Loop-mediated Isothermal Amplification assay (LAMP) based detection of Pasteurella multocida *in cases of haemorrhagic septicaemia and fowl cholera*

Mayurkumar P. Bhimani*, Bharat B. Bhanderi & Ashish Roy

Department of Veterinary Microbiology, College of Veterinary Science and A.H., Anand Agricultural University, Anand, Gujarat, India

** Corresponding author at: Department of Veterinary Microbiology, College of Veterinary Science and A.H., Anand Agricultural University, Anand-388001, Gujarat, India. Tel.: +91 9033794615, e-mail: mayur_bhimani@yahoo.co.in.*

Veterinaria Italiana 2015, **51** (2), 115-121. doi: 10.12834/VetIt.242.812.4
Accepted: 18.02.2015 | Available on line: 30.06.2015

Keywords
Fowl cholera, Haemorrhagic septicaemia, Loop-mediated Isothermal amplification (LAMP), *Pasteurella multocida,* Polymerase Chain Reaction.

Summary
Twenty two isolates of *Pasteurella multocida* were obtained from different tissues of dead birds and animals (cattle, buffalo, sheep, and goat) suspected of fowl cholera and haemorrhagic septicaemia. The isolates were confirmed as *P. multocida* by various biochemical tests and PM PCR. An attempt was made to standardize Loop mediated isothermal amplification (LAMP) using newly designed primer sequences of KMT1 gene. Loop mediated isothermal amplification was conducted using 6 sets of primers at 65°C for 30 minutes and the result was confirmed by visual observation using SYBR green fluorescence dye as marker of positive reaction under UV transilluminator. On electrophoretic analysis of the products on 2% agarose gel, a ladder like pattern was observed, which suggested a positive amplification, whereas no amplification was observed in negative controls. Additionally, product of positive reaction yielded a green fluorescence following addition of SYBR green under UV transilluminator. It was observed that LAMP is a more sensitive test than polymerase chain reaction (PCR), as the former could detect DNA to lower limit of 22.8 pg/µl, while the latter could detect DNA to lower limit of 2.28 ng/µl, thus LAMP could detect 100 times lesser concentration of DNA in comparison to PCR. Loop mediated isothermal amplification is a rather newer molecular technique, which can be used for rapid detection of infectious agent at field level and which does not require sophisticated instrument, *i.e.* thermal cycler. Furthermore, unlike the conventional PCR technique, LAMP requires lesser time to perform and result can be read visually.

Rilevamento di Pasteurella multocida *mediante amplificazione isotermica mediata da loop del DNA in casi di setticemia emorragica e colera aviare*

Parole chiave
Amplificazione isotermica del DNA mediata da loop, Colera aviare, *Pasteurella multocida*, PCR (Reazione a catena della polimerasi), Setticemia emorragica.

Riassunto
Ventidue ceppi di *Pasteurella multocida* ottenuti da tessuti e organi di uccelli deceduti per sospetto colera aviare e da animali (bovini, bufali, pecore e capre) morti per sospetta setticemia emorragica sono stati utilizzati in questo studio. I ceppi sono stati identificati come *P. multocida* mediante l'utilizzo di test biochimici e di una PCR specifica. Questo studio ha avuto lo scopo di sviluppare e standardizzare la tecnica di amplificazione isotermica del DNA mediata da loop [*Loop-Mediated Isothermal Amplification* (LAMP)] per l'amplificazione del gene KMT1 di *P. multocida* utilizzando nuovi primers. L'amplificazione LAMP è stata condotta utilizzando un set di 6 primers mantenuti a 65 °C per 30 minuti mentre la visualizzazione dei risultati è avvenuta attraverso il transilluminatore UV aggiungendo il colorante SYBR green per colorare le reazioni positive. Inoltre l'analisi elettroforetica su gel d'agarosio al 2% degli amplificati LAMP ha rilevato il classico profilo multibanda per i campioni positivi, mentre nessuna banda di amplificazione è stata osservata nei controlli negativi. Lo studio ha rilevato che il LAMP test è più sensibile della PCR, infatti il test permette di rilevare il DNA fino al limite minimo di 22,8 pg/µl, mentre la PCR permette di rilevare il DNA fino al limite di 2,28 ng/µl. Si può verosimilmente concludere che il LAMP test permette di rilevare concentrazioni di DNA fino a 100 volte minori rispetto alla PCR. Il LAMP è una nuova metodica molecolare che può essere utilizzata per il rilevamento rapido di agenti infettivi in campo perché non necessita di strumenti sofisticati. Inoltre, rispetto alla PCR, la tecnica LAMP ha tempi di esecuzione più brevi ed è possibile rilevare la reazione positiva ad occhio nudo.

Introduction

Pasteurella multocida is a heterogeneous species of gram-negative bacteria and it has been recognized as an important veterinary pathogen for over a century. The organism can occur as a commensal in the naso-pharyngeal region of apparently healthy host and it can be either a primary or secondary pathogen in the disease processes. Five capsular serotypes are routinely identified in *P. multocida* (A, B, D, E, and F) and each is generally associated with, but not completely restricted to, a specific host (Harper *et al.* 2006). *Pasteurella multocida* causes a number of diseases in various domestic and wild animals. The most important diseases are haemorrhagic septicaemia (HS) and septicemic pasteurellosis affecting sheep and goat; pneumonia, atrophic rhinitis, and septicaemia affecting pig and fowl; cholera or avian cholera in poultry/turkey. The organism is also known to be the causative agent for snuffles in rabbit and pasteurellosis in American bison, yak, deer, elephant, camel, horse, elk, and other wild animals. Fowl cholera is of significant economic importance. It is caused by infection with *Pasteurella multocida*, is a disease of many avian species. The majority of fowl cholera strains belong to serotype A (Christensen and Bisgaard 1997) and mostly affect domestic avian species, like chicken, turkey, duck, quail, and emu. The definitive diagnosis of *P. multocida* in field condition is difficult to achieve and in most of the cases it results in faulty diagnosis. Accurate and early diagnosis is the most effective tool to frame the strategy for control of the disease. Conventional diagnostic system is not effective, since it is time consuming and less sensitive as compared to molecular techniques including Loop-Mediated Isothermal Amplification (LAMP).

LAMP is rather recently developed and adapted technique based on nucleic acid amplification method which is rapid, simple, and easy to perform compared to other nucleic acid based detection techniques, *i.e.* polymerase chain reaction (PCR). In LAMP amplification of target gene takes place under isothermal condition and in presence of *Bst* DNA polymerase enzyme with 4 specific set of primers. However, Nagamine and colleagues (Nagamine *et al.* 2002) stated that the addition of 2 loop primers helps in decreasing the reaction time and also increases the specificity of the test. For these reasons, LAMP is preferred for early and specific detection of various infectious diseases in human as well as in animals. Compared to PCR, LAMP has higher specificity and amplification efficiency, hence has been employed for detection of various pathogenic organisms like protozoa, virus, fungi, and bacteria, like *Leishmania infantum* (Chaouch *et al.* 2013), Yellow fever virus (Kwallah *et al.* 2013), *Fusarium graminearum* (Niessen 2013), and *Salmonella* (Wang *et al.* 2008). In the present study we propose LAMP assay for the early and rapid detection of *P. multocida*.

Materials and methods

Bacterial strains

A total of 22 isolates of *P. multocida* earlier isolated from emu, cattle, buffalo, poultry, sheep, goat and maintained at the Department of Veterinary Microbiology, Veterinary College, Anand, Gujarat, India, were used in this study. Isolates were confirmed to be *P. multocida* by using various biochemical tests as well as PCR, conducted using species specific primers (Townsend *et al.* 1998). Three known strain of *P. multocida* capsular type A (PAP-11-88/2013), *P. multocida* capsular type D (PAS-8-85/2013), and P_{52} strain of *P. multocida* (Capsular type B) were also used in this study as positive control. *Escherichia coli* (ATCC-25922), *Proteus mirabilis* (ATCC-12453), and *Klebsiella pneumoniae* (ATCC-13883) were also used as negative control to assess the efficacy of the LAMP primer. All the strains used were grown on 5% defibrinated sheep blood agar or Brain hearth infusion broth (BHI) at 37°C.

DNA preparations

Bacterial DNA for PCR was extracted by boiling procedure. Isolates were cultured overnight in BHI

Table I. *Primers sequences used in this study to detect* P. multocida.

Test	Primer Name	Oligonucleotide sequence	Reference
PCR	KMT1-forward	GCTGTAAACGAACTCGCCAC	Townsend and colleagues, (Townsend *et al.* 1998)
	KMT1-reverse	ATCCGCTATTTACCCAGTGG	Townsend and colleagues (Townsend *et al.* 1998)
LAMP	F3	GGTGCCTATCTTGCTTCG	This study
	B3	TCGATCGCTAGCACCACA	This study
	FIP (F1c+F2)	TGCCGTAGCAGAAACTGGAC + AGGTGAGCCATGTGAGCT	This study
	BIP(B2+B1c)	CACACCGAAGCCAGGACTT + CACCAAATGTACTGGTTGCTTC	This study
	Loop F	CGACCATCGGTTGCATTTC	This study
	Loop B	TGGCATTGCATGGCTATCA	This study

Figure 1. *Primer design for LAMP to detect* Pasteurella multocida *DNA.* Nucleotide sequence of Kmt1 gene (GenBank accession no. AFF24839), used to design LAMP primers. Underlining indicates the positions of targeting sequences.

broth at 37°C and 2 ml of the culture suspension was centrifuged and the pellet was resuspended in 200 µl of doubled distilled water (ddH_2O), which was boiled for 10 minutes in a water bath and then stored at -20°C for 10 minutes. After thawing, the suspension was once again centrifuged at 8,000 rpm for 10 minutes and 200 µl of the supernatant was taken as template DNA. The concentration of DNA was measured spectrophotometrically (Nanodrop 1000, Thermo scientific, Wilmington, Delaware, USA) at 260 nm and the same DNA template was used to carry out both LAMP and PCR.

DNA oligonucleotides

In order to obtain a specific primers for the LAMP, previously identified conserved gene (KMT1) was targeted (Townsend *et al.* 1998). Six primers for LAMP were designed based on the KMT1 gene sequence (EMBL accession no. AFF24839) according to the criteria described previously (Notomi *et al.* 2000, Tomita *et al.* 2008), including 2 outer primers (F3 and B3), 1 forward inner primer (FIP), 1 backward inner primer (BIP), and 2 loop primers (Loop F and Loop B) by using LAMP Designer (Optigene, Horsham, UK) (Table I) (Figure 1). These primers

recognize 6 distinct regions on the target DNA. For the PCR, we used primers designed by Townsend and colleagues (Townsend *et al.* 1998).

Pasteurella multocida specific PCR (PM-PCR)

Pasteurella multocida strains were subjected to PM-PCR to detect the species KMT gene (Townsend *et al.* 1998). For PCR reactions 3 µl (~228 ng/µl) template DNA was added to the reaction mixture (22 µl) containing 1 µl of each primer pair (MWG-Biotech, Banglore, INDIA) in a 10 pmol primary concentration, 12.5 µl of 2x PCR Master mix (Fermentas, Thermo Fisher Scientific, Carlsbad, California, USA) and 7.5 µl of molecular grade nuclease free water. The samples were subjected to 30 cycles of amplification in a thermal cycler as per the protocol described by Townsend and colleagues (Townsend *et al.* 1998) (Veriti, Applied Biosystem, Foster City, California, USA). DNA-sequences of oligonucleotide primers and reference sequences are listed in Table I.

LAMP reaction

The LAMP reaction was carried out with 25 µL of mixture containing 5 pmol of each primer F3 and B3; 20 pmol of each primer FIP and BIP; 10 pmol of each primer Loop F and Loop B, 1x LAMP ISO-001 master mix (Optigene, Horsham, UK), and 3 µl of DNA template (~228 ng/µl). The reactions were carried out in a thermocycler at 65°C for 30 minutes and the reaction was terminated by increasing the temperature to 80°C for 5 minutes. A positive control (purified DNA of *P. multocida*) and a negative control (distilled water) were included in each run.

Detection and analysis of visual LAMP and PCR products

Amplified product of LAMP was directly visualized under the day light or UV light following addition of 1x SYBR green nucleic acid stain (Life science, Carlsbad, California, USA). To check the presence of the LAMP products, the amplified products were subjected to gel electrophoresis in 2% agarose gel along with 100 bp DNA Ladder (GeneRuler-Fermentas, Thermo Fisher Scientific, Carlsbad, California, USA) and stained with ethidium bromide (1 % solution at the rate of 5 µl/100 ml).

The PCR products were separated by electrophoresis in 2% agarose gel at 5 V/cm for 1 hour and stained with ethidium bromide (1 % solution at the rate of 5 µl/100 ml). View was captured under gel documentation system (Genetix Biotech Asia, Delhi, INDIA).

Comparison of specificity and sensitivity between LAMP and PCR

Specificity and sensitivity of LAMP and PCR were determined by using genomic DNA of *P. multocida*, which was used to conduct earlier PCR and LAMP as previously described. The results were compared with those of conventional PCR with the same template DNA at identical concentrations. To assess the species specificity, *P. multocida* capsular type A, *P. multocida* capsular type D, *P. multocida* (P_{52}-capsular type B), *Escherichia coli, Proteus mirabilis*, and *Klebsiella pneumoniae* were used. To evaluate the sensitivity of the LAMP and PCR, 10-fold serial dilutions were made using 228 ng/µl purified DNA of *P. multocida* (P_{52} strain) and DNA concentration was measured spectrophotometrically (Nanodrop 1000, Thermo scientific, Wilmington, Delaware, USA) at 260 nm. The final tube showing positive reaction was considered as detection limit and the reactions were carried out in triplicate.

Results

Detection by LAMP Assay

The result of the LAMP reaction could be observed by naked eye under daylight or UV light. Under daylight, the positive reaction (with target DNA template) turned green; whereas the negative one (without target DNA template) remained orange. Under UV light, the positive reaction emitted strong green fluorescence while the negative reaction did not emit any fluorescence (Figure 2). On electrophoresis, amplicons of positive reaction

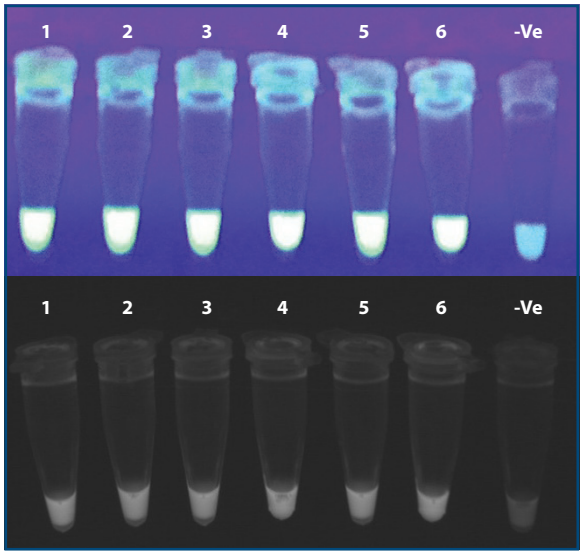

Figure 2. *Positive reaction of LAMP gives fluorescence after addition of SYBR green dye under UV light.* Tube 1-6: positive sample; -Ve: negative control.

Veterinaria Italiana 2015, **51** (2), 115-121. doi: 10.12834/VetIt.242.812.4

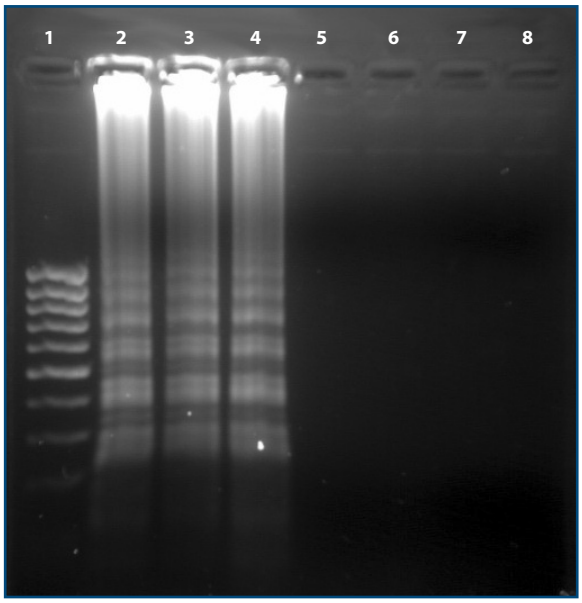

Figure 3. *Specificity of LAMP.* Lane 1: ladder; Lane 2: *P. multocida* type A; Lane 3: *P. multocida* type B; Lane 4: *P. multocida* type D; Lane 5: *E. coli*; Lane 6: *P. mirabilis*; Lane 7: *K. pneumoniae*; Lane 8: negative control (water).

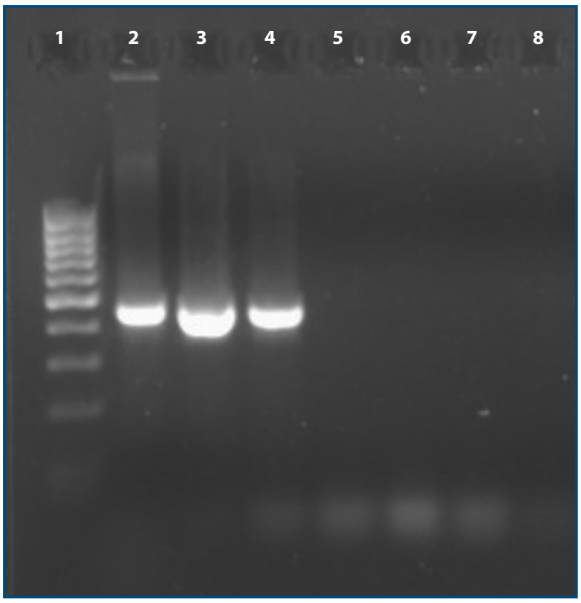

Figure 4. *Specificity of PCR.* Lane 1: ladder; Lane 2: *P. multocida* type A; Lane 3: *P. multocida* type B; Lane 4: *P. multocida* type D; Lane 5: *E. coli*; Lane 6: *P. mirabilis*; Lane 7: *K. pneumoniae*; Lane 8: negative control (water).

gave typical ladder like pattern, which were the same for all the *P. multocida* 22 isolates, as well as for the known *P. multocida* isolates, while no ladder like pattern of amplification was observed in lanes containing genomic DNA of other isolates (negative control) (Figure 3).

Detection by Pm-PCR

On electrophoresis of PCR products, it was observed that all the 22 isolate of *P. multocida*, including the known *P. multocida* isolates had generated a single 460 kb band, while other isolates failed to generate any amplified product (Figure 4).

Specificity and sensitivity of LAMP and PCR

The visual observation of LAMP reaction, as described earlier, could specifically detect presence of amplified products obtained from all the 22 *P. multocida* field isolates as well as *P. multocida* type A, *P. multocida* type D, and *P. multocida* (P_{52}- Capsular type B). While no such observation could be made for other bacterial strains and negative (no template) reaction control (Table IIA, IIB and Figure 3).

PCR results based on the observation following electrophoresis revealed similar findings by specific detection of genomic DNA of *P. multocida* isolates including known strains. It did not reveal any specific products for other bacterial strain (Table IIA, IIB and Figure 4).

On the basis of electrophoretic analysis, in assessing the comparative sensitivity for both the assay, we concluded that the detection limit of the LAMP assay was 68.4 pg/tube (containing 3 µl of DNA template) or 22.8 pg/µl of genomic DNA for 30 minutes of reaction time, while agarose gel electrophoresis of PCR products showed that the detection limit of PCR assay for the same was 6.84 ng/tube (containing 3 µl of DNA template) or 2.28 ng/µl of genomic DNA (Table III and Figure 5 and Figure 6). Thus LAMP assay was found to be more sensitive than PCR in this study.

Discussion

Pasteurella multocida is a prevalent organism responsible for causing haemorrhagic septicaemia and fowl cholera in ruminant and bird, respectively. Moreover, it causes atrophic rhinitis in pig, and snuffles in rabbit. Haemorrhagic septicaemia usually occurs as very acute disease in ruminants. It is often fatal if specific treatment is not given in primary phase. Hence efficient, rapid detection of *P. multocida* is necessary for prevention of outbreak and specific therapy for the disease. In HS very rapid death occurs, thus there is a need to develop a novel technique for rapid detection. In tropical countries like India, Pasteurellosis is a major cause of mortality in ruminant and result in high economic losses. In present study, we succeeded in designing specific sets of primer for rapid detection of *P. multocida* isolated from various animal hosts. In this assay, we targeted the conserved gene of *P. multocida*, which was earlier

reported by Townsend and colleagues (Townsend *et al.* 1998). The LAMP assay successfully detected all the isolates of *P. multocida* strains and gave negative reaction in all other bacterial species, which was in good agreement with the results of PCR assay. The specificity of the LAMP is high because it employs 6 specially designed primers recognizing 6 regions on the KMT1 gene sequence, which is specific to *P. multocida*. The sensitivity of the LAMP was found to be higher as it could detect to a lower limit of 22.8 pg/µl of genomic DNA, which is 100-fold more sensitive than that of the conventional PCR. The result is in agreement with previous results of Sun and colleagues (Sun *et al.* 2010) who reported that LAMP could detect 10 cfu/ml more than conventional PCR.

Table II. A. *Specificity of LAMP and PCR for the detection of* P. multocida. **B.** *Detail results of LAMP and PCR for the field isolates of* P. multocida.

A

Name of the isolates	Result of LAMP	Result of PCR
P. multocida field isolates (22) (Table IIA)	+	+
P. multocida (capsular type A)	+	+
P. multocida P_{52} strain (capsular type B)	+	+
P. multocida (capsular type D)	+	+
E. coli	-	-
Proteus mirabilis	-	-
Klebsiella pneumoniae	-	-
Negative Control (Water)	-	-

B

Sr. no	Isolate No.	Host Species	Capsular type	Result of LAMP	Result of PCR
1	PAB-1	Buffalo	B	+	+
2	PAB-2	Buffalo	B	+	+
3	PAB-3	Buffalo	B	+	+
4	PAB-4	Buffalo	B	+	+
5	PAB-5	Buffalo	B	+	+
6	PAB-6	Buffalo	B	+	+
7	PAB-7	Buffalo	B	+	+
8	PAB-9	Buffalo	B	+	+
9	PAB-10	Buffalo	B	+	+
10	PAB-11	Buffalo	B	+	+
11	PAB-12	Buffalo	B	+	+
12	PAB-13	Buffalo	B	+	+
13	PAB-14	Buffalo	B	+	+
14	PAC-1	Cattle	B	+	+
15	PAC-2	Cattle	B	+	+
16	PAC-3	Cattle	B	+	+
17	PAS-1	Sheep	D	+	+
18	PAG-1	Goat	F	+	+
19	PAP-1	Poultry	A	+	+
20	PAP-2	Poultry	A	+	+
21	PAE-1	Emu	A	+	+
22	PAE-2	Emu	A	+	+

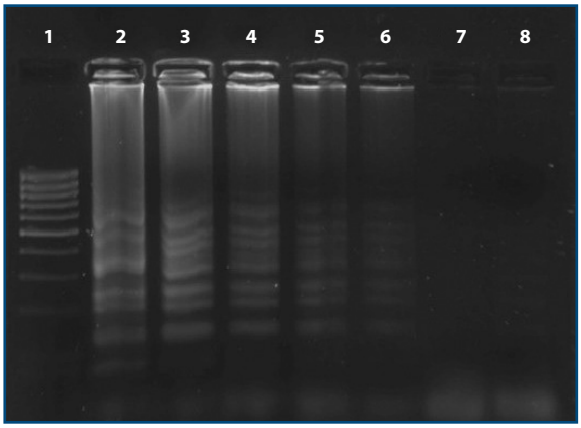

Figure 5. *Sensitivity of the LAMP to detect* P. multocida. Lane 1: ladder; Lane 2: 228 ng/µl; Lane 3: 22.8 ng/µl; Lane 4: 2.28 ng/µl; Lane 5: 228 pg/µl; Lane 6: 22.8 pg/µl; Lane 7: 2.28 pg/µl; Lane 8: 228 fg/µl.

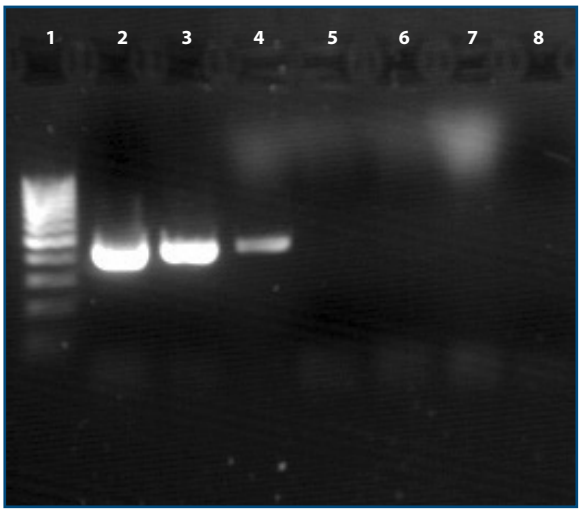

Figure 6. *Sensitivity of the PCR to detect* P. multocida. Lane 1: ladder; Lane 2: 228 ng/µl; Lane 3: 22.8 ng/µl; Lane 4: 2.28 ng/µl; Lane 5: 228 pg/µl; Lane 6: 22.8 pg/µl; Lane 7: 2.28 pg/µl; Lane 8: 228 fg/µl.

Table III. *Comparison between the sensitivity of LAMP and PCR.*

Isolate	Test	Concentration of Genomic DNA after dilution (per 3µl of genomic DNA template)						
		684 ng	68.4 ng	6.84 ng	684 pg	68.4 pg	6.84 pg	684 fg
P. multocida P_{52}-Strain	LAMP	+	+	+	+	+	-	-
	PCR	+	+	+	-	-	-	-

Loop mediated isothermal amplification is highly sensitive test, thus it is recommended that post-amplification operation should be done in separate room away from the reaction and reagent that are being used for the PCR and LAMP to reduce the chance of contamination (Zhu *et al.* 2009). This test requires only regular water bath or heating block that maintains temperature at 65°C, making LAMP assay more economical compared to conventional PCR. Moreover, addition of 2 extra loop primers increases speed (Nagamine *et al.* 2002) and specificity of amplification, which reduces the overall time and makes the LAMP faster than conventional PCR. It takes almost 3 hours to detect the *P. multocida* using PCR; while in present study we have detected the *P. multocida* in approximately 30 minutes only. Commercially available LAMP master mixes decrease the amplification speed as they contain some advanced *Bst* polymerase. The reaction time reported in this study (approximately 30 minutes) was significantly faster than the one reported by Sun and colleagues (Sun *et al.* 2010), which required 1 hour to complete the assay. An other important feature of LAMP assay is that positive amplification can be detected by naked eye, it does not require electrophoretic assessment, which is by contrast a requisite for conventional PCR. Other methods available for visual detection of LAMP reaction include addition of calcein and manganese ion to the reaction and results could be interpreted just by observing the change in colour of reaction mixture; or addition of SYBR green dye which emanates fluorescence in positive reaction under UV light. Also these techniques eliminate the need for the time-consuming electrophoresis and requirement of other sophisticated instruments. To conclude, because of its simplicity, specificity, sensitivity, and being economic, LAMP is more efficient than conventional PCR for the detection of *P. multocida* infection at field level and this technique can be used during the outbreak of haemorrhagic septicaemia and fowl cholera to ensure rapid diagnosis.

References

Chaouch M., Mhadhbi M., Adams E.R., Schoone G.J., Limam S., Gharbi Z., Darghouth M.A., Guizani I. & BenAbderrazak S. 2013. Development and evaluation of a loop-mediated isothermal amplification assay for rapid detection of *Leishmania infantum* in canine leishmaniasis based on cysteine protease B genes. *Vet Parasitol*, **198**, 78-84.

Christensen J.P. & Bisgaard M. 1997. Avian pasteurellosis: taxonomy of the organisms involved and aspects of pathogenesis. *Avian Pathol*, **26**, 461-483.

Harper M., Boyce J.D. & Adler B. 2006. *Pasteurella multocida* pathogenesis: 125 years after Pasteur. *FEMS Microbiol Let*, **265**, 1-10.

Kwallah A., Inoue S., Muigai A.W., Kubo T., Sang R., Morita K. & Mwau M. 2013. A real-time reverse transcription loop-mediated isothermal amplification assay for the rapid detection of yellow fever virus. *J Virol Methods*, **193**, 23-27.

Nagamine K., Hase T. & Notomi T. 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Mol Cell Probes*, **16**, 223-229.

Niessen L. 2013. Detection of *Fusarium graminearum* DNA using a loop-mediated isothermal amplification (LAMP) assay. *Methods Mol Biol*, **968**, 177-193.

Notomi T., Okayama H, Masubuchi H., Yonekawa T., Watanabe K., Amino N. & Hase T. 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res*, **28**, e63.

Sun D., Wang J., Wu R., Wang C., He X., Zheng J. & Yang H. 2010. Development of a novel LAMP diagnostic method for visible detection of swine *Pasteurella multocida*. *Vet Res Commun*, **34**, 649-657.

Tomita N., Mori Y., Kanda H. & Notomi T. 2008. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nat Protoc*, **3**, 877-882.

Townsend K.M., Frost A.J., Lee C.W., Papadimitriou J.M. & Dawkins H.J. 1998. Development of PCR Assays for species and type specific identification of *Pasteurella multocida* isolates. *J Clin Microbiol*, **36**, 1096-1100.

Wang L., Shi L., Alam M.J., Geng Y. & Li L. 2008. Specific and rapid detection of food borne *Salmonella* by loop-mediated isothermal amplification method. *Food Res Int*, **41**, 69-74.

Zhu R.Y., Zhang K.X., Zhao M.Q., Liu Y.H., Xu Y.Y., Ju C.M., Li B. & Chen J.D. 2009. Use of visual loop-mediated isotheral amplification of rimM sequence for rapid detection of *Mycobacterium tuberculosis* and *Mycobacterium bovis*. *J Microbiol Methods*, **78**, 339-343.