susceptible or resistant. Hence, it

eliminates the grey zone of borderline results. As stated above, the new system tends to err towards false resistance rather than false susceptibility.

In conclusion, our data demonstrate that (i) testing of susceptibility of *M. tuberculosis* to PZA with the new, nonradiometric BACTEC MGIT 960 system is easy to perform with strains grown in either liquid or solid medium; (ii) overall agreement of results generated in the BACTEC MGIT 960 system with those observed in the BACTEC 460TB system is very high (>96%); (iii) BACTEC MGIT 960 turnaround time for PZA testing is faster than that of the radiometric technique; (iv) BACTEC MGIT 960 PZA testing does not require any needles for inoculation, compared to the BACTEC 460TB procedure; and (v) there is no issue of radioactive waste disposal.

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