LAMP based assay can rectify the diagnosis of *Yersinia pseudotuberculosis* infections otherwise missed by serology

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List of abbreviations: LAMP, loop-mediated isothermal amplification; Y.pstb, *Yersinia pseudotuberculosis*; KD, Kawasaki disease; CT-SMAC, MacConkey agar with sorbitol, cefixime, and tellurite; TCBS, thiosulfate citrate bile salts sucrose; BTB, bromothymol blue lactose; SS, Salmonella-Shigella; IVIG, intravenous immunoglobulin therapy.

Abstract (241 words)

Background Despite being a well-known but seldom encountered zoonotic pathogen, diagnosis of *Yersinia pseudotuberculosis* is not necessarily easy. Infected patients occasionally present with various symptoms resembling Kawasaki disease; thus discriminating the two in the acute phase is challenging. In addition to bacterial culture and serology, novel detection methods based on loop-mediated isothermal amplification (LAMP) are reported in the literature. However, the clinical utility of LAMP based methods in comparison with the other methods is scarcely documented in the literature.

Aim To clarify the clinical utility of LAMP based method in the diagnosis of *Yersinia pseudotuberculosis* infection.

Methodology Inpatients admitted due to suspected *Yersinia pseudotuberculosis* infection during April 2008 thru March 2015 were enrolled. Results of LAMP based method as well as culture and serology were collected and compared.

Results/Key findings Among 16 eligible cases, serology proved positive in 13 (81.3%) cases, LAMP in 8 (50%) cases, and bacterial culture in 4 (25%) cases. No significant difference among the three methods could be proved statistically. Though serology was the most sensitive method, it is known to miss cases such as young patients, whereas LAMP could complement all 3 cases missed by serology. Furthermore, LAMP can return the test result within a few hours from specimen receipt, whereas serology and bacterial culture requires days to weeks of time.

Conclusion Although second to serology in sensitivity, the LAMP based method proved its utility in making rapid diagnosis, and serving a complementary role to serology.

Introduction

Yersinia pseudotuberculosis (Y.pstb) is a causative agent of acute enteritis with various complications. Approximately 8% of Y.pstb cases are known to present with manifestations resembling Kawasaki disease (KD) [1]. The clinical symptoms that Y.pstb infection share with KD are: continuous fever, conjunctivitis, strawberry tongue, cracked lips, cervical lymphadenopathy, rashes. Therefore, early stage discrimination between Y.pstb infection and KD is challenging. Indeed, no diagnostic method to distinguish between the two at the early phase is established to date.

Despite being a well-known zoonotic pathogen, rapid identification of Y.pstb is not easy. Isolation by culture requires several weeks. Furthermore, the detection rate by direct stool culture is reported to be low (approximately one third of confirmed cases), requiring cold enrichment to increase chance of detection [2, 3]. Serologic tests are cumbersome, not widely available, and also require several days. Furthermore, serologic tests are not standardized, hindering comparison among studies.

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method, which is widely used for detection of pathogenic microorganisms, i.e. central nervous system infections, respiratory infections, gastrointestinal tract infections including food poisoning [4-8]. A LAMP assay to detect Y.pstb DNA with high sensitivity and specificity is also reported [9].

Although some literature reporting the clinical utility of LAMP assay in diagnosing Y.pstb infection can be found [10], no direct comparison of the detection rates of Y.pstb among LAMP assay, serological testing, and culture has been reported yet.

The objective of this study is to compare the detection rates of Y.pstb infection among LAMP assay, serological testing, and culture test.

Materials and Methods

Study population

All patients suspected of Y.pstb infection, admitted to the Department of Pediatrics, Kawasaki Medical School Hospital during April 2008 thru March 2015 were enrolled. Patients presenting with both continuous fever and gastrointestinal symptoms, accompanied by at least one of the following five features — conjunctivitis, changes in the oral cavity and/or

lips, cervical lymphadenopathy, rash, changes in the extremities — were suspected of Y.pstb infection. Patients that underwent testing by all three testing methods described hereafter were considered eligible and included in the study. Written informed consent was obtained from the patients' guardian prior to specimen collection.

Specimen collection

Stool specimens were collected from enrolled patients on admission; each specimen was divided into two; one immediately subjected to bacterial culture, the other stored at −80°C until DNA extraction followed by LAMP assay was carried out.

Serum specimens were collected in the acute and convalescent stage and stored at −80°C until serological assay was carried out.

Diagnostic Methods

A case was diagnosed as Y.pstb infection when any of the following three methods proved positive; LAMP assay, serological testing, or culture.

LAMP assay

DNA was extracted from thawed frozen stool using QIAamp DNA Stool Mini kit (QIAGEN GmbH, Hilden Germany) according to the manufacturer supplied instructions; instead of weighing 180–220mg of stool, starting material was dispensed using the built-in spoon of 80.734 faeces container (Sarstedt K.K., Tokyo, Japan), a spoonful of which is approximately 200mg: In brief, a spoonful of stool per specimen was dispensed and subjected to DNA extraction using the kit, finally eluted with 200μL of Buffer AE (10 mM Tris-HCl, 0.5 mM EDTA (pH 9.0)). Primer sequences, primer concentrations, and reaction conditions were adopted from the literature [9]. Oligonucleotide primers were ordered from Sigma-Aldrich Japan (Tokyo, Japan), used with Loopamp DNA Amplification Kit (Eiken Chemical Co., Ltd., Tokyo, Japan). In brief, 5μL of the DNA eluate was subjected to a 25μL LAMP reaction. The reaction was carried out at 63°C for 60 minutes on Loopamp Realtime Turbidimeter LA-500 (Eiken); positive reactions were automatically determined by the build-in feature of the turbidimeter.

Stool culture

Cold enrichment was carried out along with standard bacterial isolation.

For standard bacterial isolation, the stool specimen was plated onto the following five agar plates; MacConkey agar with sorbitol, cefixime, and tellurite (CT-SMAC) (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan), thiosulfate citrate bile salts sucrose (TCBS) agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), Skirrow's Medium (Eiken), bromothymol blue lactose (BTB) agar (Kyokuto), and Salmonella-Shigella (SS) agar (Kyokuto).

For cold enrichment, 0.5mL of BHI bouillon (Nissui) was added to 9mL of pre-cooled normal saline, into which an approximately 5mm sized piece of stool was suspended. The suspension was incubated at 4°C for 3 weeks, during which 10μL of the suspension was inoculated onto BTB agar and SS agar every 7 days.

Agar plates were incubated under the following conditions: CT-SMAC and TCBS, 37°C for 2days; Skirrow's Medium, 42°C for 2days; BTB and SS, 25°C for 5days.

Species identification of grown colonies was carried out using API 20E identification kit (SYSMEX bioMérieux Co., Ltd., Tokyo, Japan).

Serological testing

Serological testing was carried out by direct agglutination. Patient serum was diluted 1:10 by normal saline following heat inactivation at 56°C for 30 min, from which a 2-fold serial dilution series of 1:10 to 1:320 was prepared.

Whole bacterial cells were used as test antigens. Test antigen suspensions were prepared from a panel of Y.pstb strains of the following subserogroups, maintained and stocked at Okayama Prefectural Institute for Environmental Science and Public Health; 1a, 1b, 2a, 2b, 3, 4a, 4b, 5a, 5b, and 6. Each strain was plated on BD BBL Trypticase soy agar (Nippon Becton Dickinson Company, Ltd., Tokyo, Japan), incubated 2 days at 25°C, before working suspensions were prepared. The two-night cultures were washed twice with normal saline, after which a working suspension of each strain, adjusted to a turbidity of McFarland standard No. 0.6, was prepared. 0.2mL of each suspension was mixed

with equal volumes of serial dilution series of patient serum, incubated at 50°C overnight. The titer of a specimen was determined as the maximum dilution in which agglutination was observed.

A case was determined serologically positive if the titer exceeded 1:160 in any serum specimen [11].

Statistical analysis

Statistical analyses were performed using R software (version 3.2.0; R Core Team [http://www.R-project.org/]).

Results

During the study period, 32 patients were admitted for Y.pstb infection. Among these patients, 16 underwent all three testing methods and were considered eligible for direct comparison and enrolled in the study. Characteristics of the 16 patients are summarized in Table 1; their median age was 64 months (interquartile range, 26.75–106.25), 12 (75%) were male, 8 met the diagnostic criteria of KD among whom 6 received intravenous immunoglobulin therapy (IVIG) whereas 2 were spared from IVIG due to defervescence within 5 days, 13 had been administered antibiotics prior to admission.

Results of LAMP assay, serological testing, and stool culture are also shown in Table 1. Among the 16 cases, 8 (50%) by LAMP assay, 13 (81%) by serological testing, and 4 (25%) by stool culture proved positive for Y.pstb. All 3 cases missed by serological testing were successfully detected by LAMP assay. One out of the 3 cases proved positive by culture as well. LAMP assay detected all 4 cases detected by fecal culture. All 4 culture positive cases proved positive with the aid of cold enrichment; none proved positive for Y.pstb by mere direct plating. LAMP demonstrated 100% sensitivity and 67% specificity for culture positive cases, whereas serology 75% sensitivity and 17% specificity. (Table 2B, 2C)

The detection rates tended to be higher in the following order, serological testing, LAMP assay, and stool culture. However, no significant difference was noted between any pair among the three methods by Fisher's exact test (serology vs. LAMP, p=0.2; LAMP vs. culture, p=0.077; serology vs. culture, p=1). (Table 2A-C)

Discussions

LAMP assay could detect half of the cases in the early stage of Y.pstb infection. Furthermore, LAMP assay bears the potency of returning results extremely rapidly; typically within a few hours from specimen reception. The detection rate of LAMP assay proved two times higher than stool culture, whereas two times lower than serological testing, though no significant differences between either LAMP assay and serological testing, or LAMP assay and stool culture was found. No cases positive by stool culture was missed by LAMP assay. In this study, LAMP proved 100% sensitivity and 67% specificity for culture positive cases, whereas serology 75% sensitivity and 17% specificity; however, due to the small number of cases, interpretation of these results deserve deliberation. Notable is the fact that LAMP assay successfully detected all 3 cases that were missed by serological analysis. Previous studies point out that serological testing can miss cases, especially young cases [3, 12, 13]; one of the missed cases in our study is an 18-month old patient. LAMP assay might serve a complementary role to serological testing in the diagnosis of such patients.

There were 8 cases of Y.pstb infection which were clinically diagnosed as KD, among which 6 cases received IVIG therapy. Among the 8 cases, 4 cases proved Y.pstb positive by LAMP assay. Early initiation of IVIG therapy, typically within 5 days of onset, is recommended for KD in order to reduce the risk of developing lifelong coronary sequelae — coronary artery aneurysms. Contrariwise, Y.pstb infections are considered benign and self-limiting, deserving no specific treatments including antibiotics, except for severe cases [1, 14]. Although whether Y.pstb infection fulfilling the diagnosis criteria of KD deserve IVIG therapy or not is yet to be determined [1, 14, 15], rapid diagnosis by LAMP assay might provide a feasible method for further investigation and/or treatment of such cases.

Furthermore, LAMP assay is advantageous to PCR based methods in the following aspects. Its utility lies in the feasibility – isothermal amplification that abolishes the use of thermal cyclers, and a far more efficient amplification surpassing that of PCR, resulting in shortened amplification time of typically 15-60 minutes, whereas typical real-time PCR requires somewhat longer reaction time exceeding 1 hour and a costly real-time PCR apparatus[4, 16-18]. The efficient amplification of LAMP also results in precipitation of magnesium pyrophosphate, which can be observed as

turbidity in reaction tubes that can be discriminated by the naked eye [19]; in contrast, amplification of DNA by conventional PCR by gel electrophoresis requires approximately another hour or more.

The authors are aware of the following limitations. First, no consensus whether stool culture is a suitable surrogate marker for systemic Y.pstb infection is established yet. The fact that serology was superior to stool culture might serve as another proof for this limitation. A case report in the literature [20] describing a case of Y.pstb infected patient from whom the bacteria was detected only from biopsied lymph node is also suggestive. Since Y.pstb grows slowly, it is likely to be obscured by outgrowth of other flora in the stool specimen. The optimal phase for stool specimen collection for successful culture of the bacteria is also not clear. Early administration of antibiotics might have further reduced the chance of collecting suitable specimen for successful culture testing. Although the detection rate of Y.pstb by culture was in concordance with the past literature [3], this might have been an underestimation, since most, if not all, subjects were administered antibiotics prior to admission. Due to continuing fever and the difficulty of establishing a definitive diagnosis at the early stage of the disease, patients with Y.pstb infection are prescribed antibiotics more often than not. Although direct plating could not prove Y.pstb in any cases, cold enrichment proved effective in culturing Y.pstb in 4 cases, among which 3 cases had been administered antibiotics prior to specimen collection. (Table 1)

Second, serological analyses were performed against 10 subserogroups covering most of the cases in Japan. Since 21 subserogroups, 18 among which with known pathogenicity against humans, are known to date [21], we might have missed cases caused by the rare untested subserogroups. Furthermore, the prevailing endemic subserogroups of the bacteria differ geographically. Thus, comparison of our results with others would require attention to the test antigens employed, and the region from which the study population were recruited as well.

The authors are also aware of the lack of healthy controls; however due to the following reasons, this is not a critical flaw in design. Healthy individuals are reported to present with antibody titers less than 1:160 [22, 23], and the titers are also known to elevate spontaneously, diminishing within 4 months [24]. Furthermore, only symptomatic subjects are known to yield positive culture [25].

Currently, Y.pstb infected patients fulfilling the diagnosis criteria of KD are recommended to undergo IVIG treatment. Further studies to investigate whether such cases deserve IVIG administration are warranted. LAMP assay in early stage diagnosis of Y.pstb infection might play an indispensable role in these studies.

In conclusion, our results suggest that LAMP assay bears the potency of rapid diagnosis in the early stage of Y.pstb infection, returning the assay result to the clinical scene within a few hours from stool specimen receipt, which neither serological testing nor bacterial culture can achieve. Its clinical utility as a complementary measure to serological testing was also suggested.

Ethics

This study was approved by the Institutional Review Board of Kawasaki Medical School. (No. 1189)

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Conflicts of interest

IM received manuscript fee from Eiken Chemical Co., Ltd. Other authors have no conflicts of interest relevant to the content of this article.

References

1. **Zakhour R, Heresi GP, Murphy JR.** *Yersinia pseudotuberculosis*. In: Kliegman RM, Stanton BF, St Geme JW, 3rd, Schor NF, Behrman RE (editors). *Nelson Textbook of Pediatrics*, 20th ed. Philadelphia, PA: Elsevier; 2015. p. 1408.
2. **Sato K, Ouchi K, Taki M.** *Yersinia pseudotuberculosis* infection in children, resembling Izumi fever and Kawasaki syndrome. *Pediatric infectious disease* 1983;2:123–126.
3. **Ouchi K, Sato K, Takahashi R, Taki M, Tateishi K.** [Significance of ruling out *Yersinia pseudotuberculosis* infection from Kawasaki Disease]. *J Jpn Pediatr Soc* 1985;89:449–454. [In Japanese]
4. **Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K *et al.*** Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* 2000;28:E63.
5. **Kimura H, Ihira M, Enomoto Y, Kawada J, Ito Y *et al.*** Rapid detection of herpes simplex virus DNA in cerebrospinal fluid: comparison between loop-mediated isothermal amplification and real-time PCR. *Med Microbiol Immunol* 2005;194:181–185.
6. **Hong TC, Mai QL, Cuong DV, Parida M, Minekawa H *et al.*** Development and Evaluation of a Novel Loop-Mediated Isothermal Amplification Method for Rapid Detection of Severe Acute Respiratory Syndrome Coronavirus. *J Clin Microbiol* 2004;42:1956–1961.
7. **Song T, Toma C, Nakasone N, Iwanaga M.** Sensitive and rapid detection of *Shigella* and enteroinvasive *Escherichia coli* by a loop-mediated isothermal amplification method. *FEMS Microbiol Lett* 2005;243:259–263.
8. **Yamazaki W, Taguchi M, Kawai T, Kawatsu K, Sakata J *et al.*** Comparison of loop-mediated isothermal amplification assay and conventional culture methods for detection of *Campylobacter jejuni* and *Campylobacter coli* in naturally contaminated chicken meat samples. *Appl Environ Microbiol* 2009;75:1597–1603.
9. **Horisaka T, Fujita K, Iwata T, Nakadai A, Okatani AT *et al.*** Sensitive and specific detection of *Yersinia pseudotuberculosis* by loop-mediated isothermal amplification. *J Clin Microbiol* 2004;42:5349–5352.
10. **Sakai N, Ohtsuka Y, Oka M, Ichimaru T, Tashiro K *et al.*** A case of *Yersinia pseudotuberculosis* infection with renal failure: the LAMP

method was helpful to make the diagnosis. *Japanese journal of pediatric nephrology* 2010;23:102–106.

11. **Tsubokura M.** Yersinioses Other than Plague. In: Balows A, Hausler WJJ, Ohashi M, Turano A (editors). *Laboratory Diagnosis of Infectious Diseases: Principles and Practice*. New York, NY: Springer-Verlag; 1988. pp. 540–549.
12. **Marks MI, Pai CH, Lafleur L, Lackman L, Hammerberg O.** *Yersinia enterocolitica* gastroenteritis: a prospective study of clinical, bacteriologic, and epidemiologic features. *J Pediatr* 1980;96:26–31.
13. **Sato K, Ouchi K, Komazawa M.** Ampicillin vs. placebo for *Yersinia pseudotuberculosis* infection in children. *Pediatr Infect Dis J* 1988;7:686–689.
14. **Gould LH.** Yersiniosis. In: Centers for Disease Control and Prevention, editor. CDC Health Information for International Travel 2016. New York: Oxford University Press; 2015. pp. 362–363.
15. **Horinouchi T, Nozu K, Hamahira K, Inaguma Y, Abe J *et al.*** *Yersinia pseudotuberculosis* infection in Kawasaki disease and its clinical characteristics. *BMC Pediatr* 2015;15:177.
16. **Njiru ZK.** Loop-mediated isothermal amplification technology: towards point of care diagnostics. *PLoS Negl Trop Dis* 2012;6:e1572.
17. **Mori Y, Kanda H, Notomi T.** Loop-mediated isothermal amplification (LAMP): recent progress in research and development. *J Infect Chemother* 2013;19:404–411
18. **Li Y, Fan P, Zhou S, Zhang L.** Loop-mediated isothermal amplification (LAMP): A novel rapid detection platform for pathogens. *Microb Pathog* 2017;107:54–61.
19. **Mori Y, Nagamine K, Tomita N, Notomi T.** Detection of Loop-Mediated Isothermal Amplification Reaction by Turbidity Derived from Magnesium Pyrophosphate Formation. *Biochem Biophys Res Commun* 2001;289:150–154.
20. **Fukushima H, Sato T, Nagasako R, Takeda I.** Acute mesenteric lymphadenitis due to *Yersinia pseudotuberculosis* lacking a virulence plasmid. *J Clin Microbiol* 1991;29:1271–1275.
21. **Fukushima H.** [Epidemiology of Pathogenic *Yersinia* and Their Detection Method]. *Japanese Journal of Food Microbiology* 2011;28:104–113. [In Japanese]

22. **Leino R, Kalliomäki JL.** Yersiniosis as an internal disease. *Ann Intern Med* 1974;81:458-461.
23. **Paff JR, Triplett DA, Saari TN.** Clinical and laboratory aspects of *Yersinia pseudotuberculosis* infections, with a report of two cases. *Am J Clin Pathol* 1976;66:101–110.
24. **Mair, NS.** Yersiniosis (Infections due to *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*). In: Hubbert WT, McCulloch WF, Schnurrenberger PR (editors). *Diseases transmitted from animals to man*, 6th ed. Springfield, IL: Charles C Thomas. Publisher; 1975. pp.174–185.
25. **Maruyama T.** Yersiniosis as zoonoses. *J. Jpn. Vet. Med. Assoc* 1982;35:2–8. [In Japanese]

Table 1. Profiles and assay results of all eligible cases.

Case No.	Age (mo)	Gender	LAMP	Serology	Culture*	Prior Antibiotics	KD
1	27	M	+	+	+	+	+
2	15	M	+	+	+	+	+
3	107	F	+	+	+	+	
4	44	M	+	+	−	+	
5	23	M	+	+	−	+	
6	18	M	+	−	+	−	+
7	76	F	+	−	−	+	+
8	106	M	+	−	−	+	+†
9	26	F	−	+	−	+	+
10	34	M	−	+	−	+	+
11	120	M	−	+	−	+	+†
12	96	F	−	+	−	−	
13	52	M	−	+	−	+	
14	147	M	−	+	−	+	
15	94	M	−	+	−	n/d	
16	116	M	−	+	−	+	

* All culture positive specimens proved positive with the aid of cold enrichment.

† Spared from intravenous immunoglobulin therapy due to defervescence within 5 days of fever onset.

LAMP, loop-mediated isothermal amplification; KD, Kawasaki disease; n/d, no data

Table 2. Comparison among the 3 methods.

A)

		LAMP +	LAMP −
Serology	+	5	8
	−	3	0

p=0.2

95%CI 0–2.21

B)

		LAMP +	LAMP −
Culture	+	4	0
	−	4	8

p=0.077

95%CI 0.81–∞

C)

		Serology +	Serology −
Culture	+	3	1
	−	10	2

p=1

95%CI 0.02–47.07

+, positive; −, negative