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Current loop-mediated isothermal amplification (LAMP) technologies for the detection of poultry pathogens

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Traditional diagnostic capabilities (serology and culture) are not enough to monitor the poultry infections efficiently. For effective control of poultry infections, a regular program incorporating simple and cost-effective molecular diagnostics is necessary. On this rationale, it is possible to present working molecular diagnostic technology that would work equally well in field as well as in the laboratory. Recently, the loop-mediated isothermal amplification (LAMP) assay has emerged as simple and inexpensive diagnostic tool for the molecular detection of various animal pathogens. To perform LAMP, no specialised instruments (*e.g.* thermal cycler) are required, permitting its use in developing countries. Various reliable LAMP assays have been reported for the detection of different poultry pathogens. However, still there is a need to improve the sensitivity, specificity, reproducibility, user-friendliness, delivery to end-user and affordability of LAMP assays. This article reviews current LAMP assays available for the molecular detection of important viral, bacterial and protozoan pathogens of poultry. It focuses on the various aspects of LAMP for the diagnosis of important poultry pathogens based upon pathogen type, specimen, target genes, LAMP primer types, detection limits, fluorescent detectors and LAMP chemistry used. This paper provides updates on principle, instrumentation, basic methodology, quantification capability, reagents and kits used currently in performing LAMP.

Keywords: LAMP; diseases; poultry; molecular diagnostics

Introduction

In developing countries, 30-50% of potential production (quality and quantity) is lost because of the poor disease control (OIE, 2007; BBSRC, 2013). Molecular diagnostics are not commonly performed in developing countries because of the limited availability of sophisticated laboratory equipment to carry out these tests. This diagnosis should be

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adopted in small poultry laboratories, especially for fastidious micro-organisms which have prioritised detection using modern molecular techniques. Conventional diagnostic tools (serology and culture) are insufficient to effectively monitor poultry infections. Many pathogens are fastidious in nature, so it is difficult to culture them. Some need longer incubation to grow ranging from days to weeks, whereas, the presence of non-specific reactors hinders the serological diagnosis. Molecular methods such as polymerase chain reaction (PCR), multiplex PCR, and real-time PCR have been used to detect different infectious diseases in chickens (Sprygin *et al.*, 2010). Although, PCR assays are rapid and precise, they require agarose gel electrophoresis which increases the risk of false positive results due to amplicon carry-over. The conventional assay is an end-point PCR reaction. Real-time PCR involves less handling of the products as compared to the conventional assay; however, the use of a costly thermal cycler with a fluorescence detector limits the effectiveness of this technique in poorly funded laboratories (Saito *et al.*, 2005). PCR methods are further limited by doubling-by-cycle amplification and most of the time cannot be used for detection of DNA or RNA directly from a variety of crude sample preparations. PCR methods are sensitive to sample matrix inhibitors and are not amenable to visual detection of amplified product, which reduces their use for pathogen detection in field settings (Takahashi *et al.*, 2014).

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification technology, recently developed and reported by Notomi *et al.* (2000). It offers simple, one step, closed-tube detection of amplification (μg yields in <15 minutes) which is compatible with multiple detection methods. The amplification is carried out under isothermal condition usually (60-65°C), in presence of *Bst* I or *Bsm* DNA polymerase which exhibits high strand displacement activity. LAMP does not require additional reverse transcription step for amplification of RNA viral gene products, and can amplify DNA with high specificity, sensitivity, and speed (Notomi *et al.*, 2000).

Various reliable LAMP assays have been reported for detection of poultry diseases including avian influenza, Newcastle disease, infectious bronchitis, infectious bursal disease, hydropericardium syndrome (HPS), Marek's disease, chicken infectious anaemia, avian mycoplasmosis, fowl cholera, and avian coccidiosis (*Eimeria spp*.). Currently, there is only one published review describing LAMP assays developed for the detection of viral infections in poultry (Mansour *et al.*, 2015). The following review aims to cover the developed LAMP assays reported to date, for the detection of different types of important pathogens infecting poultry.

The principle and major advantages of LAMP

The LAMP assay incorporates a set of four to six primers recognising six to eight sites on a conserved target gene. The assay works on the principle of auto cycling strand displacement DNA synthesis carried out by *Bst* or *Bsm* DNA polymerase. The primers can easily be generated from conserved DNA sequences of poultry pathogens. Primers may be designed by selecting the parameters defaults of LAMP primer design software named 'Primer Explorer' V4 or V5 http://primerexplorer.jp/elamp4.0.0/index.html developed by the Eiken Chemical Co., Ltd, Tokyo, Japan. The two outer primers consist of a forward outer primer (F3) and backward outer primer (B3). The inner primers are named as the forward inner primer (FIP) and backward inner primer (BIP), as described by Notomi *et al.* (2000). Loop primers (LP) are optional primers, and, if incorporated in to LAMP reaction, may further increase specificity and reduce the time of amplification (Barkway *et al.*, 2011).

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LAMP reagents, reaction mixture and kits

The LAMP reaction mixture includes the addition of four to six sets of primers, *Bst* or *Bsm* DNA polymerase large fragment, dNTPs, reaction buffer and template DNA. The reaction mixture can be incubated in a water bath or heat block. Amplification is performed at 60-65°C for 30-60 min followed by heating at 80°C for 5 min to terminate the reaction (Peng *et al.*, 2011). The whole amplification (10^9-10^{10} copies) is completed within 30 to 60 min at 63°C, isothermally. Because of the high number of DNA copies, the amplified product can be detected by visualising turbidity or fluorescence detection, thereby limiting analysis on gel.

The reagents used in LAMP assay are comparatively thermo-stable. Dried reagents fixed on the lids can save steps for preparing the reaction mixture. Such reagents are stable at room temperature for at least one year (no need to be stored in the refrigerator) as reported by Mair *et al.* (2013). The lyophilised reaction mixture (LAMP amplification kit) is commercially available as customised products from Thermo Fisher Scientific, Lithuania to be stored at 2-8°C in the dark. This lyophilised reaction mixture uses FAM™ fluorescent dye as indicator to be used in a portable equipment ESEQuant tube scanner (Qiagen) or any real-time PCR detection system. Similarly, a new WarmStart® Colorimetric LAMP 2X Master Mix, (New England Bio Labs Ltd. UK) utilises pH sensitive phenol red, as an end-point indicator. This system is designed for visual detection of amplification based on production of proton from the extensive *Bst* 2.0 WarmStart® DNA polymerase activity or WarmStart® RTx Reverse Transcriptase activity, producing a change in solution colour from pink to yellow. A novel commercial WarmStart® LAMP Kit (DNA & RNA), NEB, UK contains a blend of *Bst* 2.0 WarmStart® DNA polymerase and WarmStart® RTx Reverse Transcriptase. This WarmStart LAMP Kit is compatible with multiple detection methods, including fluorescence and or turbidity (as the end-point visualisation).

Quantitative capability of the LAMP

The LAMP method is an efficient method which allows synthesis of large amount of DNA in a short time with great specificity. As the reaction progress it produces precipitates of magnesium pyrophosphate which can be detected by spectroscopic or enzymatic analysis. The loopamp real-time turbidimeter (LA-320, Teramecs, Co., Ltd., Kyoto, Japan) and LA-500, (Eiken Chemical Co., Ltd.) are specifically designed instruments for real-time monitoring of the LAMP reaction. The reaction is considered positive when the turbidity reaches 0.1 within 60 min at 650 nm. The time needed for the turbidity of each tested sample to exceed at 0.1 is referred to as the 'threshold time' (T_T). In other words, the turbidity-versus-time curve obtained using the real-time turbidimeter represents the reaction curve of the LAMP amplification (Mori *et al.*, 2004). Such quantitative LAMP (qlamp) assays have been successfully applied for the quantitative detection of *Campylobacter jejuni* and *C. coli* in chicken carcass and water rinses using a real-time turbidimeter (Sabike *et al.*, 2016; Yamazaki *et al.*, 2009).

Another assay is the PMA-LAMP (propidium monoazide treatment) assay as reported by Youn *et al.* (2017). The PMA-LAMP assay is a rapid, accurate, and simple method for quantifying viable *Salmonella spp.* without pre-enrichment in raw samples, such as rinse water of chicken carcasses (*e.g.* treated with sanitisers). The LAMP reaction was performed with a Genie II (Optigene, UK) real-time fluorometer, which displays time to positivity and amplicon melting temperature (Tm). Youn *et al.* (2017) has reported a high correlation (R^2 = 0.99 to 0.976) between LAMP time threshold (T_T) values and

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viable *Salmonella spp.* with a quantification range of 1.0×10^3 to 1.0×10^8 CFU/ml in pure culture and rinse water of chicken carcasses.

LAMP assays available for detection of economically important chicken pathogens

APPLICATION OF LAMP FOR DETECTION OF AVIAN VIRUSES

Recently, various workers have reported the development of LAMP reactions for different poultry viral pathogens. Pham *et al.* (2005) detected the fusion protein gene of Newcastle disease (ND) virus from tracheal and lung specimens, and its improved version has been demonstrated by Li *et al.* (2009). Kirunda *et al.* (2012) detected the ND fusion protein gene from less invasive samples, such as oropharyngeal and cloacal swabs, and the assay incorporated loop primers (LP). These are added to increase specificity and to reduce the time of incubation for LAMP reaction by up to several minutes. Peng *et al.* (2011) detected avian influenza (AI) virus, hemagglutinin (HA) gene from cloacal swab samples using calcein and $MnCl_2$ as an end-point indicator for turbidity and colour change in positive samples. Later work by Ge *et al.* (2013) detected avian origin influenza virus from tracheal swabs on a lateral flow dipstick (LFD) format to provide ease in visual identification of reaction in field conditions. Helal *et al.* (2017) reported duplex LAMP for the detection of subtypes H6 and H9 of AI, and Hu *et al.* (2017) used droplet digital LAMP (dLAMP) for the detection of H5N1. In the dLAMP assay, emulsification was carried out using cross-interface emulsification (XiE) platform, which generates monodisperse droplets using a vibrating capillary, which can be easily set up and is user friendly to those who lack microfabrication facilities. Xie *et al.* (2011) detected the hexon gene of Group I avian adenovirus from a cloacal swab sample using a commercial fluorescent detection reagent (FDR; Eiken Chemical Co., Ltd, Tokyo, Japan). In addition, Xie *et al.* (2010) developed a LAMP assay incorporating LP for detection of infectious laryngotracheitis (ILT) virus by amplifying its *thymidine kinase* (*TK*) gene. Its visual detection was made by adding SYBR Green I dye. Reverse transcriptase LAMP (RT-LAMP) for avian reo virus (*S1* gene) was developed by the Xie *et al.* (2012) incorporating calcein and $MnCl_2$ to aid in visualisation of amplified product in the reaction tube.

Marek's disease virus (*meq* gene sequence) has been detected by Wei *et al.* (2012) from feather follicles using LP. Other LAMP assays for Marek's disease virus were reported by Wozniakowski *et al.* (2011) and Angamuthu *et al.* (2012) to specifically amplify the *meq* oncogene and its detection through SYBR green stain. Chen *et al.* (2010) established a RT-LAMP assay for the detection of infectious bronchitis virus (IBV) based on the nucleocapsid phosphoprotein gene of IBV. A LAMP assay to detect chicken anaemia virus (CAV) targeting the VP2 gene of CAV was developed by (Huang *et al.*, 2010). Additionally, various RT-LAMP assays have been reported for the detection of infectious bursal disease (IBD) of chicken and the key features of all LAMP assays developed so far, for the detection of important viral pathogens of chickens are detailed in *Table 1*.

APPLICATION OF LAMP FOR DETECTION OF BACTERIAL PATHOGENS OF POULTRY

In human medicine, there is a LAMP kit available for the field diagnosis of bacterial infection caused by *Mycobacterium tuberculosis* (WHO, 2013). However, the use of the LAMP method for detection of bacterial pathogens of livestock and poultry is limited. To allow its widespread application in veterinary diagnostics, there has to be a recognition and promotion of LAMP technology by the world organisation for animal health (OIE)

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and other regulatory agencies (Mansour *et al.*, 2015). *Salmonella spp*. in poultry were initially detected by LAMP assays from caecal droppings (Okamura *et al.*, 2008; Zhuang *et al.,* 2014; Gong *et al.,* 2016; Youn *et al.*, 2017) (*Table 2)*. For other pathogens, *Pasteurella multocida* from chickens was detected through LAMP by Bhimani *et al.* (2015) using SYBR green stain as an end-point indicator for visual detection of the amplification. *Campylobacter jejuni* (oxidoreductase gene) and *C. coli* (*gufA* gene) have been detected in form of real-time turbidity in the LAMP reaction from tissue, meat and faecal samples of chicken. Among other bacterial pathogens, avian mycoplasmas cause dramatic disease problems (chronic respiratory disease) with economical losses. Although mycoplasma are economically significant in poultry, only a limited number of LAMP assays have been reported for the detection of *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS), as shown in *Table 2*. A rapid conventional LAMP assay can detect MG within 30 min at 60°C. This assay targets the highly conserved *pdhA* gene of MG, resulting with 10-fold higher sensitivity as compared to PCR (Zhang *et al.,* 2015). Recently, Ehtisham-ul-Haque *et al.* (2017) reported a real-time LAMP assay for the detection of cytadhesin (*mgc2*) gene of MG using an ESEQuant tube scanner (Qiagen). For the detection of MS, Kursa *et al.* (2014) developed a sensitive LAMP assay targeting variable haemagglutinin (*vlhA*) gene of MS.

APPLICATION OF LAMP FOR DETECTION OF PROTOZOAN PARASITES OF POULTRY

LAMP has been widely used in detecting infections caused by protozoan parasites such as *Toxoplasma gondii*, *Trypanosoma* spp., *Theileria annulata*, *Neospora caninum*, Babesia caballi, *Leishmania* spp, bovine babesiosis trichinellosis, bovine trichomonosis, malaria and giardiasis as described by Ramos *et al.* (2017) and Xu *et al.* (2014). In human medicine, there has been a major LAMP field success in form of a malaria detection LAMP kit approved by WHO, as reported by Patel *et al.* (2014). Only few LAMP assays have been described for the detection of avian protozoan parasites. Barkway *et al.* (2011) and Barkway *et al.* (2015) have reported the LAMP technique for detection of all seven *Eimeria* spp. in chickens by amplification of their species-specific (SCAR markers and *Em*MIC1 gene) from purified oocysts, intestinal and blood samples using loop primers (*Table 3*). In this assay SafeView nucleic acid stain (NBS Biologicals, Cambridgeshire, UK) and hydroxynaphthol blue were used to visualize the final product. Xu *et al.* (2014) has reported the visual detection of 18SrRNA gene of *Histomonas meleagridis* infection in chicken.

Conclusions

Various LAMP assays have been developed for the molecular diagnosis of important chicken pathogens, based upon pathogen type, specimen, target genes, LAMP primers type, detection limit, fluorescent detectors and LAMP chemistry used. If the LAMP technique is properly applied, may be a useful diagnostic procedure in developing countries, as it does not require expensive equipment. Molecular diagnostics should be adopted in small microbiology laboratories, especially for fastidious pathogens like avian mycoplasma species (MG and MS) which are hard to culture via conventional techniques and are prioritised for detection using molecular techniques. It is recommended that the technique of LAMP should be adopted in a national disease control programme for avian infections in resource-limited countries. LAMP poultry disease diagnostics holds great potential for field and laboratory use. However, for its widespread application in veterinary diagnostics, there should be recognition and

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promotion of the LAMP technology by OIE, other animal health regulatory authorities and national poultry improvement plans (NPIP). This implementation will further pave the ways toward development of simple, cost effective, standardised, accurate and thermostable LAMP kits.

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Table 1 LAMP assays developed for detection of viral pathogens of chickens.

Poultry Pathogen	Target gene	LAMP Assay Format	Specimen	Detection Limit	Fluorescent reagent	Loop primers	Reference
Avian Influenza virus (AIV)	Subtype H9 Haemagglutinin (HA)	RT-LAMP	Oral swabs, and cloacal scrapings	10 copies/reaction	No	No	Chen *et al.* (2008)
	Subtype H3 Hemagglutinin (HA) gene	RT-LAMP	Cloacal swabs	0.1pg total RNA	Calcein and MnCl2	No	Peng *et al.* (2011)
	Subtype H7 Haemagglutinin (HA)	RT-LAMP	Throat and cloacal swabs	0.1-0.01 plaque-forming units PFU/reaction	SYBR Green I	Yes	Bao *et al.* (2012)
	Subtype H7N9 Haemagglutinin (HA) and Neuraminidase (NA) genes	RT-LAMP	Trachea	10 copies of synthetic RNA	Turbidity and lateral flow dipstick (LFD)	Yes	Ge *et al.* (2013)
	Subtype H7N9 Neuraminidase (NA) gene	RT-LAMP	Faecal samples	0.01 plaque-forming units PFU/reaction	Florescent detection reagent	Yes	Bao *et al.* (2014)
	Subtypes H6 and H9	Duplex RT-LAMP	Cloacal and oro-pharyngeal swab	10 copies/ reaction	Calcein and MnCl2	No	Helal *et al.* (2017)
	Subtype H5N1	droplet digital LAMP with cross-interface emulsification (XiE)	culture	10 copies/ reaction	Calcein and MnCl2	YES	Hu *et al.* (2017)
Newcastle disease virus (NDV)	Fusion protein gene	RT-LAMP	Trachea and Lungs	0.5pg or 9 X 10^4 copies/reaction	No	No	Pham *et al.* (2005)
	Fusion Protein	RT-LAMP	Lung, liver, glandular stomach, trachea, brain	1.3 ($2^{0.4}$) HA units	SYBR Green I	Yes	Li *et al.* (2009)
	Fusion protein gene	RT-LAMP	Cloacal, swab and Oro-pharyngeal swab	High relative sensitivity (98-100%)	FDR*	Yes	Kirunda *et al.* (2012)
Chicken Anaemia virus (CAV)	VP2 gene	LAMP	Liver	100fg	SYBR Green I	No	Huang *et al.* (2010)

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Table 1 Continued

Poultry Pathogen	Target gene	LAMP Assay Format	Specimen	Detection Limit	Fluorescent reagent	Loop primers	Reference
Infectious Bronchitis virus (IBV)	Nucleocapsid phosphoprotein gene	RT-LAMP	Kidney, Lung, and Blood	10^1 50% egg infection dose (EID_{50}) /50 µl of titrated viruses	SYBR Green I	No	Chen *et al.* (2010)
	Spike protein S2 gene	RT-LAMP	Kidney, trachea, caecal tonsil, allantoic fluid	10^1 (EID_{50})	Propidium Iodide staining	No	Chandrasekar *et al.* (2015)
Infectious Bursal Disease virus (IBDV)	VP2 gene	RT-LAMP	Bursa	1-10^{-5} dilution of RNA template	No	NK**	Xue *et al.* (2009)
	VP3 gene	RT-LAMP	Bursa, heart, lung, liver, kidney and serum	478fg	Pico Green	No	Xu *et al.* (2009)
	VP5 gene	RT-LAMP	Bursa	28 copies/µl	FDR*	Yes	Wang *et al.* (2011)
	VP2 gene	One step RT-LAMP	Bursa	0.01 fg of IBDV viral RNA	SYBR Green I	No	Lee *et al.*, 2011
	VP2 gene	RT-LAMP	Bursa	10^{-1} plaque-forming units (PFU)	Lateral flow dipstick (LFD)	Yes	Tsai *et al.* (2012)
Group I Avian Adenovirus	Hexon genes	LAMP	Cloacal swab	238 copies	FDR*	Yes	Xie *et al.* (2011)
Infectious laryngotracheitis virus (ILTV)	Thymidine kinase (TK)	LAMP	Trachea	46 copies per reaction	SYBR Green I	Yes	Xie *et al.* (2010)
Avian reo virus (ARV)	*S1* gene segment	RT-LAMP	Isolates propagated in chicken embryo fibroblast cells	10 fg total RNA	Calcein and MnCl2	Yes	Xie *et al.* (2012)

Table 1 Continued

Poultry Pathogen	Target gene	LAMP Assay Format	Specimen	Detection Limit	Fluorescent reagent	Loop primers	Reference
Marek's disease virus (MDV)	*meq* oncogene	LAMP	Feather tips	2 log 10 plaque-forming units (PFU)/ml	SYBR Green I	No	Wozniakowski *et al.* (2011)
	meq gene	LAMP	Feather tips	10 copies/ reaction	SYBR Green I	Yes	Angamuthu *et al.* (2012)
	meq gene sequences	LAMP	Feather follicles	3.2 copies/ million cells	NK**	Yes	Wei *et al.* (2012)

*FDR= Fluorescent detection reagent, Eiken Chemical Co. Ltd, Tokyo, Japan; **NK=Not Known; RT-LAMP= Reverse transcriptase-Loop-mediated Isothermal Amplification

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Table 2 LAMP assays developed for detection of bacterial pathogens of chickens.

Poultry Pathogen	Target Spp./gene	LAMP Assay Format	Specimen	Detection Limit	Fluorescent reagent	Loop primers	Reference
Salmonella Spp.	Fragments of the O9-Group Salmonella-specific insertion element in Salmonella serovars including *S. enteritidis* and *S. pullorum*	LAMP	Caecal dropping samples	10^3 CFU/ml	No	No	Okamura *et al.* (2008)
	O4 Group of S. enterica O4 specific gene (*rfbJ* gene)	LAMP	Faeces, fluff, dust, and swabs	103 CFU/ml	Electrophoresis	Yes	Okamura *et al.* (2009)
	bcfD gene a conserved fimbrial operon gene	LAMP	Faecal samples	5 CFU/reaction from pure culture or 200 CFU/reaction from spiked faeces	Real-time turbidity	YES	Zhuang *et al.* (2014)
	SefA gene a fimbrial operon gene	LAMP	Faecal samples	4 CFU from pure culture or 400 CFU from spiked faeces	SYBR Green I stained gel	YES	Gong *et al.* (2016)
	Salmonella invasion (*invA*) gene	PMA**-LAMP	Chicken carcass rinse water	8.0×10^1 CFU/reaction	Real-time Fluorometer (Genie II (Optigene, UK)	YES	Youn *et al.* (2017)
Pasteurella multocida	*KMT1gene*	LAMP	Trachea and nasal samples	22.8 pg/µl	SYBR Green I	YES	Bhimani *et al.* (2015)
Campylobacter jejuni/ Campylobacter coli	oxidoreductase gene/*gufA* gene	LAMP	Liver, heart, gizzard, and meat samples	7.9CFU per reaction/ 3.8CFU per reaction respectively	Real-time turbidity	YES	Yamazaki *et al.* (2009)

Table 2 Continued

Poultry Pathogen	Target Spp./gene	LAMP Assay Format	Specimen	Detection Limit	Fluorescent reagent	Loop primers	Reference
	oxidoreductase gene/ *gufA* gene	LAMP	Cloacal swabs/ faecal samples	3.69 and 3.60 log CFU/g of faecal sample respectively	Real-time turbidity	YES	Sabike *et al.* (2016)
Mycoplasma gallisepticum (MG)	*mgc2* gene	LAMP	Culture/ swab samples	10fg/µl	Real-time fluorescence	No	Ehtisham-ul-Haque *et al.* (2017)
	pdhA gene	LAMP	Trachea	10 fg/µl	Commercial dye	No	Zhang *et al.* (2015)
	NK	LAMP	Trachea	10 fg/µl	NK*	YES	Luo *et al.* (2012)
Mycoplasma synoviae (MS)	*vlhA* gene	LAMP	MS field Isolates	10^{-1} CFU/ml	SYBR Green I	YES	Kursa *et al.* (2014)

*NK=Not Known; **PMA= propidium monoazide treatment

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Table 3 LAMP assays developed for detection of protozoan pathogens of chickens.

Poultry Pathogen	Target Spp./gene	LAMP Assay Format	Specimen	Detection Limit	Fluorescent reagent	Loop primers	Reference
Eimeria spp. (Poultry)	*All 7 Eimeria* species-specific SCAR markers and *Em*MIC1 gene	LAMP	Intestinal samples	Between 1-10 genome copies	Hydroxynaphthol blue	YES	Barkway *et al.* (2015)
	All 7 Eimeria species-specific SCAR markers and *Em*MIC1 gene	LAMP	Purified oocysts and Blood	10^1 genome	SafeView nucleic acid stain (NBS biologicals, Cambridgeshire, U. K.) and Hydroxynaphthol blue	YES	Barkway *et al.* (2011)
Histomonas meleagridis	*18SrRNA gene*	LAMP	Liver and caeca	10 Copies of plasmids	SYBR Green I	No	Xu *et al.* (2014)

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