

Laboratory diagnosis for *Giardia lamblia* infection: A comparison of microscopy, coprodiagnosis and serology

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OBJECTIVE: To evaluate newer techniques such as coproantigen detection and serology in the diagnosis of symptomatic *Giardia lamblia* infection.

DESIGN: Blinded comparison of copro-antigen detection (by ELISA), serology (immunoglobulin IgG and IgM anti-*G lamblia* by ELISA, and IgG, IgM and IgA by immunoblot) and microscopy in clinical samples. Microscopic findings for three preserved stools were considered the gold standard.

SETTING: Travel medicine clinic.

POPULATION STUDIED: Adults, post-travel, with gastrointestinal symptomatology.

MAIN RESULTS: For 152 previously collected stools, copro-antigen detection had a sensitivity of 73 of 74 (98.6%) and a specificity of 78 of 78 (100%). In clinical samples of 62 patients, eight of the 62 patients (13%) were diagnosed with *G lamblia* infection on microscopy. Copro-antigen diagnosis was accurate in symptomatic patients, with sensitivity of seven of eight (87.5%) and specificity of 52 of 54 (96.8%). Serology was less accurate. IgG response to *G lamblia* had sensitivity of four of seven and specificity of 24 of 50 (48%), and IgM response had sensitivity of three of six and specificity 27 of 48 (56%). Western blot had a sensitivity of five of seven and a specificity of 38 of 49 (78%).

CONCLUSIONS: Copro-antigen diagnosis of *G lamblia* is highly accurate in patients with chronic gastrointestinal complaints, while serology is less accurate and appears to be less useful diagnostically.

Key Words: Copro-antigen, Diagnosis, *Giardia lamblia*

Analyse diagnostique de l'infection à *Giardia lamblia* : comparaison de la microscopie, du coprodiagnostic et de la sérologie

OBJECTIF : Évaluer les plus récentes techniques, telles que le dépistage des coproantigènes et la sérologie, dans le diagnostic de l'infection symptomatique à *Giardia lamblia*.

MODÈLE : Une comparaison à l'insu du dépistage des coproantigènes (par ELISA), de la sérologie (immunoglobulines IgG et IgM anti-*G lamblia* par ELISA et l'IgG, IgM et IgA par immunotransfert) et examen à la microscopie d'échantillons cliniques. Les observations microscopiques de trois spécimens de selles conservés ont été considérées comme étalons.

CONTEXTE : Clinique de médecine des voyageurs.

POPULATION ÉTUDIÉE : Adultes revenant de voyage, souffrant de symptômes gastro-intestinaux.

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PRINCIPAUX RÉSULTATS : Pour 152 spécimens de selles recueillis précédemment, le dépistage des coproantigènes s'accompagnait d'une sensibilité de 73/74 (98,6 %) et d'une spécificité de 78/78 (100 %) pour des échantillons cliniques provenant de 62 patients, 8 des 62 patients (13%) ont reçu un diagnostic d'infection à *G. lamblia* à la microscopie. Le diagnostic par analyse des coproantigènes s'est révélé précis pour ce qui est des patients symptomatiques, avec une sensibilité de 7/8 (87,5 %) et une spécificité de 52/54 (96,8 %). La sérologie s'est révélée moins précise, la réponse pour les IgG à *G. lamblia* a donné une sensibilité de 4/7 et une spécificité de 24/50 (48 %) et pour les IgM, une sensibilité de 3/6 et une spécificité de 27/48 (56 %). Le buvardage de Western a offert quant à lui une sensibilité de 5/7 et une spécificité de 38/49 (78 %).

CONCLUSIONS : Le diagnostic par l'analyse des coproantigènes de *G. lamblia* est très précis chez les patients qui souffrent de symptômes gastro-intestinaux chroniques alors que les épreuves sérologiques sont moins précises et semblent moins utiles au diagnostic.

Giardia lamblia is the most common pathogenic gastrointestinal parasite worldwide, with a prevalence ranging from 1% to 7% in industrialized countries to as high as 50% in developing countries (1,2). Asymptomatic excretion is common in some populations, such as children attending day care centres where prevalence rates of 21% to 26% have been reported (2). It is recognized as an important cause of waterborne and, more rarely, foodborne outbreaks of gastroenteritis. In addition, it has been reported to cause up to 6% of traveller's diarrhea (3).

Despite its prevalence, controversy still surrounds the best means of diagnosis. Microscopy of direct fecal smears or smears prepared following formol-ether concentration and iodine staining has been reported to reach 97% sensitivity if three stool samples are examined (4). Yang and Scholten (5) described the utility of sodium acetate-acetic acid-formalin (SAF) preservation in diagnosing intestinal protozoans and found both an increased yield with the concentration of preserved samples and a further increase of about 20% with permanent staining. However, false negatives can occur, with reasons including intermittent excretion of cysts, use of anti-diarrheal medication and barium use for diagnostic imaging (6-8). The string test, duodenal aspirate, intestinal impression smear and intestinal biopsy have all been proposed as techniques to improve microscopic diagnosis (1,9). Results have been conflicting, with some reports finding microscopy of direct smears without preservation as low as 50% sensitive (10), while others suggest that there is little diagnostic gain from more invasive and expensive testing (1,4,11-13).

Recently, immunological testing of stool and serum have been reported as more sensitive means to diagnose giardiasis. Copro-antigen diagnosis, the direct detection of antigens in stool, was first demonstrated for *G. lamblia* by Craft and Nelson (14), using counterimmunoelectrophoresis. Since then, the isolation of a *Giardia*-specific antigen (GSA) 65 (15) has facilitated the development of other antibody-associated modalities of antigen detection, such as ELISA (16,17) and immunofluorescence assay (18). This has led to the recently licensed monospecific commercial ELISA test kits (19). The accuracy of this technique has been compared with that of microscopy in patients with gastrointestinal symptomatology, with sensitivities and specificities of 95% to 100% and over 90%, respectively (9,17,19,20-23). The technique's accuracy and simplicity have been cited as its major advantages, while cost concerns appear to be the principal disadvantage.

Serology has also been used to detect giardia infection (24-31). Several studies using ELISA to detect a serological response suggest that, compared with immunoglobulin (Ig) G, the IgM antibody response is shorter and more indicative of active infection (25,28,32-34). Reported sensitivities and specificities for IgM-ELISA range from 63% to 99% and 79% to 96%, respectively. Another approach to serodiagnosis is Western blot analysis. The molecular weights of the antigenic determinants to serum IgG, IgM and IgA responses in patients with giardiasis have been determined using sodium-dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (35).

Studies evaluating diagnostic tests for *G. lamblia* have had certain limitations. Some were performed on asymptomatic individuals, such as populations in underdeveloped countries or children in day care centres (10,11,19,22,36,37), thereby analyzing parameters for screening rather than diagnosis. Some studies were flawed by problems with defining the gold standard (13,14,20,38,39) and a lack of blinding (16,37,38,40), while others have failed to follow published recommendations, such as those of the National Committee for Clinical Laboratory Standards (41) and Centers for Disease Control and Prevention (42), by not analyzing three fresh or preserved stools (15,17,22,30,39).

Therefore, the current study examined five different diagnostic modalities in a population of patients who had presented with persistent gastrointestinal complaints at a geographical medicine clinic.

MATERIALS AND METHODS

The study was performed in two phases. In phase 1, 74 microscopically positive and 78 microscopically negative stools that had been preserved in SAF were examined for the presence of giardia-soluble antigen using the ProSpecT copro-antigen kit (Alexon, California). For purposes of comparison, the clinical standard, microscopy, was defined as the gold standard. In phase 2, patients presenting to the McGill University Centre for Tropical Diseases (MUCTD) with symptomatology compatible with a diagnosis of giardiasis were prospectively enrolled into a study comparing stool microscopy, copro-antigen detection and serology. Patients were required to meet the following inclusion criteria: intermittent or continuous diarrhea of greater than two weeks' duration, increased gas and no history of fever, or blood or mucus seen in the stool during the diarrheal period. Clinical data obtained at presentation included duration of diarrhea (in weeks), number of bowel move-

ments per day and country of travel. All participants were instructed to bring three separate stool samples in SAF preservative collected on alternate days to the MUCTD. At the MUCTD, one of the three samples was relabelled with a fictitious name for blinding, to prevent bias by a technologist in the reading of samples.

Microscopy in both phases 1 and 2 included stools collected in SAF preservative and, after saline washing, divided in two parts. One part was permanently stained with hematoxylin, while the other was concentrated in formol-ethyl acetate for preparation of an iodine wet mount (42). After microscopic examination of each stain at 500 \times oil for 10 mins (150 fields), results were graded semiquantitatively (0 – negative, 1 – trophozoites, 2 – one to five cysts per coverslip, 3 – six to 20 cysts per coverslip, 4 – less than one cyst per low power field, 5 – more than one cyst per high power field, 6 – more than one cyst per oil immersion field). For purposes of comparison, microscopy was considered the gold standard.

The copro-antigen assay was performed according to instructions accompanying the ProSpecT kit. Stools were diluted in specimen dilution buffer, and added to polystyrene tubes coated with anti-GSA 65. Anti-GSA coupled to horseradish peroxidase was added to form a sandwich ELISA. Results were read visually and graded as 0 to 4 based on colour being negative, slight, light, moderate, or intense. Results were also read as an optical density (OD) by spectrophotometer at $\lambda=492$ nm. Results from phase 1 were used to determine the test parameters of visual examination and instrumental reading, and the correlation between instrumental positivity and the degree of positivity on microscopy. In phase 2, copro-antigen results were read using a visual determination only because this technique had been determined in phase 1 to perform as well as the instrumental reading.

Sera were drawn on all patients, and stored at -70°C until used in serological testing. The testing was performed at one time at the McGill Institute for Parasitology, and technicians were blinded to the microscopic and copro-antigen kit results. Serological testing included ELISA for both IgG and IgM and Western blot analysis for IgG, IgM and IgA.

For antigen preparation, *G lamblia* (ATCC 30957) trophozoites were axenically transferred twice weekly in filter-sterilized modified Diamond's TYI-S-33 medium (pH 7.0) with 10% adult bovine serum and antibiotics added (43). Tubes were incubated at 37°C in the slanted position, and after 72 to 96 h growth, trophozoites were harvested by cooling the culture tube on ice for 15 mins and centrifuging at 800 *g* for 10 mins. Cells were washed three times and resuspended in phosphate-buffered saline (PBS) at pH 7.4. These cells were disrupted by sonication in ice water bath at 50% maximum power (VCX 400 Ultrasonic processor, Vibracell, Sonics & Materials, Fisher Scientific) for 5 mins. The suspension was then centrifuged at 23,000 *g* for 15 mins, and the supernatant was retained. Protein concentration was determined by the Bradford method (44).

ELISA determination was performed as described in principle by Voller et al (45). Polyethylene microtitre plates (Falcon, Becton Dickinson, Maryland) were coated with 1 μg per well of

trophozoite crude extract and left at room temperature overnight. After washing with PBS, wells were blocked with 100 μL per well of PBS containing 1% dried milk for 1 h at 37°C . To each well, 100 μL of a 1:100 dilution (in PBS-0.1% Tween 20 and 1% milk) of human serum was added, and the plates were incubated at 37°C for 1 h. Next, 100 μL of a 1:1500 dilution (in PBS-0.1% Tween 20) of goat antihuman IgG or IgM horseradish peroxidase-conjugated antibodies (Bio/Can Scientific) was added to each well. The substrate was 2,2'-azino-bis-3-ethylbenzothiazoline sulphonic acid (Sigma, Missouri). After 15 to 30 mins, the optical density was read at 400 nm by using a microplate autoreader (Bio-Tek Instruments, Mandel Scientific Company). Positive and negative controls were included, with twice the absorbance of the SD of the negative control considered positive.

Electrophoretic separation was performed as described in principle by Laemmli (46). Trophozoite antigens were boiled for 5 mins in Laemmli reducing sample buffer and 200 μg was loaded in a Mini Protean II dual slab gel (Bio-Rad, California). After electrophoresis, antigens were transferred onto nitrocellulose (NC) membranes as described previously by Towbin et al (47), NC membranes were washed in washing buffer (10 mM Tris-HCl, 140 mM NaCl, 0.1% Tween 20) and blocked overnight at 4°C in blocking buffer (30 mg/mL glycine, 9 mg/mL NaCl, 0.01 M Tris pH 7.5, 10% fetal bovine serum [FBS]). After washing, they were incubated with human sera diluted 1:50 in a dilution buffer (0.05 M Tris-HCl pH 7.5, 0.2 M NaCl) containing 10% FBS for 2 h at room temperature. Dilution buffer without human serum was run as a control. The blots were next incubated 1 h at room temperature with a 1:1500 dilution (in dilution buffer containing 10% FBS) of goat anti-human IgA, IgG and IgM horseradish peroxidase-conjugated antibodies (Bio/Can Scientific) and after washing, bands were detected using 0.5 mg/mL of 4-chloro-1-naphthol (Sigma) in methanol/dilution buffer (1:5) containing 0.67% of 3% hydrogen peroxide. Bands were read visually as present or absent, both at the Institute and independently by one of the authors blinded to the results. Each patient's serum was characterized by the number of bands present and the spectrum of molecular weights represented.

Statistical analysis involved the binomial method to calculate 95% CI of point estimates where sample sizes were appropriate. Correlation between degrees of positivity was done using the Spearman's correlation analysis on Statistical Applications Software (SAS, North Carolina). A receiver operating characteristic curve (ROC) was drawn for Western blots and analyzed using the Fortran program ROCFIT developed by Dorfman and Alf (48). Using methods described by Hanley (49), 95% CI for the area under the curve were calculated.

RESULTS

Phase 1: A total of 74 positive and 78 negative stool samples were examined for giardia copro-antigen. Results for visual reading of copro-antigen by ELISA are shown in Table 1. Sensitivity was 98.6% (95% CI 92.8% to 100%), and specificity was 100% (lower limit 95% CI 95.4%). Instrumental reading of copro-antigen, using a cut-off of OD=0.10, gave similar re-

TABLE 1
Comparison of microscopy with visual reading of *Giardia lamblia* copro-antigen

	Microscopy <i>G lamblia</i> - positive	Microscopy <i>G lamblia</i> - negative
Copro-antigen positive	73	0
Copro-antigen negative	1	78
Totals	74	78

Sensitivity 73 of 74 (98.6%) (95% CI 92.8% to 100%). Specificity 78 of 78 (100%) (lower limit C: 95.4%)

TABLE 2
Comparison of microscopy with copro-antigen detection and serology

Technique	Sensitivity	Specificity
Copro-antigen	7/8 (87.5%)	52/54 (96.8%)
ELISA – IgG	4/7 (57%)	24/50 (48%)
ELISA – IgM	3/6 (50%)	27/48 (56%)
Western blot, ≥ 2 bands +	5/7 (71%)	38/49 (78%)
Western blot, ≥ 3 bands +	4/7 (57%)	43/49 (88%)

Ig Immunoglobulin; + Positive

sults, with sensitivity 74 of 74 (100%, lower limit 95% CI 95.2%) and specificity 74 of 78 (94.9%, 95% CI 87.6% to 98.6%). In the 74 *G lamblia* positive samples, four had trophozoites noted on microscopy, and 70 had varying intensity of cysts. Among the 70 with cysts only, the correlation between the level of infection on microscopy and the ELISA copro-antigen reading was poor, with a Spearman's correlation coefficient of 0.233 (visual reading) or 0.306 (instrumental reading). The Spearman's correlation coefficient between visual and instrumental reading of ELISA was 0.831.

Phase 2, patient population: Sixty-two patients were enrolled and had the following characteristics. The median number of daily diarrheal movements was three (range one to 25), and the median duration of symptoms was six weeks (range: two to 60). Patients' travel histories were Latin America (n=13), the Caribbean (n=12), Africa (n=12), the Indian subcontinent (n=12), Southeast Asia (n=7), camping in North America (n=4), Lebanon (n=1) and Portugal (n=1).

Phase 2, microscopy: Fifty-seven patients submitted three stool samples, and five submitted only two samples. The five patients were kept in the study even though three stool samples had not been examined because it was extremely unlikely that any misclassification bias would have occurred given the unequivocal test results. Four patients were negative for *G lamblia* by both microscopy and stool antigen by visual ELISA, and one was positive by both techniques.

On microscopy, eight of 62 patients (13%) were positive for *G lamblia*. Seven patients had all three samples positive at a grade of 5; the eighth patient had only one of three samples positive at grade 2 (one to five cysts per coverslip). Because of the low number of positive samples, further results emphasize specificity, because sensitivity could not be accurately assessed.

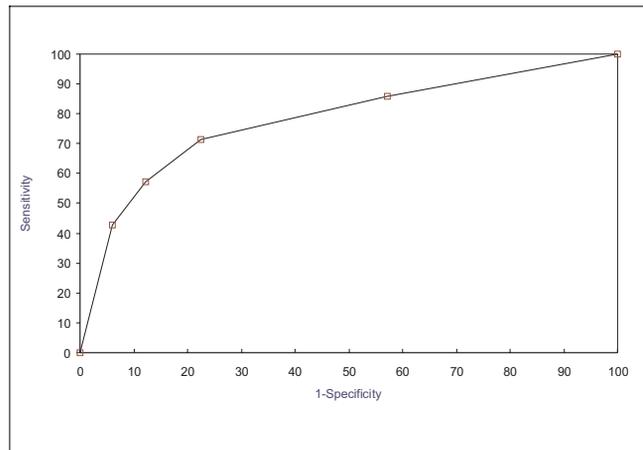


Figure 1) Receiver operator characteristic curve of Western blot analysis. Area under the curve = 0.77 (95% CI 0.58 to 1.0)

Phase 2, copro-antigen: Copro-antigen ELISA was read only by the visual technique because the results from phase 1 showed no additional advantage to instrumental reading. Visual ELISA for copro-antigen correctly identified seven of eight positive patients. The single misclassification occurred in the patient who had only one of the three samples positive at grade 2. A positive copro-antigen result was obtained in one of three samples of two patients whose stools were negative on microscopy. The resulting specificity was 52 of 54 (96.8%, CI 89% to 99.6%), with sensitivity 7 of 8 (87.5%) (Table 2).

Phase 2, serology: Because of losses to follow-up, insufficient sera and test failure, sera were collected from 57 of 62 patients, of whom 54 had results of ELISA for IgM, 57 had ELISA for IgG and 56 had immunoblots. Fifty-four patients had all five tests performed (microscopy, copro-antigen, IgG-ELISA, IgM-ELISA, IgG-Western blot).

ELISA for IgG anti-*G lamblia* antibody was performed in 57 patients, and was positive in 28 patients; specificity was 24 of 50 (48%, 95% CI 29% to 67%) and sensitivity was four of seven (57%) (Table 2). ELISA for IgM response was performed on 54 patients; specificity was 27 of 48 (56.3%, 95% CI 42% to 70%) and sensitivity was three of six (50%) (Table 2).

Fifty-six sera were analyzed for IgG, IgM and IgA using Western blot. Because as many as 11 bands were identified on a single blot, positivity results differed depending on the number of positive bands used as the cut-off criterion. No single band was more dominant or discriminatory, as observed by Char et al (35). If two or more bands were considered positive, the specificity was 38 of 48 (77.6%, 95% CI 62% to 87%) with sensitivity five of seven (71.4%) (Table 2). If three or more bands were considered positive, the specificity increased to 43 of 49 (87.8%, 95% CI 76% to 96%) while the sensitivity dropped to four of seven (57%). A ROC curve for the Western blot results is shown in Figure 1.

Patients with true positive results and patients with false positive copro-antigen or Western blot results were analyzed to determine whether results were associated with symptomatology, cross-reactions with other parasites or variations in antigenicity caused by different strains of *G lamblia* acquired

in different parts of the world. True positive patients were not different from *G lamblia*-negative patients in terms of duration of symptoms (three to 24 weeks), diarrheal frequency (one to five bowel movements per day), and travel history (India [n=3], Africa [n=2], Latin America [n=2], unknown [n=1]). Two of these patients also had *Blastocystis hominis* on stool microscopy, and one had *Endolimax nana* and *Entamoeba hartmani*. The two patients with false positive copro-antigen had travelled to India and gone camping in Canada, respectively; both had *B hominis* on stool microscopy. The six microscopy-negative patients with three or more positive bands on Western blot had the following clinical characteristics: symptoms lasting from two to eight weeks, symptom frequency of two to six bowel movements per day and places of travel such as India (n=2), Latin America (n=2), Thailand (n=1) and Africa (n=1). Of those with false positive Western blots, four of six had *B hominis* on stool microscopy; of all patients with *B hominis*, 11 of 16 had two or fewer bands, and five were completely negative on Western blot.

DISCUSSION

While it has previously been shown that microscopy is not 100% sensitive in diagnosing *G lamblia* infections, it is generally accepted as the gold standard against which new tests are compared (1,4). Some of the reported variability in the utility of microscopy may stem from different microbiology laboratories with varying expertise in the diagnosis of parasitic infections. At the MUCTD, approximately 6000 stools are processed annually for ova and parasites, and the fact that phase 1 results of copro-antigen almost completely overlap with microscopy results from samples previously read and refrigerated in SAF (only one stool in 152 misclassified) reinforces the reliability of microscopy in our laboratory.

Using microscopy as a comparison, this study supports previous data showing both high sensitivity and specificity for detection of *G lamblia* antigen in stools (13,16,17,19-23). In known *G lamblia*-positive and -negative samples, the kit performed at 98.6% sensitivity and 100% specificity. This high sensitivity agrees with previous reports, which have all described sensitivities of over 90%. Issues of specificity are more complex because of varying definitions of the gold standard. If copro-antigen assays are more sensitive than microscopy, then copro-antigen-positive/microscopy-negative cases would falsely lower the observed specificity of copro-antigen diagnosis in a study using microscopy as gold standard. Addiss et al (19) and Chapell and Matson (21) felt that there was corroborating evidence for the theory that many such cases were due to be false negative microscopy. Therefore Addiss (19) estimated specificity at 100%. The present study, however, found very few such cases (none of 78 in phase 1 and two of 54 in phase 2), suggesting that with good laboratory technique and microscopic skill, few undiagnosed cases will be detected by copro-antigen assay.

Reading by visual and instrumental methods gave virtually identical results. The degree of positivity by instrumental and visual reading correlated poorly with degree of positivity by microscopy, and therefore, this test was valuable in detecting

the presence but not the intensity of infection. This result is not unexpected, because the copro-antigen test detects the presence of soluble *G lamblia* antigen and not the cyst.

In our defined patient population, there was a low prevalence of *G lamblia* infection (13%), but other intestinal protozoans (especially *B hominis*) were commonly present, and cross-reactivity manifested by poor specificity was not observed. The sensitivity of other diagnostic modalities could not be accurately assessed with only eight positive patients. The specificity of copro-antigen testing, however, was excellent (96.8%), while serodiagnosis in this population performed poorly; IgG-ELISA had only 48% specificity, while IgM-ELISA had 56% specificity. Western blot testing gave better results: if two or more bands was used as a positive cut-off, specificity was 78%, and increasing the number of bands to three improved the specificity to 88% but may have compromised sensitivity.

Therefore, these results confirm that copro-antigen diagnosis is very specific and very reliable. The fact that copro-antigen results correlated so well with microscopy, however, suggests that it is unlikely to be more sensitive than microscopy, or more false positive results of copro-antigen detection would have been seen. As well these results do not support replacement of microscopy except in certain instances, because the inherent advantage of microscopy remains the capacity to diagnose other parasitic infections for which copro-antigen kits are not available. The cost in our laboratory for analysis of preserved stools is approximately \$17 per stool, or \$51 per patient if three stools per patient are sent. The per unit cost of the copro-antigen kit is \$14 and only one kit is needed per person, but at this price the laboratory can only test for *G lamblia*. However, if the sole objective is to determine the presence of *G lamblia* infection, such as in epidemiological studies or if screening for *G lamblia* within population groups such as children in day care centres, then batch testing using a copro-antigen kit can be less time consuming than microscopy. Moreover, the kit is a technology that is easy to incorporate into a laboratory where technologists do not have considerable parasitology expertise. Finally, as a second-line test, especially in settings where a parasitology laboratory is not available, or there is a question of false negative results because of technical reasons (21), copro-antigen kits are much less invasive and expensive than some previously advocated secondary diagnostic modalities, such as the Enterotest (HDC Corporation, California) and endoscopy for aspiration or biopsy.

These results do not support the incorporation of serological testing at this time, with the possible exception of Western blot analysis, which is nonetheless a time-consuming process with only limited diagnostic gain.

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