Loop-Mediated Isothermal Amplification (LAMP)

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Contributor: Ulrika Morris

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Keywords: loop-mediated isothermal amplification ; LAMP ; Plasmodium ; malaria ; low transmission

1. Introduction

Loop-mediated isothermal amplification (LAMP) is a sensitive molecular tool suitable for use as a near point-of-care test for the diagnosis of malaria in low resource settings [1]. The LAMP methodology—first published in year 2000—relies on isothermal deoxyribonucleic acid (DNA) amplification employing the *Bacillus stearothermophilus* (*Bst*) DNA polymerase with strand displacement activity [2]. LAMP can therefore be performed at a single temperature with a simple heating block or water bath, reducing the need for sensitive and expensive machinery such as polymerase chain reaction (PCR) thermocyclers. The LAMP reaction is primed by a specific set of four to six primers that identify distinct regions on the target DNA. The design of the primers results in DNA loop formations and several inverted repeats of the target DNA [2]. This autocycling strand-displacement DNA synthesis makes the amplification highly efficient and specific, allowing the amplification of a few DNA copies to 10^9 copies under isothermal conditions in less than one hour, reducing the time-to-result [3][4]. DNA amplification can be detected by eye by a change in turbidity caused by white precipitate of magnesium pyrophosphate formed during the reaction, or under ultraviolet (UV) fluorescence if a fluorescent indicator such as calcein is added to the reagents [3]. Visual detection avoids the need for opening the reaction tube post-amplification, hence the reaction is conducted in a closed system which reduces the risk of DNA contamination [5]. Furthermore, the *Bst* polymerase is more robust towards inhibition than *Taq* polymerase in conventional PCR, making it suitable for use with simple and field friendly DNA extraction methods, but maintaining a sensitivity comparable to PCR [1][6][7][8][9].

The first malaria specific LAMP assay targeting the *Plasmodium falciparum* 18S ribosomal ribonucleic acid (rRNA) genes was published in 2006 [10]. This was followed by *Plasmodium* genus and species-specific LAMP assays published in 2007 [7]. Since then, over 40 different LAMP methods have been developed. These developments—some of which were recently reviewed [9][11]—have aimed at improving the sensitivity of the assay by targeting mitochondrial DNA [6] or alternative gene targets [12][13][14][15]. Other methods have aimed at improving or mitigating DNA extraction processes [16][17][18][19], or improving the read-out of the results by incorporating different dyes [20][21][22] or by combining LAMP with lateral flow dipsticks providing a similar result format to malaria rapid diagnostic tests (RDTs) [23].

Recent meta-analyses evaluating the diagnostic accuracy of LAMP for malaria have detailed high sensitivity and specificity of LAMP when compared to microscopy, PCR, and RDTs in both endemic and non-endemic settings (Table 1). The pooled sensitivity and specificity of LAMP has largely remained greater than 95% whichever the comparator [1][24][25], with an area under the curve of greater than 0.98 demonstrating that malaria LAMP is a test with excellent diagnostic performance [24][25]. These meta-analyses concluded that the LAMP method is a robust tool for diagnosing malaria in both symptomatic and asymptomatic individuals [24], and that LAMP is one of the most promising new diagnostic tools for use in malaria endemic settings [25].

Table 1. Pooled estimates of the diagnostic accuracy of malaria loop-mediated isothermal amplification as determined in three meta-analyses.

Comparator	Sensitivity (%; 95% CI)	Specificity (%; 95% CI)	AUC	Reference
LAMP vs. LM	**98; 94–99**	**97; 85–99**	**ND**	[1]
LAMP vs. PCR	**96; 79–99**	**91; 68–98**	**ND**	[1]
LAMP vs. LM	**97; 96–98**	**96; 94–97**	**0.98**	[24]

Comparator	Sensitivity (%; 95% CI)	Specificity (%; 95% CI)	AUC	Reference
LAMP vs. RDT	97; 92–99	96; 92–98	0.98	[24]
LAMP vs. PCR	97; 96–98	96; 94–97	0.98	[24]
Pv LAMP vs. PCR	95; 80–99	96; 86–99	0.98	[24]
Pan LAMP vs. PCR	95; 91–97	98; 95–99	0.99	[25]
Pf LAMP vs. PCR	96; 94–98	99, 96–100	0.99	[25]
Pv LAMP vs. PCR	98; 92–99	99, 72–100	1.00	[25]

95% CI: 95% confidence interval; AUC: area under the curve; LAMP = loop-mediated isothermal amplification; LM = light microscopy; PCR = polymerase chain reaction; RDT = rapid diagnostic test; Pv = P. vivax; Pan = Pan-Plasmodium; Pf = P. falciparum; ND = not determined.

2. Commercially Available LAMP Kits

Fifty-eight of the 148 identified malaria LAMP publications employed one of two brands of field-stable and CE-marked (where CE stands for Conformité Européenne" in French or "European Conformity" in English) malaria LAMP kits that are now commercially available: the Illumigene® (now Alethia ®) malaria LAMP (Meridian Bioscience Inc., Cincinnati, OH, USA) and the Loopamp™ Malaria Detection Kits (Eiken Chemical Co., Tokyo, Japan). Neither of the kits require a cold chain, as they contain ready to use lyophilized reagents that are stable at room temperature [9]. Both brands target the mitochondrial DNA of *Plasmodium* species for genus-level identification. In addition, the Loopamp Malaria Pf Detection Kit and the more recent Loopamp Malaria Pv Detection Kit can differentiate *P. falciparum* and *P. vivax* infections from other species, respectively (Table 2) [26].

Table 2. Comparison between the Illumigene® (Alethia®) malaria LAMP (Meridian Bioscience Inc.) and the Loopamp™ Malaria Pan Detection kits (Eiken Chemical Co.).

Comparator	Illumigene®	Loopamp™
Species identification	Pan-*Plasmodium*	Pan-*Plasmodium*; *P. falciparum*; *P. vivax* [1]
Sample type	Fresh/frozen blood	Fresh/frozen blood or dried blood spots
Methods of DNA extraction	Illumigene malaria; Illumigene malaria PLUS	PURE DNA Extraction Kit; Boil and spin; other methods [2]
Limit of detection [3]	2.0 p/µL for *P. falciparum* and 0.1 p/µL for *P. vivax*	1–2 p/µL
Required equipment	Illumipro-10™ incubator	Centrifuge, heat block/water bath, UV light, (or LA-500 turbidimeter/HumaLoop M)
Number of samples per run	10	16, 46 or 94 [4]
Read out of results	Turbidity in Illumipro-10™ incubator or by eye	Turbidity in turbidimeter or by eye; fluorescence under UV light
Primary area of use	Malaria diagnosis in non-endemic settings	Malaria prevalence surveys in endemic settings
Cost per test [5]	28 EUR	5.2 EUR

[1] Three separate kits. [2] Including Chelex extraction, column-based extraction methods, and a high-throughput DNA extraction platform. [3] Under laboratory conditions. [4] If used together with a turbidimeter or HumaLoop M platform, regular heating block, or high-through put extraction setup, respectively. [5] Does not include cost for equipment.

Both the Illumigene and the Loopamp kits can be performed on fresh or frozen blood, in combination with simple and quick DNA extraction methods. The Loopamp kits can also be performed on dried blood spots [27][28][29]. Both brands provide high analytical sensitivity, with a time-to-result of 1–2 h [27][29][30][31]. Both kits are easy to handle but require some basic laboratory skills as they require blood sample preparation and measures to avoid DNA cross-contamination [32]. The

risk for DNA contamination is however minimized, as both kits utilize a robust closed system, where tubes with amplified products are never opened (unlike conventional or nested PCR) [33]. A drawback of the LAMP methodology is that it does not provide parasite quantification.

2.1. The Illumigene® (Alethia®) Malaria LAMP

The Illumigene Malaria LAMP kit is easy to perform, with DNA extraction and result readout included in the same kit. DNA extraction is conducted by a simple gravitation system that employs lysis buffers and columns to simplify sample preparation. In this approach, whole blood is mixed with a lysis buffer and either used directly in the LAMP assay (simple filtration assay) or passed through a column that purifies the DNA via gravity (Illumigene malaria PLUS) [31]. Both procedures rely on chemical lysis and produce amplifiable DNA within 10 minutes, requiring only the use of micropipettes, DNase/Rnase free sterile pipette tips, and latex gloves. Each test device consists of two tubes, a test tube with primers targeting the *Plasmodium* genus and a control tube with primers to detect a housekeeping human gene used as a DNA extraction and amplification control.

Result readout with an Illumigene Malaria Illumipro-10™ incubator/reader measures turbidity induced by the formation of magnesium pyrophosphate. Ten samples can be analyzed per run in this platform [34]. The detection limit of the assay is 2.0 parasites per microliter (p/µL) or 0.3 p/µL for *P. falciparum* (depending on which extraction method is used) and 0.1 p/µL for *P. vivax* [30][31]. A disadvantage with the kit is that no species differentiation is possible, and there are a few reports of occurrence of invalid results [32][34].

The Illumigene kit is preferably conducted on venous EDTA whole blood samples, making it possible to perform the LAMP assay with some delay. This blood sampling method can however be challenging in resource limiting settings [31]. Furthermore, the Illumigene kit has a high cost (~28 euros per sample plus the cost of the Illumipro-10™ incubator/reader), limiting its use to developed countries for diagnosis of imported malaria [9][30].

2.2. The Loopamp™ Malaria Detection Kits

The Loopamp Malaria Detection Kits have been endorsed by the Foundation for Innovative New Diagnostics (FIND) who has developed standardized procedures for their use [11][35]. The Loopamp Malaria Pan Detection Kit is typically used for malaria screening with an analytical sensitivity of 1–2 p/µL [27][29][36][37]. Positive samples can thereafter be retested using the Pf or Pv detection kits for further species identification [38].

DNA can be extracted with the commercially available Loopamp PURE DNA Extraction Kit, consisting of a series of interlocking plastic components providing a closed system for preparation of a blood aliquot requiring only a heat block or incubator, micropipettes, DNase/Rnase free sterile pipette tips, and latex gloves [39]. The Loopamp kits can also be used with a cheaper, quick and easy, boil and spin DNA extraction method, but requires additional equipment such as a centrifuge [9][35]. The LAMP reaction can either be conducted in a simple heat block or water bath, or in a real-time turbidimeter. The formation of magnesium pyrophosphate resulting in turbidity, or fluorescence produced by the release of calcein upon amplification in positive samples, can immediately be read by eye or by using a simple UV light source, respectively [40]. However, the use of a UV-lamp for reading results is comparably less objective than a turbidimeter [36][41].

The LA-500 real-time turbidimeter (Eiken Chemical Co.) or the HumaLoop M (Human diagnostics Worldwide, Wiesbaden, Germany) platform for sample preparation, amplification, and easy visual result reading under a built in UV light can hold 16 tests per run. If a simple heating block system is used then batches of up to 46 samples can be run together with a positive and negative control (the maximum number of tubes that fit on a regular heat-block), making it a useful tool for prevalence surveys in endemic areas [38][42]. Furthermore, a high throughput DNA extraction platform that can extract up to 94 samples at a time has been assessed, albeit with varying performance [41][43]. The Loopamp kits cost about 5.20 euros per test, excluding the costs for DNA extraction and equipment.

3. Performance and Application of Commercial LAMP Kits in Malaria Endemic Settings

The performance of LAMP has been evaluated in malaria endemic field settings in over 30 publications, of which 15 studies employed the Loopamp™ MALARIA Detection kits and two studies the Illumigene® (Alethia) malaria LAMP. Overall, LAMP has shown ≥95% pooled sensitivity and specificity for detecting both *P. falciparum* and *P. vivax* infections when used in endemic settings [25]. The consensus among studies that have employed one of the commercial kits in malaria endemic settings is that the methods are easy to perform after only 3–5 days laboratory training [31][38][39][42][44][45][46][47][48][49], even in the remotest of field settings [47].

3.1. Performance of LAMP in Asymptomatic and Low-Density Infections

As malaria transmission declines in areas of successful malaria control, the relative proportion of asymptomatic and low-density malaria infections increases [50]. These infections are often subpatent, i.e., falling below the detection limit of conventional malaria diagnostic tools such as microscopy or RDTs. Hence, as malaria transmission decreases, new and more sensitive field applicable screening tools are needed for the detection and management of very low-density infections, especially if malaria elimination is to be considered possible [25][51].

The improved analytical sensitivity of LAMP, resulting in the detection of significantly more infections compared to RDT or microscopy [42][46][47][48][52][53], and the reduced time-to-result compared to PCR [31][38][39][41][42][47][48], has put LAMP forward as a promising tool for near point-of-care detection of low parasite density infections in asymptomatic carriers, especially in low endemic and pre-elimination areas [3][9][24][25]. However, despite its improved analytical sensitivity, LAMP may still miss a substantial proportion of the asymptomatic reservoir of very low-density infections [41][46][52], especially in low transmission settings (Table 3). Although LAMP may provide a better understanding of the prevalence and distribution of low-density asymptomatic infections than conventional diagnostic tools, a rapid turn-around time may not necessarily be the highest priority in malaria epidemiological surveys where highly sensitive PCR-based methods are still often the method of choice [54]. On the other hand, results should be made available within 48 hours of testing in detect-and-treat approaches such as reactive case detection (i.e., screening and treatment of household members and neighbors of passively identified index cases), and mass or focal testing and treatment strategies. Further studies are however needed to assess if employing LAMP will significantly improve the impact such resource-intensive elimination strategies have on malaria transmission [52][55].

Table 3. Performance of malaria loop-mediated isothermal amplification for detecting asymptomatic low-density infections.

Setting	Sample Size [1]	Sample Type [2]	LAMP Method	Comparator	Prevalence (%)	Mean Parasite Densities (p/µL)	Sensitivity (%; 95% CI)	Specificity (%; 95% CI)	Reference
Zanzibar, Pre-elimination	996	Fresh blood + Boil and Spin	Loopamp Pan/Pf	RDT; Ref: qPCR	RDT: 1.0 LAMP/PCR: 1.8	26 (range: 0–4626).	Pan LAMP: 83.3; 59–96 RDT: 55.6; 31–79	Pan-LAMP: 99.7; 99–100 RDT: 100; 99.6–100	[38]
Zanzibar, Pre-elimination	3983	Fresh blood + Boil and Spin	Loopamp Pan/Pf	RDT; Ref: LAMP	RDT: 0.5; LAMP 1.6	ND; 71% of LAMP positives <LOD of RDT	RDT: 24.6; 15–37	RDT: 99.9; 99.7–100	[42]
Zanzibar, Pre-elimination	3008	Filter device +HTP extraction	Loopamp Pan/Pf	RDT; Ref: qPCR	RDT: 0.4; qPCR: 1.6; HTP-LAMP 0.7	1.8 (range: 0.1–770)	HTP-LAMP: 40.8; 27–56 Chelex-LAMP: 49; 34–64 HTP_LAMP >2: 54; 25–81 HTP_LAMP ≤2: 36; 21–54	HTP-LAMP: 99.9; 99.8–100	[41]
Eswatini (formally Swaziland), Very low transmission	10890	DBS + Chelex extraction	Loopamp Pan/Pf	RDT Ref; LAMP LAMP Ref: nPCR	RDT: 0.6; LAMP: 1.7	ND; 67% of LAMP positives <LOD of RDT	LAMP: 72.2; 63−80 RDT: 33.4; 33–35	LAMP: 98.0; 97−98 RDT: >90.0	[52]
Namibia, Low transmission	2642	DBS and used RDTs + Chelex extraction	Loopamp Pan	RDT Ref: nPCR	RDT: 0.9; LAMP 1.8	ND; 51% of LAMP positives <LOD of RDT	LAMP on RDT: 95.4; 84–99 LAMP on DBS: 95.5; 85–99 RDT: 9.3; 2.6–22	All > 99	[48]

Setting	Sample Size [1]	Sample Type [2]	LAMP Method	Comparator	Prevalence (%)	Mean Parasite Densities (p/µL)	Sensitivity (%; 95% CI)	Specificity (%; 95% CI)	Reference
Colombia, Varied transmission	980	Fresh blood + Boil and Spin	Loopamp Pan/Pf	LM Ref: qPCR	LM: 0.2; LAMP: 6.6; qPCR: 7.2	ND, (range: 1–897)	Pv: 90.9; 80–97 Pf: 100; 78–100	All > 99	[53]
Peruvian Amazon, Low to moderate transmission	1167	Fresh blood + Boil and Spin	Loopamp Pan/Pf	LM Ref: qPCR	LM: 4.9; LAMP: 21.9	10 (CI95% 7.5–13)	LAMP: 91.8; 88–95 LM: 20.3; 16–26	91.9; 88–95 LM: 98.0; 95–99	[47]
Uganda, High transmission	554	DBS + Chelex extraction	Loopamp Pan	LM Ref: qPCR	LM: 18.2; LAMP: 37.2; qPCR: 48.9	LAMP neg: 0.1 (CI95% 0.07–0.2) LAMP pos: 5.7 (CI95% 3.0–10.8)	LAMP: All LM negs: 44.7 ≥0.01–<0.1 p/µL: 10.8 ≥0.1–<1 p/µL: 40.9≥1 p/µL: 81.5	All LM negs: 94.0	[46]

[1] All asymptomatic; [2] All samples from finger prick; p/µL = parasite per microliter; 95% CI = 95% confidence interval; Ref = reference method; Pf = P. falciparum; Pan = Pan-Plasmodium; RDT = rapid diagnostic test; LM = light microscopy; qPCR = quantitative PCR; nPCR = nested PCR; LAMP = loop-mediated isothermal amplification; DBS = dried blood spot; HTP = high through put; <LOD = below the limit of detection; ND = not determined.

3.2. Application of LAMP in Prevalence Surveys in Malaria Endemic Settings

Eight studies—all of which were conducted in sub-Saharan Africa—have applied commercial LAMP kits as a screening tool in malaria prevalence surveys. For instance, LAMP has been used in several studies in Uganda to investigate the effect of HIV infection on malaria incidence [56]; the effect of indoor residual spraying on the prevalence of asymptomatic infections [57]; for assessing the prevalence of microscopic and submicroscopic malaria infections in different transmission sites [58]; and the prevalence of submicroscopic infections among schoolchildren [59]. In these studies, dry blood spots on filter paper were collected at point-of-care. DNA extraction was conducted by a Chelex-based method, followed by the Loopamp Malaria Pan Detection Kit. Depending on age, prevalence of asymptomatic infections, and malaria transmission intensity, the LAMP positivity rate was two to ten times greater than the positivity rate by microscopy or RDT [56][57][58][59].

LAMP has also been applied for assessing risk factors and spatial clustering of asymptomatic malaria though active and reactive surveillance in low transmission settings [60][61][62]. In these studies, dry blood spots were collected for later Chelex extraction followed by screening with the Loopamp Pan Detection Kit. LAMP detected significantly more infections than RDTs, with clustering of asymptomatic low-density infections around index cases [60][62]. In the study by Smith et al. RDT identified only 17% of the index and neighbor cases detected by LAMP, suggesting that infections missed by RDT during reactive case detection may be responsible for 50–71% of transmission from humans to mosquitoes [62].

A single study performed in Congo has applied the Illumigene Malaria kit. Venous blood (4 mL) with EDTA was taken from 1088 children aged between 6 and 59 months for assessing the prevalence of anemia and its relationship with asymptomatic submicroscopic *Plasmodium* infection. Malaria prevalence was 16% and 34% by microscopy and LAMP, respectively and submicroscopic *Plasmodium* infection was found in 22% of the children [63].

4. Pros and Cons of Commercially Available LAMP Kits in Low Resource Settings

It is unlikely that LAMP will replace conventional diagnostic tools such as RDTs and microscopy in the point-of-care diagnosis of clinically symptomatic malaria in endemic settings [1][9][33][64]. LAMP in its current formats does not completely fulfil the World Health Organization (WHO) ASSURED criteria (i.e., being Affordable, Sensitive, Specific, User-friendly, Robust and rapid, Equipment free, and Deliverable) for identifying appropriate diagnostic tests for resource-constraint settings, and some improvements are likely to be necessary for this tool to become readily available [1][9]. Whilst the commercially available diagnostic kits certainly offer user-friendliness, with high specificity and robust, rapid, and deliverable assays that are independent of a cold chain, they are not equipment free and require a reliable source of electricity. Despite the ease of use, LAMP still requires good enough facilities for preparing basic molecular assays, ideally

with separate workstations for avoiding DNA-cross-contamination [64]. In addition, the technicians running the assays require sufficient knowledge to manage DNA contamination, an issue with all nucleic acid amplification-based methods that needs to be addressed [28][36][42][45][65]. Efforts to simplify DNA isolation methods [19], or reduce the requirement of electricity dependent equipment [45], have succeeded only on behalf of the sensitivity or specificity of the assay. In addition, the high cost of these commercially available assays has been put forward as a limiting factor in their uptake [33][44].

4.1. Lack of Specific Species Identification Is a Limitation with Currently Available Kits

Malaria species identification is important in areas where co-endemicity of non-falciparum species occurs, e.g., in areas where *P. vivax* infections—requiring additional liver-stage treatment—are prevalent [1][37]. Although the Loopamp Pf and Pv detection kits provide specific identification of *P. falciparum* and *P. vivax*, it is at an additional cost of 5.20 euros per additional test and it still does not provide a very detailed and/or accurate description of the *Plasmodium* species composition [38]. This has also shown to be of importance in areas where *P. knowlesi*—which has the ability to cause severe disease even at low parasite densities—is present. Molecular testing for quality assurance of microscopy-confirmed cases in Indonesia recently found that microscopy was unable to identify or miss-classified up to 56% of confirmed malaria cases, half of which were later determined to be *P. knowlesi* infections [66]. Although the Loopamp Pan Detection kit successfully detected these infections in dried blood spots (unlike a more standard 18S rRNA nested PCR reference targeting the four human-only species), further species identification was limited by the unavailability of species-specific testing with the platform used. This highlights the difficulties of malaria species identification at the point-of-care and reference laboratory levels in settings where co-endemicity of non-falciparum species occurs [66].

4.2. The Loopamp Malaria Detection Kits Provide Several Advantages in Low Resource Settings

The lower cost of the Loopamp Malaria Detection Kits, together with the endorsement from FIND, is likely the main reason as to why primarily these kits have been used in malaria endemic settings [67]. The simple and cheap boil and spin extraction method that can be used together with the Loopamp kits reduces the costs of extraction and can be performed near point-of-care reducing the time to result. A downside with the boil and spin method is that the extracted DNA is most likely not suitable for conventional PCR due to the presence of *Taq* polymerase inhibitors such as hemoglobin [4], and is not recommended for freezing due to the instability of the DNA [36][42][68]. However, the Loopamp assay has successfully been conducted on finger prick blood samples collected in extraction buffer and stored at −80 °C for up to one year before the boil and spin extraction was performed [49].

An additional advantage with the Loopamp kits is the possibility of using dried blood spots on filter paper, as supposed to venous blood samples or whole blood from finger pricks [27][28]. This allows easy transport and storage of samples at room temperature, allowing the assays to be conducted at a central laboratory rather than at point-of-care. Furthermore, if biological material is stored on filter papers, then LAMP positive samples can easily be transferred to more sophisticated laboratory facilities for more detailed analysis by PCR, e.g., for *Plasmodium* species identification and parasite density quantification.

4.3. Improving Throughput Will Aid Large Prevalence Surveys

Improved throughput for screening of larger numbers of samples simultaneously would benefit routine prevalence surveys in areas aiming at malaria elimination [38][42]. High-throughput performance primarily depends on the DNA extraction capacity, which is considered the main bottleneck with large number of samples [47]. The centrifugation-free methods available for both commercial kits have shown good clinical sensitivity, but at a greater cost per sample and they are not compatible with testing of large number of samples [47]. The high throughput extraction platform developed for use with the Loopamp kits requires highly specific equipment [43], without significantly improving the time-to-results compared to boil and spin extraction of the same number of samples [41]. Simplifying the sample processing protocol, e.g., by reducing the number of transfer steps, may increase throughput. This would also reduce the amount of plastic consumables needed, which is not only a benefit from an environmental point-of-view [19], but also given that sterile filter tips suitable for molecular assays are a commodity that are not readily available in low-resource-settings [64].

Finally, the use of the naked eye or a UV-lamp for the read-out of LAMP results is comparably less objective than the use of specialized equipment such as the Illumipro-10™ incubator or the LA-500 real-time turbidimeter [26][36][41][65]. However, these instruments have limited capacity of 10 or 16 samples per run. Providing a more objective format for result readout on a larger scale could be a useful, especially for use in low prevalence areas where the occurrence of positive samples is rare and might be missed.

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