Table 1. LAMP primer sequences for simultaneous detection of *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Staphylococcus aureus* and *Streptococcus suis*

Primers	Sequence 5′–3′
FIP	CGCTTTCG(C/A)(A/G)C(A/C)TCAGCGTCATGGAGGAA CACC(A/G)GTGGC
BIP	CACGC(C/T)GTAAACGATGAGTGCTAGGC GGAGTGCTTAATGC
F3	CATGTGTAGCGGTGAAATGC
B3	TCAACCTTGCGGTCGTACT

S. agalactiae (Fig. 1b). The name, positions and nucleotide sequences of all four primers are shown in Fig. 1b and Table 1. The DNA sequence alignment of 16S rRNA gene of these four species indicated a low variation among these species.

Sensitivity and specificity of LAMP assay

The sensitivities of the broad range LAMP assay were performed by running 10-fold serial dilutions of target bacteria (from 10^7 to 10^0 CFU mL^{-1}). The detection limit was 100 CFU mL^{-1} of *S. pneumoniae* by both real-time turbidimeter and electrophoresis of LAMP products, and 10 000 CFU mL^{-1} by conventional PCR method (Fig. 2). Similarly, the broad range LAMP assay detected *S. suis*, *S. agalactiae* and *S. aureus* at 100 CFU mL^{-1}, while conventional PCR assay only detected these bacteria with more than 10^4 CFU mL^{-1} (Table 2). The results of all the positive samples detected by the LAMP assay were achieved within 60 min.

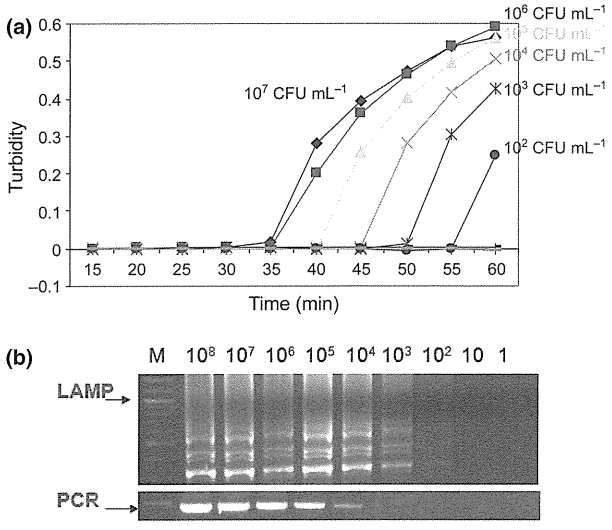

Fig. 2. Sensitivity of broad range LAMP assay for the detection of *Streptococcus pneumoniae*. LAMP reactions detected by real-time turbidity (a) and electrophoresis of LAMP products and conventional PCR products (b). The assay was performed in 10-fold serial dilutions (from 10^7 to 10^0 CFU mL^{-1}). *Streptococcus pneumoniae* with more than 100 CFU mL^{-1} was detected by both real-time turbidimeter and electrophoresis. The conventional PCR only detected *S. pneumoniae* with more than 10^4 CFU mL^{-1}.

Table 2. Sensitivities of LAMP and conventional PCR assays

	The limit of detection (CFU mL^{-1})	
Bacteria	LAMP assay	PCR assay
S. pneumoniae	10^2	10^4
S. suis	10^2	10^4
S. agalactiae	10^2	10^5
S. aureus	10^2	10^4

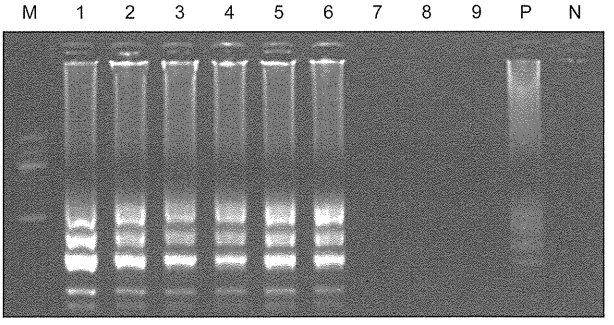

Fig. 3. Specificity of broad range LAMP assay. Two microlitre of the DNA template (10^5 CFU mL^{-1}) extracted from *Staphylococcus aureus* (lane 1), *Streptococcus pneumoniae* serotype 3 (lane 2), *S. pneumoniae* serotype 10 (lane 3), *Streptococcus suis* serotype 2-8-01 (lane 4), *S. suis* serotype 2-8-02 (lane 5), *Streptococcus agalactiae* (lane 6), *Haemophilus influenzae* (lane 7), *Escherichia coli* (lane 8) and *Neisseria meningitidis* (lane 9). Lane P, Positive control; lane N, Negative control; and lane M, DNA size markers.

The specificity of the LAMP assay was evaluated by cross-reactivity test using DNA extracted from *N. meningitidis*, *H. influenzae* and *E. coli*. There were ladder-like products amplified from *S. pneumoniae*, *S. suis*, *S. agalactiae* and *S. aureus* but not from *N. meningitidis*, *H. influenzae* and *E. coli* cultures (Fig. 3), suggesting that the broad LAMP assay was specific for *S. pneumoniae*, *S. suis*, *S. agalactiae* and *S. aureus*.

Visual detection of the LAMP products

LAMP products were further explored by visual inspection based on the intercalation of fluorescent dye SYBR Green I into amplified DNA. As shown in Fig. 4, the product of positive reaction became visible under ultraviolet lamp and was green colour under naked eye, while the negative product was not seen under ultraviolet lamp and remained orange colour under day light.

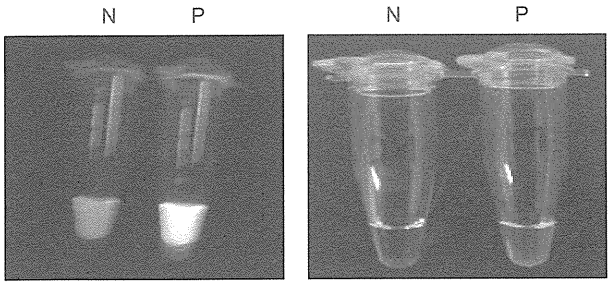

Fig. 4. Visual detection of LAMP products. Representative visual inspection of *Streptococcus pneumoniae* by fluorescence under ultraviolet lamp (left *Streptococcus*) and day light (right). (N), negative control without DNA template; (P), positive reaction in the presence of *S. pneumoniae* DNA template.

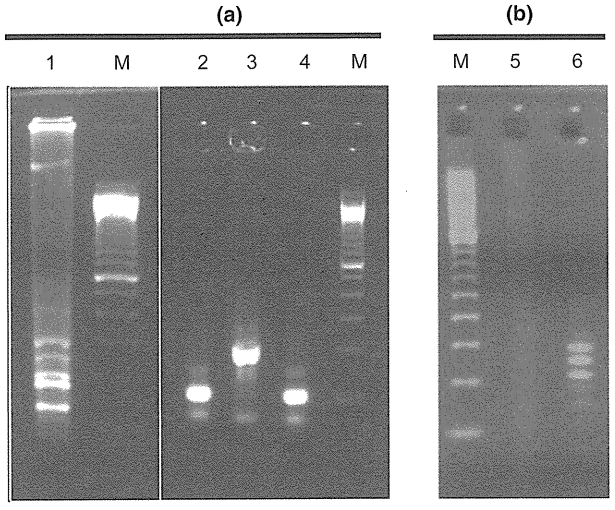

Fig. 5. The digested pattern of the LAMP products with restriction enzymes using 2% agarose electrophoresis. (a) DdeI digestion patterns of the LAMP products from *Staphylococcus aureus* (lane 1), *Streptococcus agalactiae* (lane 2), *Streptococcus pneumoniae* (lane 3) and *Streptococcus suis* (lane 4). (b) HaeIII digestion patterns of the of the LAMP products from *S. suis* (lane 5) and *S. agalactiae* (lane 6). Lane M is 100-bp ladder size markers.

Identification of bacterial species

To identify bacterial species, the LAMP product was digested with specific restriction enzyme and analysed by gel electrophoresis. After digested with DdeI, all LAMP products were digested into several fragments. *Staphylococcus aureus* gave five bands at 55, 150, 197, 230 and 263 bp (Fig. 5a, lane 1). *Streptococcus pneumoniae* produced three bands at 55, 150 and 200 bp (Fig. 5a, lane 3). *Streptococcus agalactiae* (lane 2) and *S. suis* (lane 4) gave similar pattern. Thus, the LAMP products of *S. agalactiae* and *S. suis* were further digested with HaeIII. The result showed that *S. agalactiae* was digested into four bands at 70, 216, 254 và 292 bp (Fig. 5b, lane 6), while *S. suis* was not digested by HaeIII (Fig. 5b, lane 5).

To our knowledge, this is the first study that developed a broad range LAMP assay for simultaneous detection of more than four different bacterial species. The sensitivity of our LAMP assay was 100–1000 times higher compared with the conventional PCR assay. The bacterial species could be distinguished among *S. pneumoniae*, *S. suis*, *S. agalactiae* and *S. aureus* based on the digested pattern of the LAMP products with restriction enzymes of DdeI and HaeIII. In addition, our method has several advantages over the current diagnostic methods. Firstly, the method is rapid (*c.* 1 h) as compared with the real-time PCR method which requires 6 h to run (Nadkarni *et al.*, 2002). Secondly, the LAMP method does not require expensive fluorimeter and fluorogenic primers and probes. Thirdly, the assay is simple and does not require highly experienced technician. More importantly, the assay can be performed in a water bath at bedside or in rural areas. These advantages suggested that our broad range LAMP assay would improve the early diagnosis and treatment of BM, helping to reduce morbidity and mortality. Furthermore, the assay could detect bacterial species, helping to select an appropriate antibiotic therapy.

One limitation of our LAMP assay was that only four species could be detected. A single-tube LAMP assay for the detection of more than four species is under development using a mixture current broad range LAMP primers and specific LAMP primers of other bacteria species. Additional clinical studies are also required to validate this new assay.

Conclusions

Four common pathogen of BM including *S. pneumoniae*, *S. suis*, *S. agalactiae* and *S. aureus* could be simultaneously detected using a broad range LAMP assay in single tube in < 1 h. The assay is highly sensitive, rapid and simple and can be performed at bedside in healthcare facilities.

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Authors' contributions

N.T.H and L.T.T.H. contributed equally to this work.

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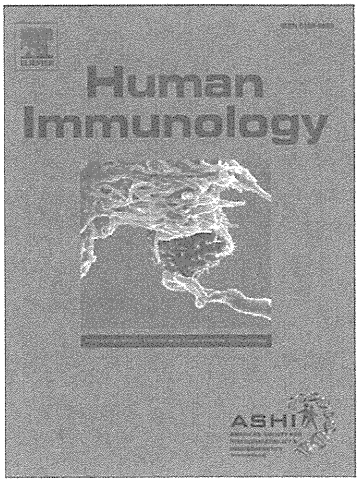

21-Hydroxylase gene mutant allele CYP21A2*15 strongly linked to the resistant HLA Haplotype B*14:02-DRB1*01:02 in Chronic Chagas Disease

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21-Hydroxylase gene mutant allele CYP21A2*15 strongly linked to the resistant HLA Haplotype B*14:02-DRB1*01:02 in Chronic Chagas Disease

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The authors have not any conflict of interest.

ABSTRACT

We previously reported protective haplotype HLA-B*14:02-DRB1*01:02 against chronic Chagas disease in Bolivia. The V281L mutant allele of the 21-Hydroxylase gene, CYP21A2*15, is reported to be located in the Class III region of the Human Leukocyte Antigen region and linked to the haplotype HLA-B*14:02-DRB1*01:02. The mutant allele might play a primary role in the pathogenesis of chronic Chagas disease in the associated HLA region. We analyzed the frequency of this allele in the same subjects for the previous one. The statistical analysis showed a significant association of the CYP21A2*15 with resistance to severe chronic Chagas disease (OR=0.207273; Pv = 0.0041). However, there is no significant tendency of the mutant gene contribution to the resistance after the elimination of the HLA-B*14:02-DRB1*01:02 linked mutants (OR=0.38; Pv = 0.1533). Although the frequency of the CYP21A2*15 was small, we found no primary contribution of this mutation to the protection against chronic Chagas disease.

Keywords: Chagas disease, Human Leukocyte Antigen, 21-Hydroxylase, Haplotype, Linkage

INTRODUCTION

Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi* and it is estimated that 10 million people are infected with this parasite worldwide but mostly in Latin America [1].Around 10-30% of infected individuals develop chronic Chagas disease which involves cardiac and/or gastrointestinal complications 10-20 years post infection. The cardiac manifestation of chronic Chagas disease vary from mild symptom to heart failure, inflammation with accompanying fibrosis scattered throughout the myocardium results in severe heart lesions. These abnormalities can be detected by characteristic changes in ECG. Chronic Chagas disease can also manifest as megacolon due to destruction of the autonomic enteric innervations of the gastrointestinal system leading to loss of gastrointestinal motility [2].

The strong interconnection between immune and neuroendocrine systems may optimize the defensive response of the host, but also set the

basis for an altered regulation of inflammation when pathogens cannot be cleared, such as in chronic Chagas. However, integrative immunoendocrine response in the course of Chagas disease remains poorly characterized at the experimental as well as in humans [3] . Cortisol has a crucial role in maintaining homeostasis, influencing differentiation, suppressing inflammation, and affecting cross-talk among the immune, nervous and endocrine systems [4]. Cortisol acts both through intracellular receptors and through poorly characterized membrane-bound receptor that are expressed in almost every cell of the body. They intracellularly bind to receptors either directly to specific sites in the DNA, thereby altering transcription, or interact with other transcription factors, such as NFκB, to modulate their function. Cortisol can also act directly on cellular processes, leading to a much more rapid production of anti-inflammatory proteins. This causes exaggerated responses, which have both beneficial and toxic effects [5].

To produce cortisol, the major glucocorticoid in human, CYP17 (P450c17, 17α-hydroxylase/17, 20 lyase) in the endoplasmic reticulum of the zona fasciculata and zona reticularis converts pregnenolone to

17α-hydroxyprenenolone. 3ß-Hydroxysteroid dehydrogenase in the zona fasciculata utilizes 17α-hydroxypregnenolone as a substrate producing 17α-hydroxyprogesterone. The latter is 21-hydroxylated by CYP21A2 to form 11-deoxycortisol, which is converted to cortisol by CYP11B11 (P450c11, 11ß-hydroxylase) in mitochondria [6].

The structural gene encoding human CYP21A2 and a pseudogene (CYP21A1P) are located in the Human Leukocyte Antigen (HLA), the major histocompatibility complex. In human chromosome 6p21.3, CYP21A2 and CYP21A1P contain 10 exons and 9 introns covering a distance of 3 kb showing a high homology with a 98% nucleotide identity in the exonic sequences and a 96% in the intronic ones [6]. Deficiency by mutations of the 21-Hydroxylase, is the most common cause of disorders of cortisol biosynthesis. The degree to which each mutation in CYP21A2 compromises enzymatic activity correlates with the clinical severity in the Congenital Adrenal Hyperplasia, the most common cause of genital ambiguity [7]. A non classic 21-Hydroxylase deficiency is caused by a single mutation called CYP21A2*15 that is given by an amino acid substitution of valine with leucine (V281L) because of a nucleotide

substitution (1683 G > T) in the exon 7 [8]. And this V281L mutation is almost invariably linked to the HLA-B*14:02-DRB1*01:02 haplotype in studies based on Ashkenazi Jews and other Caucasians [9, 10, 11].

In our previous study we reported the strong association of the Haplotype HLA-B*14-DRB1*01 with the resistance to chronic Chagas disease in Bolivia. Therefore, we perform the genetic analysis of the V281L mutation in the previous study subjects to see the primary contribution of the CYP21A2*15 to the chronic Chagas disease.

SUBJECTS AND METHODS

Subjects

Two hundred and ninety one patients with chronic Chagas disease (136 men and 155 women, mean age 45 years) were recruited from: Centro Nacional de Enfermedades Tropicales (CENETROP) (91 men and 119 women), Hospital Primero de Mayo (12 men and 7 women) and from post-operative patients at the Hospital Universitario Japonés (HUJ) (33 men and 29 women) in Santa Cruz, Bolivia. The selection criteria,

grouping and clinical manifestations were previously described by del Puerto et al 2012 [12]. Briefly, 229 seropositive Chagas outpatients in Santa Cruz, Bolivia, were examined by Electrocardiogram and Barium enema colon X-ray. The residual 62 post-operational patients from HUJ were confirmed to be Chagas Megacolon during admission period.

The experimental protocol was approved by the Institutional Ethical Review Committee of the Institute of Tropical Medicine, Nagasaki University, Japan (No. 0210170018) and the Centro Nacional de Enfermedades Tropicales (CENETROP), Bolivia.

Identification of the mutant allele by RFLP

DNA extraction and handling were described by del Puerto et al. 2012 [11]. PCR was carried out in a total volume of 30 µl containing 1X buffer, 0.2 mM dNTPs, and 1 µM each primer, 0.15 units of *Taq* polymerase (Takara Bio INC, JPN) and 150 ng of sample DNA. Cycling condition: 95 °C for 5 min initial denaturation, 35 cycles of 1min denaturation at 95 °C, 1 min annealing, at 70 °C, 1min extension at 72 °C, and a final extension at 72 °C for 7 min.. Primer sequence 5' GGA CCT

GTC CTT GGG AGA CTA C and 5' GCC GTG TGG TGC GGT GGG GCA AGG CTA were used and analysis of restriction fragments were performed according Pucci L, et al, 2010 [13].

The RFLP technique was performed with the BsiHKAI enzyme. The reaction mixture was heated overnight at 65 °C according to manufacturer instruction (New England, BioLab® Inc). The digested product was ran on a 1.5% agarose gel stained with ethidium bromide and visualized by UV light. 2.3.

Statistical analysis

Statistical analysis was performed by Chi square and Fisher's exact tests using the StatsDirect software (StatsDirect Ltd, UK) and interval confidence of 95%. Hardy-Weinberg Equilibrium, linkage disequilibrium (LD) and Haplotype analyses were calculated with PyPopWin32.0.7.0 software [14].

RESULTS

Linkage

After the detection of the mutant alleles by RFLP as shown in Figure 1, we observed a linkage between the CYP21A2*15 and the alleles B*1402 and DRB1*0102, in the subjects [12] (Table 1). Out of the 291 samples analyzed, we could obtain results in 285 of them, the CYP21A2*15 was present in a frequency of 5.96 % (17 out of 285) of the studied population (data not shown).

Statistical analysis

We found a statistical significant association of this V281L to Chronic Chagas disease (Table 2) by independent analysis by comparing the three groups: ECG+ &/or Megacolon+, ECG alterations+ and Megacolon+ with the Indeterminate group; (OR = 0.207273, Pv = 0.0041), (OR = 0.141968, Pv = 0.007) and (OR = 0.222874, Pv = 0.0118) respectively. However, in the table 3, statistical significance disappeared after the elimination of B*14:02-DRB1*01:02 haplotype positives from the analysis (OR = 0.38; Pv = 0.1533).

DISCUSSION

In the present study, the CYP21A2*15 was closely linked to the HLA

haplotype B*14:02-DRB1*01:02 in Bolivia as was previously reported in other ethinic groups [15] We also found significant linkage between the CYP21A2*15 and the other low frequency alleles of HLA-B or DRB1 such as B*53:05, B*40:01, B*13:09, B*49:02, B*51:02, B*42:01, B*15:09, B*39:02, B*35:43, B*45:01 and DRB1*03:20. Therefore, we could identify the subjects who were positive for CYP21A2*15 with or without the resistant HLA haplotype. Independent analysis of the V281L mutation by comparing the chronic Chagas groups to the indeterminate gave a significant result positioning the mutation as a protective factor. But we consider this association as a mere coincidence due to the strong linkage to the resistant HLA haplotype under such a low frequency of this mutation in the population studied (5.96 %). Therefore, we analyzed the negative effect of the V281L to the chronic Chagas complications after the exclusion of HLA-B*14-DRB1*01 haplotype positive persons as shown in Table 3. If such a protective role of the V281L mutation itself is real, the other patients carrying other neutral alleles of the HLA-DRB1 and HLA-B also should have a tendency to be free from those complications. Due to the low frequency of mutant allele (N=17 out of 285, Supplementary), we

could not definitely deny the tendency. However, there was no significant effect of protection after the exclusion of the HLA Haplotype (Table 3).

This result is consistent with an immunoendocrine analysis in patients with progressive forms of Chronic Chagas disease where they observed normal cortisol levels among the studied groups (Healthy, Indeterminate, Mild to moderate cardiac, and Severe cardiac); whereas a progressive diminution of DHEA-s levels, the sulfate ester of DHEA, was found as Chagas disease severity progressed in a previous study [3]. Furthermore, the DHEA and testosterone therapy in *T. cruzi* infected rats, improved the effectiveness of the host's immune response [16].

The 21-Hydroxylase encoded by the CYP21A2 gene plays an important role in the metabolic pathway for the conversion of cholesterol to cortisol while DHEA derived from Cholesterol through the conversion of the pregnenolone to 17α-Hydroxypregnenolone by the CYP17A1 enzyme, coded in the CYP-17 gene. This CYP17A1 gene is located on the chromosome 10 and is independent from CYP21A2 gene on the chromosome 6. Our observation that the functional mutant allele of CYP21A2 did not show any significant effect on the progression of

chronic Chagas disease is consistence with the DHEA association, although, we had not measured the cortisol and DHEA levels in our subjects.

In conclusion, we confirmed the Chronic Chagas disease resistant HLA haplotype HLA-B*14:02-DRB1*01:02 is linked to CYP21A2*15. The CYP21A2*15 protective effect was not clarified after the exclusion of the Haplotype.

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