Development of a high throughput PCR to detect *Coxiella burnetii* and its application in a diagnostic laboratory over a 7-year period

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Abstract

Q fever is a worldwide zoonotic infectious disease due to *Coxiella burnetii*. The clinical presentation may be acute (pneumonia and/or hepatitis) or chronic (most commonly endocarditis). Diagnosis mainly relies on serology and PCR. We therefore developed a quantitative real-time PCR. We first tested blindly its performance on various clinical samples and then, when thoroughly validated, we applied it during a 7-year period for the diagnosis of both acute and persistent *C. burnetii* infection. Analytical sensitivity (< 10 copies/PCR) was excellent. When tested blindly on 183 samples, the specificity of the PCR was 100% (142/142) and the sensitivity was 71% (29/41). The sensitivity was 88% (7/8) on valvular samples, 69% (20/29) on blood samples and 50% (2/4) on urine samples. This new quantitative PCR was then successfully applied for the diagnosis of acute Q fever and endovascular infection due to *C. burnetii*, allowing the diagnosis of Q fever in six patients over a 7-year period. During a local small cluster of cases, the PCR was also applied to blood from 1355 blood donors; all were negative confirming the high specificity of this test. In conclusion, we developed a highly specific method with excellent sensitivity, which may be used on sera for the diagnosis of acute Q fever and on various samples such as sera, valvular samples, aortic specimens, bone and liver, for the diagnosis of persistent *C. burnetii* infection.

Keywords: Blood donations, *Coxiella burnetii*, high throughput, PCR, Q fever

Original Submission: 5 July 2013; **Accepted:** 22 July 2013

Article published online: 18 September 2013

New Microbe New Infect 2013; **1:** 6–12

Introduction

Q fever is a worldwide zoonosis due to *Coxiella burnetii*, a small pleomorphic gram-negative intracellular bacillus. Human infection is caused by inhalation of contaminated aerosols that can spread by wind over some distance [1]. When infected pregnant small ruminants abort, billion of *C. burnetii* end up in the environment and fewer than ten organisms are sufficient to seed an infection [2]. The clinical presentation may be (i) acute with influenza-like illness, hepatitis or pneumonia, or (ii) chronic with mainly endocarditis [3]. Diagnosis still remains challenging and relies mainly on serology, mostly performed by indirect immunofluorescence assay. However with serology it is difficult to make an early diagnosis, especially in acute settings because it may occur during an epidemic.

In Switzerland, an important outbreak of human Q fever occurred in 1983 in a Swiss Alpine valley [4] caused by flocks of sheep infected by *C. burnetii*. Some villages located near the flocks were strongly affected and the percentage of inhabitants with acute Q fever was about 21%. Since that time, several new outbreaks have been described, including a huge epidemic in the Netherlands, with more than 4000 human cases notified between 2007 and 2010 [5]. This large outbreak demonstrated the importance of recognizing an epidemic early, through strict veterinary surveillance and early recognition of acute Q fever among humans. Serology is one of the current diagnostic methods for Q fever, but it is obvious that the lag phase in antibody response of 7–15 days after onset of clinical symptoms, is an important drawback of serology [5]. Conversely, for the diagnosis of persistent *C. burnetii* infection, serology is associated with a very high negative predictive value whereas PCR may be applied to various samples such as cardiac valves, aortic fragments and liver tissue, according to the respective organ involved, so representing a pivotal tool to confirm which organ is affected.

In this context, we developed in 2005 an in-house diagnosis real-time PCR that may be applied to various clinical specimens including lower respiratory tract samples, blood, sera and biopsies. We then assessed its analytical performance and all respiratory specimens submitted to our diagnostic laboratory for *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and *Legionella pneumophila* PCRs from 2005 to 2012 were

prospectively analysed with the *C. burnetii* PCR. Specimens from patients with suspected persistent *C. burnetii* infection were also tested prospectively using our real-time PCR during this 7-year period. Finally, in July 2012, in the context of a local Q fever outbreak, we used this PCR to test all blood donations to prevent transmission of *C. burnetii* via blood transfusion. Hence, we report here our 7-year experience with this new PCR initially validated at the analytical and clinical levels.

Material and Methods

Development of the real-time *C. burnetii* PCR

Primers, probe and PCR settings. Primers and probe were selected using Primer express software (Applied Biosystems, Foster City, CA, USA) from alignments of the available sequences of the *ompA* gene using Clone Manager 9 software (Scientific & Education software, Cary, NC, USA). A forward primer COX_F (5′-CAGAGCCGGGAGTCAAGCT-3′), a reverse primer COX_R (5′-CTGAGTAGGAGATTTGAAT CGC-3′), and a minor-groove binder probe labelled with 5′ TET(6-carboxyfluorescein) COX_probe (5′-CAGCCCTGC-AGCGAGGAGCC-3′) were selected to amplify an 82-base-pair fragment of the highly conserved region of the *ompA* gene.

The PCR were performed in a final volume of 20 μL containing 0.4 μM of each primer, 0.1 μM of probe, 10 μL of 2× Universal master mix (Applied Biosystems) and 5 μL of extracted sample. The cycling conditions were 2 min at 50°C, 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C, during 45 cycles. The PCR products were detected with an ABI Prism 7900 instrument (Applied Biosystems). Since 1 April 2012, the Fast ABI Prism 7900 instrument was used, 7.5 μL of Fast advanced Master Mix (Applied Biosystems) was used in each reaction and the cycling conditions were 2 min at 50°C, 10 min at 95°C, followed by 45 cycles of 1 s at 95°C and 20 s at 60°C, during 45 cycles. This fast PCR reduced the time of the PCR from 2 h and 20 min to 1 h.

Analytical sensitivity and specificity. To allow quantification, a plasmid containing the target was constructed as performed previously [6]. To trace possible plasmid contamination the insert was synthesized with four base modifications (R&D Biotech, Besançon, France). These plasmids were run to evaluate the analytical sensitivity and the intra-run and inter-run reproducibility with samples containing ten-fold dilution from 1000 to ten copies of the positive control (plasmid DNA). When the TaqMan Fast instrument was introduced, these controls were run again to reassess the analytical sensitivity and the robustness of the system. Moreover, the plasmids were used routinely as positive controls in each run of PCR (1000, 100, 10 copies) and the cycle thresholds were introduced in a database (multiQ system, developed by Philippe Marquis, France) in order to daily check the analytical sensitivity of the PCR.

DNA extraction. DNA was extracted using the MagnaPure LC automated system (Roche, Rotkreuz, Switzerland) and the MagNA Pure LC DNA isolation kit I (Roche) from 2005 to 1 February 2011. From this date, to increase the diagnostic efficiency of our molecular diagnosis platform, the samples were extracted using a full automation of nucleic acid extraction with the dual STARlet (Hamilton®, Bonaduz, Switzerland)-MagNAPure 96 (Roche) system using the DNA and viral NA small-volume kit with the Pathogen Universal 2.0 protocol. This new system was evaluated before its use as described earlier [7]. DNA was extracted from 200 μL of specimen, i.e. EDTA-blood, sera, ground biopsies.

Validation of the *C. burnetii* PCR on clinical samples

Patients. First, we retrospectively analysed blindly eight paired sera taken from four patients with acute Q fever, infected during the 1983 Swiss outbreak and diagnosed as Q fever by indirect immunofluorescence assay. All four paired sera were initially negative by serology and all seroconverted for IgM and/or IgG phase 2 antigens.

To evaluate the sensitivity and specificity of the real-time PCR, 123 extracted DNA from 66 EDTA-blood, 45 sera and 12 cardiac biopsies specimens (provided by Prof. Didier Raoult) were tested blindly and the results were compared to a nested PCR targeting the IS 1111 [8].

Pneumonia is the predominant presenting syndrome in acute Q fever, we therefore analysed prospectively all 1046 respiratory specimens (111 sputa, 132 bronchial aspirations, 752 bronchoalveolar lavages, 20 nasopharyngial secretions, 19 endotracheal secretions, 12 pleural fluids) for which *Chlamydia pneumoniae*, *M. pneumoniae* or *L. pneumophila* were requested by clinicians and that were referred to our laboratory from 2005 to 2012. Other specimens (48 EDTA-blood, 29 serum, 59 cardiac biopsies, 13 liver biopsies, 9 pulmonary biopsies and 19 other different specimens) from 101 patients with suspected acute or persistent *C. burnetii* infection were additionally tested prospectively, when requested by clinicians.

Blood donors. From 14 July to 20 August 2012, we had to test in our high throughput platform, 2393 blood donations in a prospective reactive way, to prevent Q fever transmission by blood transfusion in the setting of a local *Coxiella* outbreak.

We received a mean of 100 specimens per day (minimum 50 and maximum 179 specimens) to be tested for *C. burnetii* by

PCR within 24 h so that the blood could be used as quickly as possible. This overwork was challenging and had to be carried out in parallel with the routine diagnostic workload. In practice, when the bar-coded EDTA blood tubes arrived in the laboratory they were scanned using the STARlet liquid handling system (Hamilton®) and then 200 μL was transferred to the bar-coded MagnaPure 96 Processing cartridge (Roche). This step took 15 min to be completed for 95 specimens and one negative extraction control. DNA was then extracted (1 h for 96 specimens) and eluted in another bar-coded plate, i.e. MagnaPure 96 output plate. Generally two runs per day were completed.

The master mix was prepared manually each day and distributed in a bar-coded microamp optical 384-well reaction plate (Applied Biosystems) by the liquid handling system Tecan Freedom Evo through the 2.4 SDS software (Applied Biosystems). The DNA distribution in the 384-well plate was carried out according to the Hamilton® Robot EXCEL master file through Gemini (Tecan, Männedorf, Switzerland). For each plate, two sets of diluted plasmid-positive controls were added (1000, 100, 10 copies/PCR corresponding to 100 000, 10 000 and 1000 copies/mL of the initial sample). For each specimen, duplicates were performed. Moreover a third well used as an inhibition control was prepared by adding 200 copies of plasmid DNA (in 1 μL) to the DNA of each specimen (4 μL). One 384-well plate was needed for 119 specimens, because three wells were required per specimen and as many as 27 wells were used for the positive and negative controls of the PCR. The set up of the plate was performed in 100 min and finally the 384-well plate was placed in the Fast ABI-Instrument for 55 min. The results were read and checked on the 2.4 SDS in less than 15 min.

Results

Reproducibility, analytical sensitivity and specificity of the PCR

To detect *C. burnetii*, a new quantitative real-time PCR was developed that targets the *ompA* gene. This gene was chosen given its high sequence conservation, as assessed by *in silico* comparison of the seven available sequences in 2005, confirmed with 64 *ompA* sequences available in 2012. Analytical specificity was excellent and no cross-amplification was observed when we tested the genomic DNA of humans (Human genomic DNA, Roche), fungi (*Candida albicans* ATCC10231, *Aspergillus fumigatus* clinical isolate), bacteria (*Bordetella pertussis* clinical specimen, *Chlamydia pneumoniae* ATCC VR-1310, *Parachlamydia acanthamoebae* ATCC VR-1476, *Escherichia coli* ATCC 35218, *Klebsiella pneumoniae* ATCC 27736, *Haemophilus influenzae* ATCC 49247, *Haemophilus parainfluenzae* ATCC 7901, *Legionella pneumophila* ATCC 33152, *Mycoplasma pneumoniae* ATCC 15293, *Staphylococcus aureus* 29213, *Staphylococcus epidermidis* ATCC 14990, *Streptococcus pneumoniae* ATCC 49619, *Streptococcus pyogenes* ATCC 19615, *Streptococcus mitis* ATCC 6249, *Moraxella catharralis* ATCC 25238, *Mycobacterium tuberculosis* clinical specimen, *Pseudomonas aeruginosa* ATCC 27853), and viruses (adenovirus QAL-London, cytomegalovirus QAL-London, herpes simplex type 1 clinical specimen, herpes simplex type 2 clinical specimen, varicella zoster virus clinical specimen).

Then, a plasmid was constructed to assess the reproducibility of the PCR, to define the analytical sensitivity and to be used as positive control. Sensitivity was first determined by testing duplicates of ten-fold serial dilutions of the plasmid in independent experiments. The ten-fold diluted plasmids corresponding to 1000, 100 and 10 copies per reaction were always positive when tested in ten different runs with Ct of 28.04 ± 0.33, 31.56 ± 0.24 and 35.11 ± 0.71, respectively. The 1 and 0.1 copy positive controls were only positive in 5/10 and 3/10 PCRs with cycle threshold of 37.78 ± 2.04 and 40.1 ± 2.44, respectively. Hence the analytical sensitivity is close to one copy and clearly better than ten copies. To assess the intra-run and inter-run variation observed during routine work, the data of the plasmid positive controls were introduced from 1 January 2008 to 31 January 2012 in a MULTIQC WINDOWS software application, a program that allows monitoring of the analytical sensitivity of the PCR. During this 4-year period, 468 runs were performed with the conventional ABI 7900 and the 1000 copies, 100 copies and 10 copies/reaction exhibited medium cycle threshold values corresponding to 27.76 ± 0.44, 30.97 ± 0.51, 34.18 ± 0.73, respectively; with the new TaqMan Fast 7900, 80 runs were performed from 1 January 2012 to 1 August 2012 and the medium cycle threshold values were 27.96 ± 0.42, 31.13 ± 0.42 and 34.42 ± 0.78, showing the robustness of the analytical sensitivity of the PCR run on two different instruments with two different master mixes (Table 1).

TABLE 1. Analytical sensitivity and reproducibility of the real-time PCR

	Initial validation of the PCR		468 runs 1 January 2008 to 1 January 2012		80 runs 1 January 2012 to 1 August 2012	
Copies/reaction	Ct mean	SD	Ct mean	SD	Ct mean	SD
1000	28.04	0.33	27.76	0.44	27.96	0.42
100	31.56	0.24	30.97	0.51	31.13	0.42
10	35.11	0.71	34.18	0.73	34.42	0.78
1	37.78	2.04	nd	–	nd	–
0.1	40.1	2.44	nd	–	nd	–

Ct, Cycle threshold; SD, standard deviation; nd, not done.

TABLE 2. **Retrospective blind validation on 123 specimens of the new real-time PCR targeting the *ompA* gene using as gold standard a nested PCR targeting the IS 111 gene**

	Nested PCR IS 111	Real-time PCR *ompA*	*n*	Sensitivity, %	Specificity, %	min–max Ct	mean Ct	median Ct
66 EDTA-blood	+	+	3	100	100	19.82–34.04	26.01	27.17
	–	–	63					
45 serum	+	+	18			33.74–42.4	36.46	35.59
	+	–	5					
	–	+	1	78.3	95.5	34.88		
	–	–	21					
12 valvular biopsies	+	+	5			15.41–36.4	31.32	34.88
	+	–	1	83.3	83.3			
	–	+	1			37.94		
	–	–	5					
All	+	+	26					
	+		6					
		+	2	81.2	98			
	–	–	89					

The mean, median and minimum/maximum Ct values are also shown. Ct, Cycle threshold; *n*, number of samples.

TABLE 3. **Prospective evaluation on 1046 respiratory samples***

Respiratory specimens	*n*	*Coxiella burnetii*	*Legionella pneumophila*	*Mycoplasma pneumoniae*
Sputa	111	0	18	8
Bronchial aspirates	132	0	7	0
Bronchoalveolar lavage	752	0	9	4
Nasopharyngeal secretions	20	0	0	1
Endotracheal secretions	19	0	3	0
Pleural fluids	12	0	2	0

*Note that all these 1046 samples have also been tested for *Chlamydia pneumoniae*, but none were positive [31].

Retrospective validation on clinical samples

Among eight sera from four patients infected during the outbreak of Q fever in 1983, all 4 initial sera negative by serology were positive by PCR (cycle threshold : 32.04, 36.84, 38.04 and 40.69) whereas all four convalescent sera positive by serology were all negative by PCR. This confirmed that real-time PCR may be useful for early diagnosis of acute Q fever before seroconversion [5,8,9]. These sera had been stored for 20 years and this long storage did not prevent the positivity of the PCR.

We then assessed the sensitivity and specificity of the real-time PCR on 66 EDTA-blood, 45 sera and 12 valvular specimens. These 123 samples were tested blindly and the results were compared with the nested PCR targeting the IS 1111 performed in Marseille as described elsewhere [8] (Table 2). Real-time PCR was positive in three out of three EDTA-blood samples that were positive with the nested PCR and negative in 63 out of 63 EDTA-blood samples that were negative by nested PCR. This shows a sensitivity and specificity of 100% in EDTA-blood samples. Cycle threshold values for the three positive samples were 19.82, 27.17 and 34.04, corresponding to high copy numbers per mL. For serum samples, 18 were positive by both PCR methods, whereas five were only positive by the nested PCR and one was only positive by real-time PCR, the other 21 serum samples were negative by both PCR, showing a sensitivity of 78.3% and a specificity of 95.5% for serum samples when compared with the nested PCR. For positive serum samples the mean Ct value was 36.46 (minimum 33.74, maximum 42.4) clearly lower than the values of EDTA-blood samples, suggesting that EDTA-blood is a better specimen than serum. Regarding the 12 valvular samples, five were positive by both PCR, one was positive only by nested PCR and another one only by real-time PCR whereas five were negative by both PCR. Sensitivity and specificity were both 83.3% but these are small numbers of specimens. For specimens that were positive by both PCR, cycle threshold values were 15.41, 34.88, 34.04, 35.90, 36.40, respectively, corresponding to a mean 31.32 (minimum 15.41; maximum 36.40), median of 34.88. The relatively high mean and median of cycle threshold values of serum samples could explain the lower sensitivity of the real-time PCR for those specimens.

Prospective application to respiratory samples

During 7 years (2005 to 2012), 1046 respiratory samples (111 sputum, 132 bronchial aspirates, 752 bronchoalveolar lavage, 20 nasopharyngeal secretions, 19 endotracheal secretions and 12 pleural fluids) were prospectively analysed by this new *C. burnetii* real-time PCR. All were found to be negative whereas 39 were found positive for *Legionella pneumophila* PCR, 13 were positive for *Mycoplasma pneumoniae* PCR and none were positive for *Chlamydia pneumoniae* PCR (Table 3).

Prospective application to specimens other than respiratory samples

Among a total of 177 samples, the *C. burnetii* real-time PCR was positive in 12 samples taken from six different patients: seven cardiac biopsies, one serum, two inguinal biopsies, one

TABLE 4. Clinical characteristics and results of laboratory investigations of the six patients with a positive *Coxiella* PCR

Patient no.	Age	Sex	Clinical presentation	Laboratory findings	Sample	*Coxiella burnetii* PCR DNA copies/mL (mean value Ct)	Serology*
1	65	M	Aortic prosthesis infection Ileofemoral abscess	Serum and EDTA-blood negative by *C. burnetii* PCR	Femoro-iliac abscess	260 (35.3)	Ph1: IgG: 6 400; IgM: 0; IgA: 3 200
					Aortic stent 1	19 900 (29.7)	Ph2: IgG: 6 400; IgM: 0; IgA: 800
					Aortic stent 2	184 000 (26.1)	
					Inguinal fragment 1	124 (37.9)	
					Inguinal fragment 2	3100 (33.0)	
					Intraoperative groin biopsy	123 (36.6)	
2	71	F	Endocarditis		Periaortic valve abscess	3500 (32.3)	Not available
					Pus around aortic valve	1 357 900 (24.7)	
3	43	F	Endocarditis	Eubacterial PCR positive for *C. burnetii* Serum negative by *C. burnetii* PCR	Aortic valve	1 000 000 (16.7)	Ph1: IgG: 6400; IgM: 0; IgA: 200 Ph2: IgG: 12 800; IgM: 0; IgA: 400
4	53	H	Aortic aneurysm		Pseudoaneurysm fragment	380 (36.0)	Not available
5	57	M	Spondylodiscitis	Paraffin-embedded tissue was also positive by PCR	L3–L4 vertebral biopsy	18 969 (31.2)	Ph1: IgG: 8192; IgM: 0; IgA: 0 Ph2: IgG: 4096; IgM: 0; IgA: 0
6	47	M	Granulomatous hepatitis		Serum**	390 (35)	**Ph1: IgG: 0; IgM: 0; IgA: 0 Ph2: IgG: >640; IgM: 0; IgA:0

*Only highest titres are shown.
**Serology on initial serum was negative for *C. burnetii*. The PCR was only positive on first serum

intraoperative groin biopsy and one vertebral biopsy. The clinical, microbiological and serological characteristics of these six patients are summarized in Table 4. Four patients (patients 1–4) presented with endocarditis, one patient with a spondylodiscitis and the last one had acute Q fever with isolated granulomatous hepatitis. For this last patient, the first serum did not exhibit any anti-*Coxiella* antibodies but was positive by PCR confirming that PCR was positive earlier than serology.

High throughput application of the *Coxiella* PCR to blood from blood donors

For 7 weeks (from 14 July until 30 August 2012) in the context of a local outbreak (see Introduction) we had to test 2393 samples. Time to results had to be minimal because the stock of blood was very low during the summer. Thanks to the automated format of our molecular diagnostic platform, time to get the results was less than 4 h for up to 96 specimens (one run of extraction, Tecan distribution and TaqMan), less than 5 h for up to 119 specimens (two runs of extraction, one run of Tecan distribution and Taqman) and less than 7 h for up to 179 specimens (two runs of extraction, Tecan distribution and TaqMan). All the results were negative.

Discussion

Since the first report of molecular methods for the detection of *C. burnetii*, different PCR mainly targeting the IS 1111 have been developed [10–16]. In Lausanne, a novel real-time PCR targeting the *ompA* gene was developed to specifically detect *C. burnetii* DNA in clinical samples. This new real-time PCR was sensitive with an analytical sensitivity of at least ten plasmid DNA copies per reaction and was highly reproducible. The high analytical sensitivity of our real-time PCR was expected given the small PCR products of the TaqMan format and the efficient detection thanks to a fluorescent probe. Regarding the analytical specificity, all DNA from other pathogens were negative, confirming the good specificity of the primers and probe. However, when tested blindly the overall specificity was 98% (Table 2). Conversely, the overall sensitivity ranged from 78.3% on serum samples to 100% on EDTA-blood. This higher sensitivity on EDTA-blood may be because the centrifugation steps used to remove blood cells, when preparing sera, may also decrease the total number of bacteria whereas EDTA-blood does not require such a centrifugation step.

Early diagnosis of Q fever is important because delayed diagnosis may lead to severe chronic infections such as endocarditis and endovascular infections with high morbidity and mortality [17–21]. As a matter of fact, despite the poor performance of PCR on serum [9], sera are often used instead of EDTA-blood because serum is often already available for serology. To further validate our PCR on sera from patients with acute Q fever, we also tested retrospective samples. All four seronegative initial sera from the former Swiss outbreak of Q fever, were positive by PCR whereas all the seropositive convalescence stage sera were negative. This confirmed that real-time PCR may be useful at the onset of the disease [5,8,9,22] and that PCR positivity is directly related to the absence of phase II antibody [23]. Regarding the valvular biopsies, the sensitivity was 83.3%, which is

excellent because sensitivity of eubacterial PCR is generally as low as 60% [24]. Hence, valve biopsy represents an ideal sample, when available, for the diagnosis of *Coxiella* endocarditis [13].

During 7 years of clinical use, our PCR was positive on various non-respiratory samples such as cardiac valve, aortic aneurysm, liver biopsy and vertebral biopsy (Table 4), congruent with manifestations of Q fever already reported in the literature [3,21,25–28]. All these positive results were also positive by serology, confirming the excellent specificity of our test over a 7-year period. Specificity of the PCR is also supported by the negative results obtained on 1046 respiratory tract samples taken from patients with lung infections of unknown aetiology.

Finally it has been shown that *C. burnetii* can survive and remain infectious in blood products [29]. As this pathogen may be present in blood during the first weeks of infection, *C. burnetii* may be present in donated human blood [30]. For this reason, it was decided when an outbreak of Q fever occurred close to Lausanne in June 2012 to test all blood donations during a 2-month period. All tests were negative, confirming again the high specificity of our PCR. This large screening prevented the exclusion of blood donations as a precaution. Moreover this blood testing demonstrated the usefulness of the automated format of our PCR platform.

In conclusion, we have developed a specific and sensitive real-time PCR for the direct detection of *C. burnetii* DNA in clinical samples that is compatible with our high throughput Taqman real-time diagnostic PCR platform.

Acknowledgements

We are grateful to all the technicians of the Molecular Diagnostic Laboratory of the Institute of Microbiology, particularly René Brouillet, Zahera Nazeri and Gregory Gonzalez for their technical help.

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