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Leptospiral 3-hydroxyacyl-CoA dehydrogenase as an early urinary biomarker of leptospirosis

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Abstract

Leptospirosis, caused by spirochetes of the genus *Leptospira*, is a globally widespread, neglected and emerging zoonotic disease. The currently used diagnostic tests are time-consuming, require technical expertise or require the use of sophisticated equipment. Clinicians have pointed out the urgent need to develop a rapid test for the diagnosis of acute leptospirosis with a non-invasive and easy sampling method. In this study, we have focused on a leptospiral enzyme, 3-hydroxyacyl-CoA dehydrogenase (3-HADH), as a urinary biomarker of acute leptospirosis. A specific antiserum for pathogenic *Leptospira* spp. was produced, targeting a peptide corresponding to amino acids 410 to 424 of 3-HADH. The antiserum was used to investigate whether 3-HADH is excreted in the urine by Western blotting. Among 70 suspected leptospirosis patients, 40 were laboratory confirmed by microscopic agglutination test (MAT) using paired sera samples and/or polymerase chain reaction (PCR). In the acute phase of the laboratory-confirmed leptospirosis cases, sensitivity for 3-HADH, blood PCR and

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urine PCR were 52.5%, 57.5% and 12%, respectively. 3-HADH was detected from 2 days post-onset of illness (p.o) and could be detected at least until 9 days p.o. The combination of PCR and 3-HADH detection increased sensitivity of diagnosis to 100% in samples collected between 1 and 3 days p.o., and to 82% in samples collected between 4 and 9 days p.o. Our results suggested that the detection of 3-HADH can support a clinical diagnosis of leptospirosis, especially when serological methods are negative during the acute phase.

Keywords: Infectious disease, Microbiology

1. Introduction

Leptospirosis is a zoonotic disease of major public health concern due to its epidemic potential. It is distributed worldwide, and its occurrence is higher in tropical and subtropical regions. Pathogenic *Leptospira* spp. colonize the proximal renal tubules of reservoir hosts and are excreted into the environment through urine. Humans and animals become infected through the contact with soil and water contaminated with the urine of infected animals [1]. Flooding and heavy rainfall have been associated with numerous outbreaks of leptospirosis around the world, and leptospirosis is considered to be an important disaster-related infectious disease [2]. On the other hand, in Okinawa (a subtropical island of Japan), outbreaks are commonly reported after water-related recreational activities [3]. The rapid detection of leptospirosis is a critical step toward effectively managing the disease and controlling outbreaks [4]. However, the clinical recognition of leptospirosis is complicated because the infection causes a wide range of symptoms that are similar to other febrile infectious diseases. Additionally, even though leptospirosis is suspected due to the clinical symptoms and patient history of water-related activities, a rapid diagnostic test (RDT) that could be used directly at the bedside is not available. The currently used diagnostic tests, such as serological tests, isolation of *Leptospira* by culture, or detection of leptospiral gene by polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP), are time-consuming, require technical expertise or require the use of sophisticated equipment [5, 6]. There is, therefore, a need to develop an RDT for leptospirosis that is affordable and easy to use [7]. Immunochromatographic (IC) RDTs have demonstrated significant potential for use as point-of-care diagnostic tests in many infectious diseases [8], and several IC methods have been developed to detect either anti-*Leptospira* antibodies or leptospiral antigens [9, 10]. However, serological diagnostic ICs have low sensitivity in the early phase of infection [9]. Because urine is a more accessible sample for rapid diagnosis, leptospiral lipopolysaccharides or outer membrane protein LigA in urine samples have been evaluated with promising results [10, 11], but they still need further improvement.

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Chronically infected rats with *Leptospira* excreted pathogen-derived proteins including Loa22, a virulence factor, as well as the GroEL [12, 13]. Because the detection of leptospiral antigens excreted in the acute phase of illness may be useful in the developing of an RDT for leptospirosis, Segawa et al. analyzed the proteins that are excreted in the urine of infected hamsters and identified an enzyme, 3-hydroxyacyl-CoA dehydrogenase (3-HADH), which is released in the urine of experimentally infected hamsters before the onset of illness [14]. They suggested that 3-HADH, which is a lipid-metabolising enzyme from the *Leptospira* species, might be a possible candidate leptospiral antigen for use in the early diagnosis of human leptospirosis. However, the presence of this antigen in human urine samples has not been investigated yet. The aim of this study was to evaluate whether 3-HADH is a suitable urinary biomarker for the diagnosis of acute human leptospirosis.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Leptospira interrogans serovar Manilae strain UP-MMC-NIID was used for the cloning of 3-HADH. Other bacterial strains used in this study are listed in Table 1. *Leptospira* spp. were cultivated in Ellinghausen-McCullough-Johnson-Harris (EMJH) broth at 30 °C.

2.2. Cloning and purification of 3HADH

The gene encoding 3-HADH was amplified using oligomers rHADH-F 5′-GGAATTCCATATGAGAGAAATCAAAACAGTAACAG-3′ and 5′-CCGCTCGAGACCTTTGAAAAGAGAACGAGC-3′ designed based on *L. interrogans* serovar Manilae 3-HADH (locus_tag, LIMLP_16880), GenBank accession CP011931 [17]. Cloning procedures were performed as previously described [14] with some modifications. Briefly, the plasmid for the expression of recombinant 3-HADH

Table 1. Bacterial strains used in this study.

Bacterial species	Serovar	Strain (Reference)
Leptospira interrogans	Manilae	UP-MMC-NIID [15]
	Lai	Lai (Institut Pasteur, France)
	Ratnapura	K5 [16]
	Icterohaemorrhagiae	Oki-1 (This study)
	Hebdomadis	Oki-2 (This study)
	Pyrogenes	Oki-5 (This study)
Leptospira borgpetersenii	Javanica	K6 [16]
Leptospira biflexa	Patoc	Patoc I (Institut Pasteur, France)

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(r3-HADH) was generated in pET-28a (+) (Novagen), and after confirming the presence of the correct insert by nucleotide sequencing, the plasmid was transformed into *E. coli* BL21-AI (Invitrogen). Protein expression was induced by 0.2% arabinose and 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 3 hours at 37 °C. Histidine (His)-tagged r3-HADH was purified under denaturation conditions with TALON Metal Affinity Resin (Clontech) by using BugBuster Master Mix (Novagen) as the lysis buffer.

2.3. Preparation of polyclonal antiserum

Anti-3-HADH peptide polyclonal antiserum was generated by immunizing rabbits with peptide 5 (EVLAKAKPGKPFYEL), corresponding to amino acids 410 to 424 (Table 2). The peptide (3-HADH$^{410-424}$) was conjugated to keyhole limpet haemocyanin as a carrier protein (Sigma Aldrich, Japan). The conjugated 3-HADH$^{410-424}$ was mixed with complete Freund's adjuvant for the first immunization and 3 subsequent booster injections were given at two-week intervals by using incomplete Freund's adjuvant. Animal experiments were conducted in accordance with the guidelines of Sigma Aldrich for the care and use of laboratory animals and the immunization protocol was approved by the ethics committee for animal experiments of Sigma Aldrich Japan (#SAJ520722).

2.4. Ethics approval for clinical research

The Ethics Review Committee of the University of the Ryukyus (Clinical Investigation Program, CIP#1000), the National Institute of Infectious Diseases (CIP#723) and the Okinawa Prefectural Institute of Health and Environment (CIP#488-2) approved this study. Informed consent of patients was obtained through an opt-out methodology or written consent.

2.5. Urine collection and clinical case definition

The urine samples included in this study were those within a collection of clinically suspected cases of leptospirosis submitted to the National Institute of Infectious

Table 2. List of immunogenic peptides specific for *L. interrogans*.

Peptide No.	Sequence	Amino acid position
1	MSKTPDGKKEKL	285−296
2	GADLYEPVPKF	301−311
3	RQANKRISEADYTGAM	317−332
4	DAIQLIEKAKLPVPEVLAK	396−414
5	EVLAKAKPGKPFYEL	410−424

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Diseases or the Okinawa Prefectural Institute of Health and Environment for laboratory confirmation between January 2016 and April 2017 and were not collected specifically for this study. These reference laboratories received specimens routinely for laboratory confirmation which is done by nested-PCR (PCR) for the flagellar *flaB* gene, bacterial culture in EMJH and Korthof's media and/or microscopic agglutination test (MAT). A positive laboratory diagnosis of leptospirosis required at least one of the following criteria: 1) PCR positive, 2) culture isolation or 3) serological diagnosis by MAT (at least 4-fold rise in titer between acute and convalescent serum samples). Among 70 clinically suspected cases of leptospirosis, 40 were laboratory-confirmed cases and 30 were negative for these tests. Forty-nine urine samples from healthy volunteers were used as negative control. Urine samples were anonymized and stored at −80 °C until analysis.

2.6. Western blotting (WB)

Whole-cell lysates of cultivated bacteria or urine samples were loaded onto 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for the detection of 3-HADH with anti-3-HADH$^{410-424}$ antiserum, followed by incubation with horseradish peroxidase (HRP)-goat anti-rabbit IgG conjugate. Albumin was detected with anti-human albumin mouse monoclonal antibody (Santa Cruz, SC-271605). For detection of LigA, samples were loaded onto 7.5 % SDS-PAGE and probed with anti-LigA mouse polyclonal antiserum [5], followed by incubation with HRP-donkey anti-mouse IgG conjugate. The bands were detected by ECL Western Blotting Analysis (GE Healthcare). Urine samples were concentrated eight-fold with a centrifugal filter device (Amicon Ultra 4 molecular mass cut off, 10-kDa; Merck Millipore) as described by Segawa et al. [14].

3. Results

3.1. Anti-3-HADH$^{410-424}$ antiserum reacts with pathogenic *Leptospira* strains

An ideal biomarker for the diagnosis of infectious diseases should be specific for pathogenic species, but 3-HADH is a 436-amino acid protein present both in pathogenic and non-pathogenic *Leptospira.* To prepare pathogenic species-specific antiserum, we compared *L. interrogans* and *L. biflexa* 3-HADH sequences and identified five immunogenic peptides specific for *L. interrogans* (Table 2). The region corresponding to peptides 4 and 5 (amino acid position 396−424) showed the highest predicted antigenic value (Fig. 1) and was conserved among the pathogenic *Leptospira* species (*L. interrogans, L. kirschneri, L. borgpetersenii, L. santarosai, L. noguchii, L. weilii, L. alexanderi, L. alstonii*). Peptide 5 (amino acid position 410−424) was selected as the peptide for immunization because of its higher hydrophilicity

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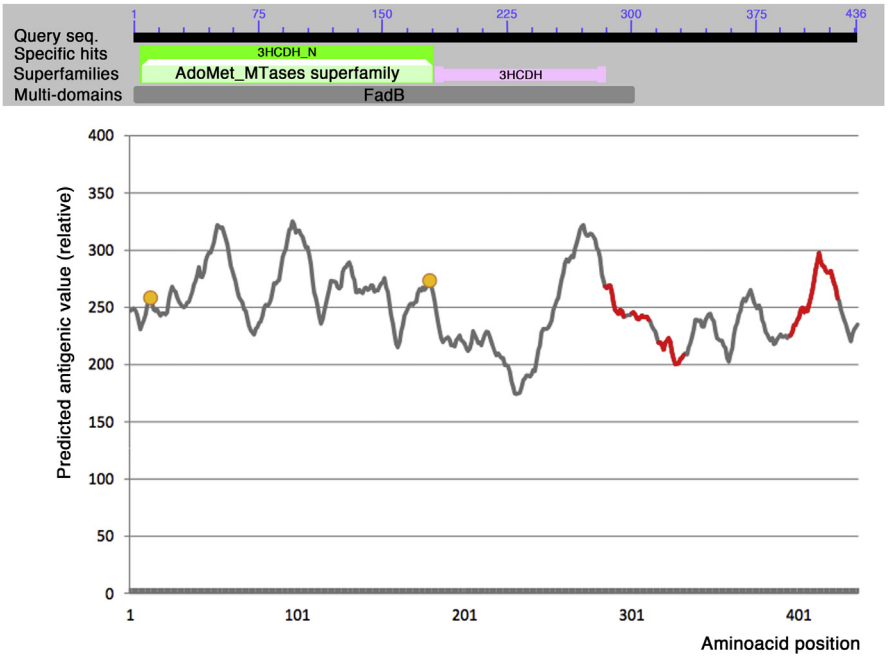

Fig. 1. Peptide design to prepare pathogenic *Leptospira* species-specific antiserum. Predicted antigenic value (relative) in each amino acid position is shown. Pathogenic *Leptospira* specific regions are showed in red. Yellow circles show putative glycosylation sites.

(Table 2). The anti-3-HADH$^{410-424}$ antiserum recognized the purified His-tagged r3-HADH protein and reacted with a protein band of ~52 kDa in whole-cell lysates of pathogenic strains, while it did not react with the non-pathogenic *L. biflexa* (Fig. 2).

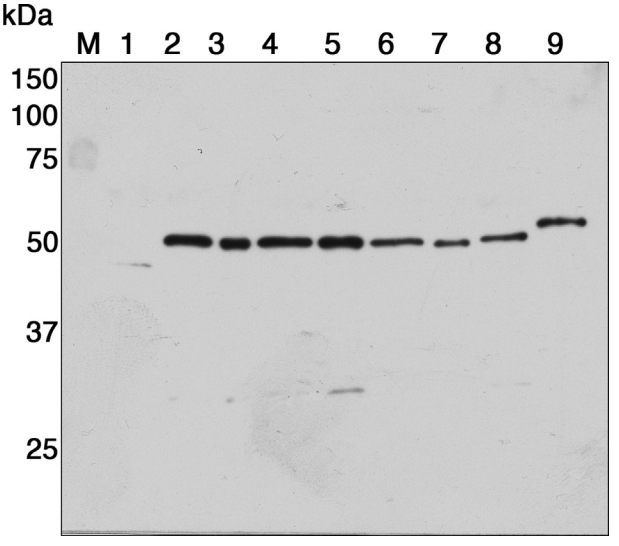

Fig. 2. Detection of 3-HADH in bacterial whole cell lysates. Lanes: (M) Potein molecular weight markers (1) *L. biflexa*, (2) *L. borgpetersenii* ser. Javanica (3) to (8) *L. interrogans.* Serovars (3) Manilae, (4) Lai, (5) Ratnapura, (6) Icterohaemorrhagiae, (7) Hebdomadis, and (8) Pyrogenes. Lane 9: purified His-tagged rHADH.

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3.2. Detection of excreted proteins in urine samples

A total of 70 patients who presented to hospital before 11 days post-onset of illness (p.o.) were included in this study. Among them, 40 were considered definite leptospirosis cases based on the laboratory results. Thirty samples were defined as non-leptospirosis cases based on the laboratory-negative results (MAT and/or PCR negative). Detection of 3-HADH was positive in 12 samples of the laboratory-confirmed cases (30%) (Table 3). No correlation was found between the leptospiral serogroup associated with infection and detection of 3-HADH, suggesting that the low detection rate was due to the low amount of 3-HADH excreted in the urine. We then, performed WB using concentrated urine samples to increase the sensitivity of 3-HADH detection. The detection rate increased to 52.5% (21 out of 40 samples). Fig. 3 shows representative samples of WB analysis. Albumin was detected in all urine samples, while the outer membrane protein, LigA, was not detectable in urine.

Among the 3-HADH-positive samples, 6 cases were negative for PCR (blood and urine) (Table 4). These cases were confirmed as leptospirosis in the convalescent phase by MAT, except for 1 case that was laboratory confirmed by bacterial culture (Table 3, patient 18). In the urine samples of clinically suspected cases of leptospirosis with negative laboratory tests, a ~52 kDa protein was detected in 3 out of 30 samples. All urine samples from healthy volunteers were negative for 3-HADH. The sensitivity, specificity, positive predictive value and negative predictive values for 3-HADH detection were 52.5%, 90%, 87.5% and 58.69%, respectively.

3.3. Comparison of 3-HADH Western blotting and PCR

Because a urine biomarker excreted into the urine from the early stage of infection, will be useful in the development of an RDT, we compared the detection of 3-HADH with that of leptospiral DNA by PCR using blood or urine in regard to days of p.o in the laboratory-confirmed cases (Table 3, Fig. 4). As shown in Fig. 4, sensitivity for PCR from blood was 89% before 3 days p.o.; however, sensitivity decreased to 32% for samples collected between 4 and 9 days p.o. When considering urine samples, sensitivity for PCR was 32% between 4 to 9 days p.o., but PCR was not able to detect leptospiral DNA in samples collected between 1 and 3 days p.o. On the other hand, 3-HADH was detected from day 2 p.o., and showed a wider window of detection than blood-PCR.

Considering that any PCR positive is considered criteria for laboratory confirmed cases of leptospirosis, we calculated if 3-HADH detection increased sensitivity of laboratory diagnosis in the acute phase. As shown in Fig. 4, a combination of PCR and 3-HADH detection increased sensitivity of diagnosis to 100% in samples collected between 1 and 3 days p.o, and to 82% in samples collected between 4 and 9 days p.o.

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Table 3. Results of MAT, PCR and detection of 3-HADH in urine samples from laboratory-confirmed leptospirosis patients.

Patient No.	Urine collection[a]	PCR (Blood)	PCR (Urine)	MAT Serogroups	3-HADH (1x)	3-HADH (8x)
1	1	+	−	Hebdomadis	−	−
2	1	+	−	Hebdomadis	−	−
3	2	+	−	Hebdomadis	+	+
4	2	+	−	Hebdmadis	−	−
5	2	+	−	Pyrogenes	−	+
6	2	+	−	Hebdomadis	−	−
7	2	+	−	Hebdomadis	+	+
8	2	+	−	Hebdomadis	−	+
9	2	+	−	ND[b]	−	−
10	2	+	−	ND	−	−
11	2	+	−	ND	−	+
12	3	+	−	Canicola/Pyrogenes	−	−
13	3	−	−	Hebdomadis	+	+
14	3	+	−	Hebdomadis	+	+
15	3	+	−	Hebdomadis	−	−
16	3	+	−	Hebdomadis	+	+
17	3	+	−	Pyrogenes	−	+
18[c]	3	−	−	ND	−	+
19	4	+	+	Icterohaemorrhagiae	−	+
20	4	−	+	Pyrogenes	−	−
21	4	−	−	Autumnalis	+	+
22	4	−	−	Hebdomadis	−	−
23	4	−	−	Australis	−	−
24	4	−	−	Hebdomadis	−	−
25	4	+	−	ND	−	−
26	5	−	+	Canicola/Hebdomadis	+	+
27	5	+	−	Grippotyphosa	+	+
28	5	−	−	Hebdomadis	−	−
29	5	+	+	Autumnalis	+	+
30	5	+	−	ND	−	−
31	5	+	+	ND	+	+
32	5	+	+	ND	+	+
33	6	−	−	Hebdomadis	−	−
34	6	−	−	Autumnalis	−	−
35[d]	6	−	−	Hebdomadis	−	+
36	7	−	−	Hebdomadis	−	+

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Table 3. (*Continued*)

Patient No.	Urine collection[a]	PCR (Blood)	PCR (Urine)	MAT Serogroups	3-HADH (1x)	3-HADH (8x)
37	7	–	–	Hebdomadis	+	+
38	7	–	–	Hebdomadis	–	–
39	8	–	–	Hebdomadis	–	–
40[d]	9	–	+	Hebdomadis	–	+

[a] Day of urine collection p.o.

[b] Not done.

[c] Diagnosed by bacterial culture.

[d] PCR positive for cerebrospinal fluid.

4. Discussion

In this study, we detected the leptospiral 3-HADH for the first time in the urine of leptospirosis patients. The comparison of 3-HADH detection with blood-PCR and

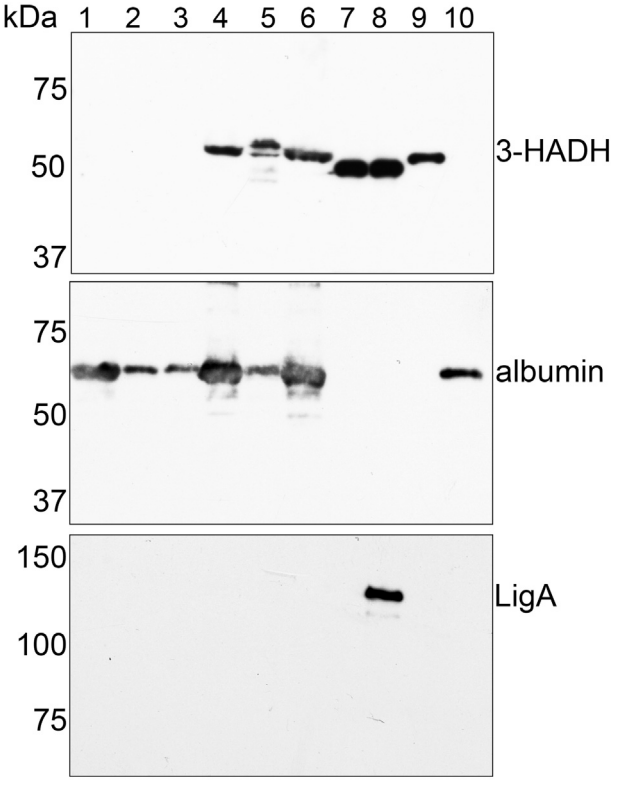

Fig. 3. Analysis of urine samples by Western Blotting. Representative urine samples collected up to 3 days p.o. are shown. Lanes 1 to 6: urine samples of laboratory confirmed leptospirosis patients. Lanes 1: patient 12, lane 2: patient 4, lane 3: patient 6, lane 4: patient 16, lane 5: patient 7, lane 6: patient 13. Lanes 7 and 8: whole cell lysates of *in vitro* cultivated *L. interrogans* serovar Manilae. Lane 7: culture-attenuated strain (3-HADH-positive, LigA-negative) [15]. Lane 8: virulent strain (3-HADH-positive, LigA-positive). Lane 9: purified His-tagged rHADH (10 ng). Lane 10: human albumin (125 ng).

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Table 4. Comparison of PCR results with 3-HADH detection.

3-HADH	PCR (blood &/or urine)		
	Positive	**Negative**	**Total**
Positive	15	6	21
Negative	10	9	19
Total	25	15	40

urine-PCR showed that blood-PCR is the most sensitive method for diagnosing leptospirosis within 3 days p.o.. However, due to the transient nature of *Leptospira* in the bloodstream, the window of optimal specimen collection is very narrow for DNA detection and the sensitivity decreased from 3 days p.o. Our results showed that 3-HADH is excreted from the early phase of infection when leptospiral DNA in urine is negative and can be detected at least until 9 days p.o. Thus, while blood-PCR and urine-PCR sensitivities are dependent on the time p.o., 3-HADH detection has a wider window of detection and allows diagnosis of leptospirosis when other laboratory tests are negative. Our results suggested that a combination of PCR and 3-HADH detection increases sensitivity of diagnosis in the acute phase (Fig. 4).

One limitation of our study was that samples were not specially collected for this study, and were not immediately analyzed after collection; thus, transportation or long-term storage might cause the decreased in the detection rate of 3-HADH. Another limitation is that we focused on a 15-amino acid peptide for 3-HADH

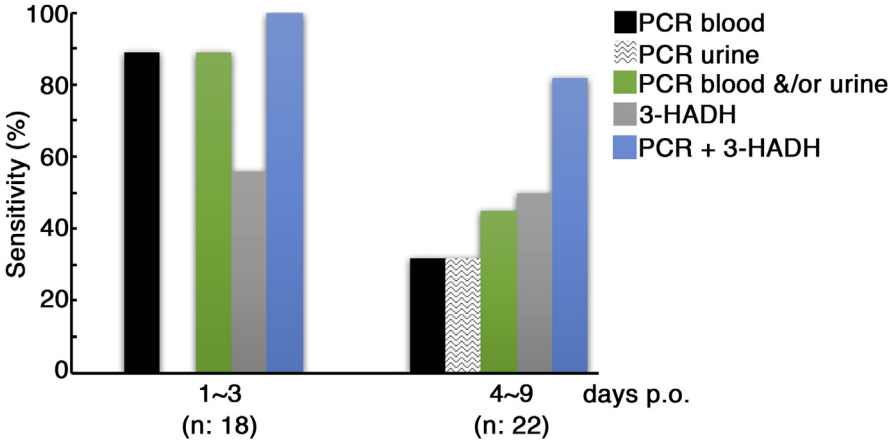

Fig. 4. Sensitivity of 3-HADH detection and PCR in laboratory confirmed cases. Sensitivity for each method (PCR blood, PCR urine and 3-HADH WB) or sensitivity when several methods are combined (PCR blood and PCR urine, PCR + 3-HADH) are shown based on the days post-onset of illness. PCR + 3-HADH: combination of PCR (blood and/or urine) with 3-HADH detection as the criteria for laboratory confirmation of leptospirosis.

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detection, but this region might be masked in some biological samples giving a false negative result in our WB assay. Thus, the efficacy of 3-HADH diagnosis needs to be further evaluated on several sets of well-defined cases from different regions on a large number of clinical samples with additional antibodies raised to different regions of 3-HADH. Interestingly, 3-HADH from urine showed different migration behavior on SDS-PAGE than 3-HADH from *in vitro* cultivated bacteria (Fig. 3). We also observed apparent molecular weight variation in the urine samples, which may be caused by the presence of several isoforms during infection. Recently, Nally et al. reported that pathogenic leptospires modulate protein post-translational modifications in response to mammalian host signals [18].

In the clinically suspected cases of leptospirosis without laboratory confirmation, 3-HADH was detected in 10% of the cases. A recent study by Agampodi et al., showed that due to the lack of seroconversion in some patients, paired-sample MAT negative cases were positive with a more sensitive such as qPCR [19]. We were not able to detect leptospiral DNA in the MAT-negative/3-HADH-positive cases, however, because a negative PCR in a symptomatic patient will not rule out the diagnosis of leptospirosis, further study is needed to conclude if our antiserum is cross−reacting with a host protein. The detection of 3-HADH in urine from 2 days p.o., suggested that during the septic phase, lysis of bacteria causes cytoplasmic proteins released. We were not able to detect LigA, a 120 kDa outer membrane protein. Thus, it is probable that high molecular weight proteins are not released into urine until kidney function is markedly decreased [20].

Because an RDT capable of diagnosing leptospirosis using urine will be useful in remote health centres and large outbreaks situations, several studies have focused on the urine by targeting structural leptospiral antigens, such as lipopolysaccharides [10]; outer membrane proteins LigA [11], LipL32 [21] or DNA [22]. More recently, Chaurasia et al., detected additional pathogen specific antigens (LipL41, Fla1, HbpA and sphingomielinase) in the urine of leptospirosis patients collected in Kerala, India [23]. These antigens were also detected in patients with pyrexia of unknown origin with MAT-negative results. Thus, the combinational use of 3-HADH detection with other reported antigens can be explored to develop a multiplex lateral flow IC assay, which will be very useful for the clinicians especially when serological methods are negative during the acute phase.

Declarations

Author contribution statement

Claudia Toma: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Nobuo Koizumi, Tetsuya Kakita: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Takayoshi Yamaguchi: Contributed reagents, materials, analysis tools or data.

Idam Hermawan, Naomi Higa: Performed the experiments.

Tetsu Yamashiro: Analyzed and interpreted the data.

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Competing interest statement

The authors declare the following conflict of interests: Claudia Toma, Takayoshi Yamaguchi: A patent application related to the subject of the present article has been applied for. All other authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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