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Original Article

Comparison of real-time PCR, indirect immunofluorescence antibody assay, and different staining techniques for the diagnosis of *Pneumocystis jirovecii*

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Abstract

Aim: *Pneumocystis* pneumonia (PCP), caused by *Pneumocystis jirovecii (P. jirovecii)*, is an opportunistic infection with a severe progression, often observed in immunocompromised patients. The diagnosis is difficult due to the non-specific clinical and radiological findings. Therefore, rapid and accurate diagnosis of the agent is important in terms of timely implementation of the treatment. In this study, it was aimed to detect *P. jirovecii* by Giemsa staining, Modified Toluidine Blue O staining (MTolB), indirect immunofluorescent antibody (IIFA) assay, and real-time polymerase chain reaction (PCR) in clinical samples obtained from patients suspected of having PCP.

Material and Methods: Respiratory tract samples (23 oral wash, 19 bronchoalveolar lavage fluid, and eight induced sputum samples) of 50 patients referred to the Microbiology Laboratory of Gazi University Health Application and Research Hospital with the suspicion of PCP were analyzed. The presence of *P. jirovecii* in the respiratory tract samples was investigated by Giemsa staining, MTolB staining, IIFA (Pneumocell, Cellabs Pty Ltd, Australia), and real-time PCR (the primers targeting the DHFR gene).

Results: Of the 50 samples included in the study, four (8%) with MTolB, five (10%) with Giemsa, seven (14%) with IIFA, and seven (14%) with real-time PCR were positive. When real-time PCR was accepted as the gold standard, the sensitivity and specificity values were found to be 85.7% and 97.7%, respectively for IIFA, 71.4% and 100%, respectively for Giemsa and 57% and 100%, respectively for MTolB. There was almost perfect agreement between the results of real-time PCR and IIFA (κ=0.92). In the comparison between PCR and cytochemical staining methods, Giemsa had almost perfect agreement with PCR (κ=0.92) and had a higher coefficient compared to MTolB (κ=0.88).

Conclusion: It is considered that it would be more beneficial to use IIFA and real-time PCR tests together in the diagnosis of *P. jirovecii*.

Keywords: Pneumocystis jirovecii; giemsa; MTolB; IIFA; real-time PCR

INTRODUCTION

Pneumocystis jirovecii, (P. jirovecii, previously called *Pneumocystis carinii),* is an atypical fungus that causes severe *Pneumocystis* pneumonia (PCP) in immunocompromised patients, especially in those infected with HIV (1,2). From its discovery until the late 1980s, *Pneumocystis* was considered to be a protozoan based on morphologic characteristics, proposed life cycle, and drug susceptibilities (3,4). However in 1988, ribosomal RNA and DNA studies demonstrated that it was closely related to fungi (4,5). In 2001, it was officially reclassified as a fungus belonging to the phylum Ascomycota and was renamed *Pneumocystis jirovecii* (2,6,7).

Although the incidence of PCP is significantly decreased due to the extensive use of highly active anti-retroviral therapy (HAART) and *Pneumocystis* chemoprophylaxis, PCP remains an important cause of morbidity and mortality in HIV-positive patients (8,9). However, in recent years, the

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use of new immunosuppressive and immunomodulatory therapies, especially in hematologic malignancies, solid tumors, organ transplantations, and connective tissue and chronic inflammatory diseases has increased the incidence of PCP in immunosuppressed patients (9,10).

The diagnosis of PCP is difficult due to the non-specific symptoms and signs, concomitant use of prophylactic drugs, and co-infections (11). Since *P. jirovecii* cannot be grown in culture, the laboratory diagnosis is based on the microscopic examination of the cyst or trophozoite forms of the organism by using cytochemical staining or immunofluorescence staining with monoclonal antibodies in induced sputum, bronchoalveolar lavage (BAL), endotracheal aspirate (ETA) and lung tissue samples (8,11-13).

The rapid diagnosis of PCP is especially important for HIV-negative patients because early treatment in these patients has been reported to significantly reduce mortality (14,15). However, due to the low organism load in the samples of patients receiving highly active anti-retroviral therapy, those with PCP chemoprophylaxis, and HIV-negative cases, an accurate diagnosis with conventional microscopy is difficult since it largely depends on the skill and experience of the microscopy expert. Therefore, there is a need for rapid identification techniques that can detect even low organism loads (15,16).

In recent years, the advances in the detection of *P. jirovecii* DNA in clinical specimens by Polymerase Chain Reaction (PCR) assay has brought about important benefits in the diagnosis, epidemiology, and management of PCP (17). Different methods and target genes, such as major surface glycoprotein (MSG), dihydropteroate synthase (DHPS), dihydrofolate reductase (DHFR), mitochondrial large subunit rRNA (mtLSUrRNA), 5S rRNA, 18S rRNA, and cdc2, have been developed the molecular diagnosis of PCP. Over the last few decades, real-time PCR has gradually begun to replace conventional PCR (10,18-19).

The aim of this study was to evaluate the diagnostic value of the real-time PCR method compared with indirect immunofluorescent assay (IIFA) and cytological staining for the diagnosis of *P. jirovecii* infections.

MATERIAL and METHODS

A total of 50 patients suspected of PCP with underlying diseases, who were followed-up in outpatient and inpatient clinics in Gazi University Health Application and Research Hospital, were included in the study. The patients were either HIV-infected or HIV-negative with a known risk factor for PCP, such as hematological malignancies, cancer, bone marrow or organ transplantation, and long-term immunosuppressive drug or corticosteroid therapy. Of the samples included in the study (n=50), 23 were obtained from oral wash, 19 from bronchoalveolar lavage (BAL) and eight from induced sputum. All procedures followed were in accordance with the ethical standards of the Ethics Committee of Gazi University Faculty of Medicine (Protocol Number 13.02.2013/31).

Each sample was aliquoted and stored at +4 °C until use. BAL and oral wash specimens were centrifuged at 1,300 rpm for five minutes. Mucus containing specimens was treated with equal volumes of 0.01 M dithiothreitol (DTT), vortexed and incubated at 37 °C for 15 min until liquefaction. Then, one volume of 70% ethanol was added. The suspension was centrifuged at 2000 rpm for 15 min, and the supernatant was discarded. The pellet was washed twice with sterile deionized water and centrifuged at 13,000 rpm for five minute. The supernatant was discarded. The resuspended pellet was stored at +4 ºC until DNA extraction and the indirect immunofluorescent antibody (IIFA) assay.

The samples were cytocentrifuged, and then the slides were stained using Giemsa and Modified Toluidine Blue O (MTolB) as described previously for the examination of the presence of *P. jirovecii* (20,21).

IIFA test was performed according to the manufacturer's instructions using a commercially available monoclonal antibody labeled with fluorescein isothiocyanate (Pneumo-cel Indirect IF Test, Cellabs Pty Ltd., Australia). The entire specimen area was examined with a fluorescence microscope (400x magnification) using a 495-nm excitation and 515-nm barrier filter. The smears were considered to be positive for *P. jirovecii* when two or more bright to medium-bright apple-green cysts were visualized, whether isolated or in a group. For quality control, the positive and negative control slides were stained with every new kit used.

To detect *Pneumocystis* DNA in samples, 300 µl of each resuspended pellet was used for DNA extraction with the Heliosis® DNA Extraction kit (Metis Biotechnology, Turkey) according to the manufacturer's instructions. DNA was eluted in 20 µl SD buffer and stored at -20 ºC until use. The quality and quantity of DNA was evaluated using a Nanodrop (ND-1000) spectrophotometer (Thermo Scientific, USA).

A TaqMan probe (PCJ-1P) (5′-TGCGTGAAACAGATACATGGAGCTCTACCC-3′), where FAM was 6-carboxy fluorescein and TAMRA was 6-carboxytetramethylrhodamine, was used with the following primers: PCJ-1A (5′-GGCTGATCAAAGCATGGATA-3′) and PCJ-1B (5′-CGGCATAGACATATTCGATACTTGTT-3′) to detect the DHFR gene of *P. jirovecii* on real-time PCR. The real-time PCR reaction master mixture consisted of 4 µl LightCycler® FastStart DNA Master^{PLUS} HybProbe kit (Roche, Germany), 20 pmol of each primer and probe, 5 µL template DNA, and distilled water completed to a total volume of 20 µL. All real-time assays were carried out on a LightCycler® 2.0 instrument (Roche Diagnostics, Germany) using the following cycling conditions: initial denaturation 95 ºC for 15 min, followed by 50 cycles of denaturation for 10 s at 95 ºC and annealing for 40 s at 60 ºC, followed by absolute quantification analysis for each sample using

LightCycler® software version 3.5 (Roche). For each run, negative (ultra-pure distilled water) and positive controls (*P. jirovecii* DNA) were included.

Statistical Analysis

The statistical analysis of the data was performed using the Statistical Package for Social Sciences (IBM SPSS® v. 19) software (22). When summarizing the data, the categorical variables were expressed as n (%). The sensitivity, specificity, positive predictive and negative predictive values were calculated from the diagnostic accuracy criteria. The statistical difference between the methods was analyzed by the McNemar test, and the agreement between the methods was investigated using the corrected Kappa (κ) statistic (Prevalence-Adjusted Bias Adjusted Kappa; PABAK). The corrections were made according to the prevalence index (PI) and bias index (BI), and a κ value of <0.20 was considered to indicate none to slight, 0.21–0.40 fair, 0.41– 0.60 moderate, 0.61–0.80 substantial and 0.81–1.00 almost perfect agreement (23).

RESULTS

Of the 50 patients included in the study, 34 (68%) were male and 16 (32%) were female. The age range of the patients was three to 77 years, and the mean age was 53.7 years.

Table 1. The underlying diseases of the patients undergoing *P. jirovecii* screening

		Pneumocystis jirovecii	
	(n=50)	Negative (n=43)	Positive (n=7)
Hematologic malignancies			
Acute myeloid leukemia	10	10	-
Acute lymphoid leukemia	3	2	1
Chronic lymphoid leukemia	8	6	2
Multiple myeloma	13	11	2
Non-Hodgkin's lymphoma	2	2	-
Solid tumors			
Lung cancer	5	3	2
Nasopharyngeal cancer	2	2	-
Kidney cancer	1	1	-
Gastric cancer	1	1	-
Solid organ transplantation			
Heart transplantation	1	1	-
Other			
HIV-positive patient	1	1	-
Chronic Obstructive Pulmonary Disease	2	2	-
Chronic Renal Failure	1	1	-

One patient (2%) was HIV-positive, 36 had hematological malignancies (72%), nine had solid tumors (18%), two had (4%) chronic obstructive pulmonary disease, one (2%) had undergone heart transplantation, and one (2%) had chronic renal failure (Table 1).

Fifty respiratory tract samples comprising 23 oral wash, 19 BAL and eight induced sputum specimens were included in the study. Of the seven (14%) samples detected to have *P. jirovecii* positivity using PCR, six were also found positive by IIFA, five by Giemsa staining, and four by MTolB staining. Of the seven positive samples according to real-time PCR, three had been obtained from BAL, two from induced sputum, and two from oral wash. Positivity was detected in four samples (one from BAL and three from induced sputum) using all four methods. In one induced sputum sample that was found to be negative by real-time PCR, Giemsa and MTolB methods, positivity was detected using IIFA. When this sample was examined by IIFA, rare empty cysts were seen. An example of these cases was an HIV-positive patient whose PCP treatment had just ended (Table 2).

Table 2. The test results and underlying diseases of the patients with *P. jirovecii*

Patient	Underlying Disease	Sample	MTolB	Giemsa	IIFA	Real-time PCR
1	Chronic lymphoid leukemia	Induced Sputum	+	+	+	+
2	Acute lymphoid leukemia	BAL	+	+	+	+
3	Lung cancer	BAL	+	+	+	+
4	Multiple Myeloma	BAL	+	+	+	+
5	Acute lymphoid leukemia	Induced Sputum	-	+	+	+
6	Lung Cancer	Oral wash	-	-	-	+
7	Multiple Myeloma	BAL	-	-	+	+
8	HIV	Induced Sputum	-	-	+	-
Total	8	8	4	5	7	7

MTolB: Modified Toluidine Blue O; IIFA: Indirect immunofluorescent antibody; Real-time PCR: Real-time Polymerase Chain Reaction; HIV: Human immunodeficiency virus; BAL: Bronchoalveolar lavage

Accepting real-time PCR as the gold standard method, the diagnostic accuracy criteria (sensitivity, specificity, positive predictive value, and negative predictive value) of the other three diagnostic methods are presented in Table 3. According to these results, the sensitivity and specificity of IIFA were 85.7% and 97.7%, respectively. For the Giemsa and MTolB staining methods, the sensitivity was 71.4% and 57%, respectively, and the specificity was 100% for both (Table 3).

When real-time PCR and IIFA were compared, the results were statistically similar (p = 1.000), and there was almost perfect agreement between the two methods (κ = 0.92). Similarly, when real-time PCR and the cytochemical staining methods were examined, the results were statistically similar, but the coefficient of the comparison between real-time PCR and Giemsa was found to be higher (κ = 0.92) compared to the MTolB method (κ = 0.88). The results indicated almost perfect agreement between PCR and both staining methods (Table 4).

Table 3. Performance of other methods when real-time PCR is accepted as the Gold standard

	SEN (95% CI)	SPEC (95% CI)	PPV (95% CI)	NPV (95% CI)	Overall accuracy
Giemsa	71.4 (29.0-96.3)	100.0 (91.8-100.0)	100.0 (46.3-100.0)	95.6 (86.9-98.6)	96.0 (86.3-99.5)
MTolB	57.1 (18.4-90.1)	100.0 (91.8-100.0)	100.0 (39.6-100.0)	93.5 (85.9-97.1)	94.0 (83.5-98.8)
IIFA	85.7 (42.1-99.6)	97.7 (87.7-99.9)	85.7 (45.8-97.7)	97.7 (87.2-99.6)	96.0 (86.3-99.5)

MTolB: Modified Toluidine Blue O; IIFA: Indirect immunofluorescent antibody; SEN: Sensitivity; SPEC: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; CI: Confidence interval. The results are presented as %

Table 4. Agreement between the methods when real-time PCR is accepted as the Gold standard

		Real-time PCR		p*	Cohen's Kappa	PABAK
		-	+			
Giemsa	-	43	2	0.500	0.81	0.92
	+	0	5			
MTolB	-	43	3	0.250	0.69	0.88
	+	0	4			
IIFA	-	42	1	1.000	0.83	0.92
	+	1	6			

*p vaue of the McNemar test; MTolB: Modified Toluidine Blue O; IIFA: Indirect immunofluorescent antibody; PABAK: Prevalence-adjusted and bias-adjusted kappa

DISCUSSION

PCP is a serious and potentially fatal disease in immunocompromised patients if adequate and timely treatment is not undertaken (19). In PCP infections, the patient's clinical condition may suddenly deteriorate, in which case, early diagnosis and initiation of treatment with high sensitivity and appropriate methods can have a positive effect on clinical outcome (12,24).

Since *P. jirovecii* is a fungus that cannot be cultured in vitro, in addition to cytochemical staining and direct immunofluorescence antibody (DFA) methods, molecular tests have also been widely used in the diagnosis (25,26).

In the diagnosis of *P. jirovecii,* various cytochemical staining methods are utilized; e.g., Gomori methenamine silver (GMS), Gram Weigert (GW), Giemsa, Diff-Quik (DQ), toluidine blue O (TBO), and calcofluor white. The walls of the cystic forms of *Pneumocystis* are selectively stained dark brown by GMS while GW and TBO staining produces a purple or blue color. Giemsa and Diff-Quick do not stain sporocyst or cyst walls, but they stain the nucleus of the life cycle stages of *Pneumocystis* (3,27).

In HIV-negative patients, bronchoscopic procedures for the diagnosis of PCP are often difficult due to rapidly progressive respiratory failure (9). In addition, HIV-negative patients have a lower *Pneumocystis* burden than AIDS patients, which makes it difficult to microscopically detect organisms. Due to its high sensitivity, PCR is increasingly used for the microbiological diagnosis of PCP (9,29).

There are several studies comparing multiple methods in patients with PCP; however, the results of these studies are contradictory (12,25,26,30,31). In one of these studies, the incidence of *P. jirovecii* was investigated using Giemsa and methenamine silver staining, IIF staining, and conventional PCR to determine the diagnostic value of these methods in 30 immunocompromised patients with respiratory system complaints (12 of whom were HIV-infected). Four, eight and 13 samples were positive with cytochemical staining, IIF and conventional PCR, respectively. PCR revealed positivity in all samples that were found to be positive by the cytochemical staining and IIF methods. The authors concluded that the IIF and PCR methods should be applied together in the analysis of sputum samples in terms of *P. jirovecii* (30).

Flori et al. investigated the presence of *P. jirovecii* in 173 BAL specimens obtained from 150 cases (19 HIV-positive and 131 non HIV-infected) using Giemsa and Gomori Grocott staining methods, conventional PCR (mtLSUrRNA gene), and real-time PCR (MSG gene) and reported the development of PCP in 11 patients, of whom seven were HIV-seropositive and four were HIV-seronegative. When the sensitivity and specificity of the diagnostic methods were compared, the sensitivity of the staining methods was 60% (6/10, insufficient for 1 sample staining), and specificity was 100% (139/139). The sensitivity and specificity of the PCR methods were determined as 100% (11/11) and 87% (121/139), respectively for conventional PCR and 100% (11/11) and 84.9% (118/139), respectively for real-time PCR. The authors noted that there was a significant difference between PCR and staining techniques in terms of both sensitivity and specificity ($p = 0.01$), that staining methods provided perfect specificity despite their low sensitivity, and that both PCR tests produced false positives despite much higher sensitivity (25).

In another study, DFA and real-time PCR were compared in 400 BAL specimens in terms of their efficacy in the diagnosis of *P. jirovecii*. Sixty-six samples were positive by real-time PCR and 31 samples were positive by DFA. None of the cases that were negative by PCR were found to be positive by DFA. The authors suggested that real-time PCR might replace DFA as a definitive diagnostic method in the diagnosis of *P. jirovecii* (31).

Samuel et al. investigated the incidence of *P. jirovecii* in 202 hospitalized children in Africa using real-time PCR and IIF. They also examined the presence of HIV in 200 patients and found 129 HIV-infected cases, of which 27 had received trimoxazole prophylaxis. A total of 349 samples were obtained from the 202 patients 147 patients had paired samples consisting of a nasopharyngeal aspirate with either BAL or induced sputum, and 55 patients produced a single respiratory sample. The authors identified *P. jirovecii* in 180 (52%) of these samples using real-time PCR. Twenty-six patients were found to have *P. jirovecii* positivity by IIF, and all of these cases were confirmed by PCR, with the IIF method producing no additional positivity. It was reported that real-time PCR was five times more sensitive than IIF in the diagnosis of *P. jirovecii* in children with PCP based on upper and lower respiratory tract samples. In addition, the use of upper respiratory tract and lower respiratory tract specimens together increased the diagnostic yield, and the upper respiratory tract specimens were found to be as reliable as those lower respiratory tract specimens for the PCP diagnosis (12).

In a study conducted with 100 patients suspected to have PCP, Tekinşen et al. examined the presence of *P. jirovecii* using Giemsa staining, DFA, and real-time PCR (primers targeting the MSG gene) in the respiratory tract specimens and (1→3)-β-D-Glucan (BDG) test and PCR in the serum samples. Positivity was detected in a total of eight (8%) BAL samples; two by Giemsa, DFA and PCR, and six by PCR alone. All the serum samples were found to be negative by PCR while 29 were positive, five were suspicious, and 66 were negative according to the BDG test. For the eight patients that PCR revealed to be positive for *P. jirovecii*, BDG also provided positive results. When the agreement between the methods in detecting *P. jirovecii* was investigated, there was high ($\kappa = 1$) agreement between Giemsa and DFA; however, the correlations between PCR and DFA ($\kappa = 0.38$), DFA and BDG ($\kappa = 0.07$), and BAL-PCR and BDG ($\kappa = 0.28$) were fair. When the DFA test was accepted as the gold standard, the

sensitivity and specificity values were calculated as 100% for Giemsa, 100% and 93%, respectively for PCR and 100% and 67%, respectively for BDG. In the diagnosis of PCP-suspected cases, the examination of clinical specimens with Giemsa and DFA had a high specificity (100%), whereas the sensitivity was increased with the addition of the BAL-PCR and BDG tests (93%); therefore, the authors concluded that in the laboratory diagnosis of *P. jirovecii*, all these tests should be undertaken (26).

Many patients with positive PCR and negative microscopy results have been reported to develop PCP with typical clinical and/or radiological symptoms. In addition, PCP is seen in many immunocompromised HIV-negative patients despite their lower burden, which reduces the sensitivity of microscopic tests. Furthermore, in HIV-infected or non-infected PCP patients, chemoprophylaxis may reduce the organism burden, making the diagnosis difficult. PCR testing is particularly helpful in such cases (17).

In this study, *P. jirovecii* was detected in five patients by Giemsa, four by TBO, seven by IIFA, and seven by real-time PCR. The patients that were positive according to PCR but negative using the other methods were re-evaluated in terms of clinical findings, and the PCP diagnosis was confirmed in all of these cases.

In sputum samples taken from a patient with AIDS, positivity was detected by DFA but not by other methods. The patient received PCP treatment, and the treatment process was completed. The exact cause of inconsistent test results is not known. Possible explanations for the inconsistent test results include the presence of an inhibitor in the PCR reaction and/or low PCP load due to the termination of *Pneumocystis* treatment (32). PCR inhibition is a current issue that limits the reliability and sensitivity of diagnostic PCR systems (33). It has been shown that inhibitors in blood, feces, respiratory samples and other complex biological samples inhibit PCR and cause false negative results (33, 34).

Döşkaya et al. analyzed the degree and frequency of inhibition of real-time PCR used to detect *P. jirovecii* in respiratory samples. In their first PCR study, the authors reported that the inhibition frequency of BAL samples was 23.80% (15/63), whereas the inhibition rate in sputum was 50% (5/10). The inhibition of sputum samples decreased to 40% (4/10) and 20% (2/10) at 1:2 and 1:5 dilutions, respectively, and at 1:10 dilution, inhibition was prevented in all samples. The inhibition frequency of BAL samples was reduced to 17.46% (11/63), 11.11% (7/63) and .34% (4/63) when the samples were diluted at 1:2, 1:5 and 1:10, respectively. Inhibition in all BAL samples was resolved at 1:20 dilution (34). In our patient, the results may been affected by the presence of a PCR inhibitor or low PCP load due to the termination of treatment.

At the end of this study, the highest positivity was obtained from real-time PCR and IIFA, and when PCR was accepted as the gold standard, the sensitivity and specificity of IIFA were 85.7% and 97.7%, respectively while the Giemsa and MTolB staining methods had a sensitivity of 71.4% and 57%, respectively and specificity of 100%. When the findings obtained from different methods were compared, there was almost perfect agreement between PCR and all the remaining methods. The coefficient of agreement was 0.92 for the comparison of PCR results with those of IIFA and Giemsa, while it was slightly lower for the MTolB method ($\kappa = 0.88$).

The cytochemical staining methods used in this study (Giemsa and MTolB) are inexpensive (~ 2.5 TL/sample) and allow multiple samples to be processed simultaneously. However, they take a long time to process (Giemsa ~ 60 min, MTolB ~ 40 min) and contain many steps. These methods can be applied to any clinical sample, but they are not specific because they show affinity for other pulmonary pathogens. Therefore, interpreting microscopic slides requires training and expertise, especially when working with patients with a low fungal load (27).

The IIFA method is more expensive (~ 40 TL) and takes longer to apply (~ 65 min) compared to the cytochemical staining methods. It also requires the use of a fluorescent microscope and evaluation of experienced staff. In the presence of a large number of samples, early results cannot be achieved with IIFA due to the time required for the microscopic examination.

The advantage of real-time PCR is that it can operate on a large number of samples at a time and has a fast turn-around time. The possible difficulties in the widespread implementation of real-time PCR include its higher cost and the need for a specialized molecular laboratory and equipment, as well as specialized personnel and standardization.

CONCLUSION

In conclusion, the most important issue in the diagnosis is to consider the possibility of *P. jirovecii* positivity in high-risk patients. In immunocompromised patients, PCP should be investigated in the presence of dyspnea and fever, and interstitial infiltrates in the lung x-ray during follow-up. In order to reduce mortality and morbidity, it is suggested that IIFA and real-time PCR with high sensitivity in diagnosis should be studied together.

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