An improved visual closed tube Loop mediated isothermal amplification (LAMP) assay for rapid identification of Orf virus in sheep and goats

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Summary
The Orf virus (ORFV) is an epitheliotropic virus causing a highly contagious skin disease mainly in sheep and goats. Several diagnostics including molecular tools like Loop mediated isothermal amplification (LAMP) assay are available to detect ORFV in affected species. However, the carry-over contamination associated with LAMP as open tube format prevents the assay applicability as point-of-care test in field diagnostic settings. In this study, the *B2L* gene based LAMP assay was optimized in a closed tube format using hydroxynaphthol blue (HNB) and calcein as pre-addition dyes and it has shown a clear positive and negative signal at 60 °C using 4 and 5 mM concentrations of $MgSO_4$ for these dyes, respectively. This optimized assay that could reveal the result within one hour is highly specific and sensitive with a limit of detection of 12.5 femtogram of viral genomic DNA or ~ 85 virus genome equivalent. This improved method can also prevent the cross-contamination of LAMP reactions in the laboratory without compromising diagnostic sensitivity and specificity when compared to open tube system. This closed tube LAMP method has potential to act as a simple visual detection assay for the rapid and specific diagnosis of ORFV in sheep and goats.

Orf or contagious ectyma is a highly contagious debilitating and economically important viral disease affecting primarily sheep and goats. It may also affect several wild ruminants and humans (Karki *et al.* 2019). This skin disease is present in many parts of the world. It is endemic in India where several outbreaks with varying degreee of severity have been reported in different states of the country (Venkatesan *et al.* 2011, Venkatesan *et al.* 2018, Nandi *et al.* 2011, Shome *et al.* 2005). It is caused by Orf virus (ORFV), of the genus *Parapoxvirus*, subfamily *Chordopoxvirinae*, family *Poxviridae* (Karki *et al.* 2019). The disease is characterized by proliferative skin lesions mainly in and around the mouth where the lesions are often confounded with other viral diseases like capripox, bluetongue, foot and mouth disease and peste des petits ruminants (Nandi *et al.* 2011). Several PCR techniques including the Real-time PCR are available to detect and identify the viral DNA in affected species (Gallina *et al.* 2006, Bora *et al.* 2011). However, these techniques are not deployable in resource limited field settings in developing countries. Loop mediated isothermal amplification (LAMP) is a simple, rapid, inexpensive, sensitive, specific and very useful field diagnostic tool developed for many infectious agents including viruses (Notomi *et al.* 2000). The LAMP assay can detect microbial pathogens in clinical samples in shorter period of time than other routinely used molecular diagnostic tools and is appropriate for poor and developing countries (Parida *et al.* 2008).

However, the possible carry-over contamination associated with LAMP assay in open tube system that involves multiple opening of caps during various processes of reaction set up hinder the test applicability and suitability in field diagnostic settings (Parida *et al.* 2008, Karthik *et al.* 2014). Detection of specific target in LAMP reaction modified into closed tube format can minimize such carry-over contamination to future reactions and can act as a point of care test in field diagnosis. In this study, we modified/improved the open tube LAMP assay to a closed tube format targeting the highly conserved *B2L* gene of ORFV using different concentration of $MgSO_4$, dNTPs, hydroxynaphthol

blue (HNB) and calcein that were used as pre-addition dyes.

The vaccine strain ORFV-Mukteswar 59/05 (Venkatesan *et al.* 2016) was used for initial optimization of the LAMP assay. In addition to vaccine strain, nine (n = 09) ORFV isolates, one sample each of Goatpox Virus (GTPV), Sheeppox Virus (SPPV), Buffalopox Virus (BPXV), Camelpox Virus (CMLV) and other viruses of goats and sheep namely Bluetongue Virus (BTV), Foot and Mouth Disease Virus (FMDV) and Peste des Petits Ruminants Virus (PPRV) were included in the evaluation of the analytical and diagnostic specificity. A total of fifty (n = 50) clinical tissues which included skin scabs/skin scrapings/swabs of sheep and goats suspected for ORFV infection were tested for evaluating the developed LAMP assay. Scab samples were triturated and freeze thawed three times before extraction of total genomic DNA (gDNA), whereas the cell culture isolates were directly processed for extraction using a commercial DNA extraction kit (Qiagen, Hilden, Germany) and used as template in the optimization and evaluation of the improved LAMP assay. The LAMP primers (Venkatesan *et al.* 2016) targeting the highly conserved major envelope, the *B2L* gene of ORFV was used in developing closed tube LAMP assay.

The LAMP reaction conditions in closed tube format were optimized at different temperatures (60-65 °C), time range (10-75 min), different concentration of $MgSO_4$ (4-9 mM), dNTPs (0.2-1.0 mM) with pre-addition of hydroxy naphthol blue (HNB) (Sigma, MO, USA) and calcein dyes using PCR thermal cycler (EppendOrf, Hamburg, Germany) as per standard protocol described earlier (Venkatesan *et al.* 2012). For visual detection, HNB and Calcein dyes at a final concentration of 120 µM and 0.5 mM, respectively, were employed. The outcome of closed tube LAMP reaction was checked initially in agarose gel electrophoresis to identify authenticity of positive visual detection. But later, the reactions were performed solely in a closed tube format as HNB master mix containing all the required reagents for LAMP. Ten-fold serial dilutions of purified viral gDNA (125 ng to 1.25 fg) were used for analytical sensitivity and the results were analyzed by HNB/calcein dye based visual detection in the optimized closed tube system. The optimized closed tube LAMP assay was later evaluated by using cell culture adapted ORFV isolates (n = 09; Mukteswar/09, Izatnagar 79/04, Shajahanpur 83/04, Assam/10, Mukteswar/18, Sundhalkal/18, Mukteswar 66/04; Assam/10, Bareilly/BB) and other pox virus isolates (n = 07) namely SPPV-Srinagar, GTPV-Uttarkashi, BPXV-Vij/96 and CMLV-Bikaner. Also, other important viruses of sheep and goats namely PPRV-Sungri, BTV-serotype 23 and FMDV-O serotype were used. These virus isolates have been tested in developed assay as known positive and known negative for ORFV. A total of twenty-nine (n = 29, positive n = 15 and negative n = 14) samples and isolates of known status for ORFV segregated by open tube *B2L* LAMP assay (Venkatesan *et al.* 2016) were used to compare the diagnostic performance of the new improved LAMP assay. Later, the developed closed tube LAMP assay was evaluated using random tissue samples (n = 50; goats: n = 39 and sheep: n = 11) from field outbreaks suspected for ORFV to identify detection rate when compared to open tube system. These clinical tissue samples (scabs/skin scrapings/swabs) were either collected from different field outbreaks or sent to poxvirus laboratory from different parts of the country after collection and now available in the repository of poxvirus laboratory, IVRI, Mukteswar. All the samples were collected from non-vaccinated sheep and goats showing typical lesions of contagious ecthyma and stored at - 80 °C until further use. Samples were selected from the repository on random basis and processed for evaluation by open and closed tube LAMP assays. Further, the total genomic DNA extraction was simplified using the protocol as described earlier (Adnan 2011) with slight modifications and employed over optimized closed tube LAMP assay to check its applicability under resource limited field settings. It involves addition of tissue lysis buffer containing 500 mM Tris, 50 mM EDTA, 500 mM- NaCl, 1.5% SDS and 0.3% β-Mercaptoethanol reagents to the sample for lysis and precipitation of lysate using 3M potassium acetate. The supernatant from precipitation by brief centrifugation will be used as template. From a total of twenty-five (n = 25) known positive ORFV samples the DNA was extracted using this modified simple technique as well as commercial kit and tested by closed tube LAMP assay.

In this study, the optimum amplification of target DNA using LAMP master mix was found to be at 60 °C for 60 min using HNB dye in the presence of 5 mM $MgSO_4$ concentration where the maximum difference in color change between positive and negative sample was observed (Figure 1A). The same LAMP system with calcein dye has been optimized at 4 mM $MgSO_4$ level (Figure 1B). Typically, LAMP master mix (2X) is composed of ThermoPol buffer (2x), 1.6 M Betaine (Sigma Aldrich, MO, USA), 1.6 mM dNTPs, 16 U *Bst* DNA polymerase large fragment (New England Biolabs, Sumido, Tokyo, Japan), 0.4 µM of each of the F3 and B3 primers, 3.2 µM of each of the FIP, BIP, LF and LB primers and 10 mM $MgSO_4$ (for HNB) or 8 mM $MgSO_4$ (for calcein). In this optimized closed tube LAMP, the final concentration of either HNB or calcein dyes was 180 µM and 0.25 mM, respectively. The results indicated that the optimum level of Mg^{2+} concentration is essential for the visible color change, and it needs to be at least 5 mM and 4.0 mM for hydroxynaphthol blue (HNB) and calcein, respectively, to give positive visible color change.

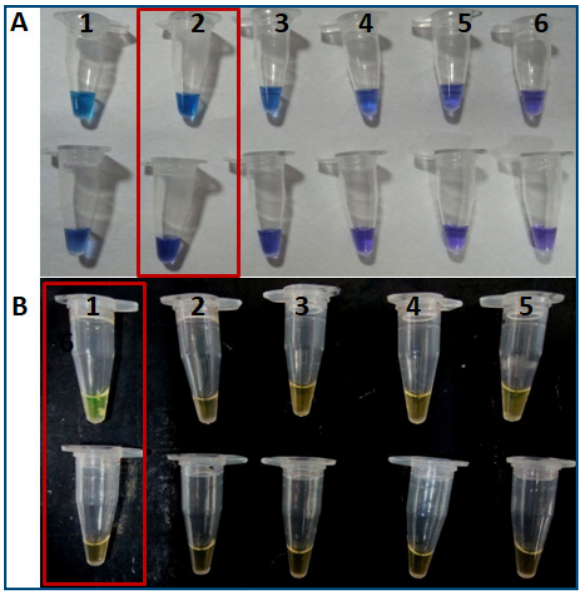

Figure 1. *Optimization of the* B2L *gene ORFV LAMP assay using different concentration of MgSO4 in closed tube format.* **A.** HNB dye showing clear demarcation at 5 mM concentration (Tube 2 as red framed) between positive (Row 1) and negative sample (Row 2); Tube 1: 4 mM $MgSO_4$; Tube 3-6: 6-9 mM $MgSO_4$ concentration, respectively. **B.** Calcein dye showing clear demarcation at 4 mM concentration (Tube 1 as red framed) between positive (Row 1) and negative sample (Row 2); Tube 2-5: 5-8 mM $MgSO_4$ concentrations, respectively.

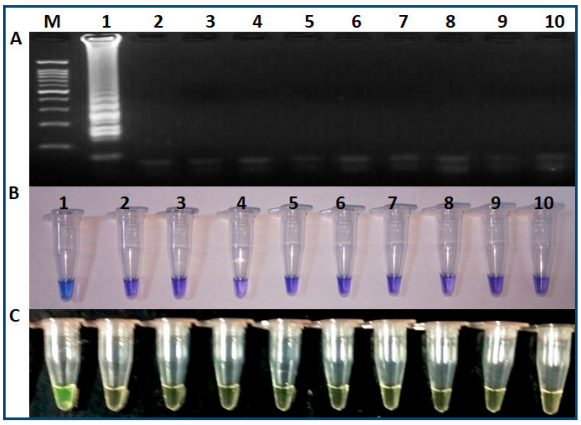

Figure 2. *Analytical specificity of* B2L *gene ORFV closed-tube LAMP assay.* Agarose gel (2%) electrophoresis (**A**) showing typical ladder-like pattern in ORFV-Mukteswar 59/05 (Lane 1) and no such signal in other related viruses namely GTPV, SPPV, BPXV, CMLV, BTV, FMDV, PPRV (Lane 2-8) and negative controls namely Vero cell DNA and NTC (Lane 9-10); HNB (**B**) and calcein (**C**) dyes based analytical specificity in *B2L* LAMP assay as visual detection format showing the similar specificity.

However, in open tube system, 8 mM $MgSO_4$ were optimum final concentration, as mentioned earlier (Venkatesan *et al.* 2016) whereas, in closed tube protocol, the optimum concentration was not more than 5 mM. Mg^{2+} concentration has shown to affect the visual detection of closed-tube LAMP assay because Mg^{2+} ion is used for HNB and calcein dyes to obtain a clear-cut differentiation between positive and negative LAMP reaction.

The closed tube LAMP system was specific for ORFV and no cross reactions with other related poxviruses were observed (Figure 2). Visual detection limit of the HNB and calcein dye closed tube LAMP assays was 10-fold (12.5 fg viral gDNA or ~ 85 virus genome equivalent) higher than ladder-like pattern observed in gel electrophoresis (Figure 3). Both dyes could be able to identify cell culture adapted ORFV isolates in new improved closed tube format (Figure 4). However, HNB based closed tube LAMP assay was selected over calcein for the final format of diagnostic evaluation, as the former dye is economical and easy to prepare. Further, HNB has shown better visual color stability than calcein at room temperature during post-LAMP reaction storage. HNB was found stable over period of time compared to calcein dye in this LAMP assay. Therefore, the HNB-LAMP in closed tube format was further evaluated for diagnostic specificity (DSp) and sensitivity (DSn) using known cell culture virus ORFV/non-ORFV isolates and tissue samples. The developed closed tube LAMP has shown the same diagnostic sensitivity and specificity of an open tube *B2L* LAMP assay (Venkatesan *et al.* 2016), since both of them were able to correctly detect the positivity of 90% of random tested samples (45/50). The new developed closed tube LAMP has shown same detection rate than the open tube system without compromising the diagnostic sensitivity and specificity. On testing over a total of twenty-nine (n = 29) samples of known status for ORFV, previously identified by the open tube system, this closed tube assay showed 100% of diagnostic sensitivity (DSn) and specificity (DSp)". The entire known positive (n = 25) ORFV DNA samples extracted by simplified extraction technique (Adnan 2011) were identified as positive in LAMP assay and same results were observed with DNA samples extracted by commercial kit.

Rapid and specific identification of an infectious pathogen at field level are imperative for an effective control and management of these infections. The LAMP assay has been proven by many reports that has great potential in clinical diagnosis of several microbial pathogens of livestock including sheep and goats (Notomi *et al.* 2000, Parida *et al.* 2008). However, post-LAMP reaction manipulations, namely agarose gel electrophoresis and addition of visual detection dyes namely SYBR green I/HNB/calcein by opening the tube, allow carrying-over contamination which ultimately leads to false positive results (Quoc *et al.* 2018). It also prevents the assay adaptability and applicability in field conditions of developing world (Parida *et al.* 2008). LAMP based ORFV DNA detection methods have been reported earlier as a simple and rapid diagnostic tool that may be useful to make

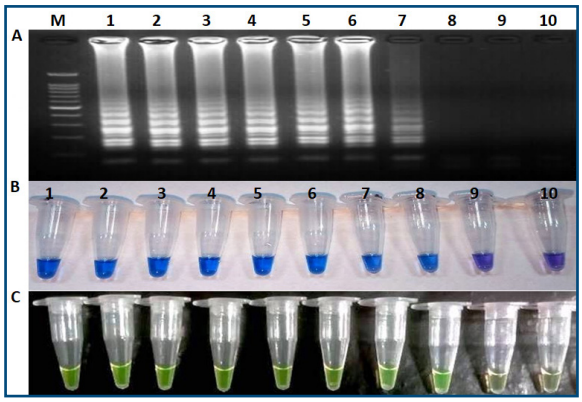

Figure 3. *Analytical sensitivity of* B2L *gene ORFV closed-tube LAMP assay.* Agarose gel (2%) analysis (**A**) of closed tube LAMP reaction for the analytical sensitivity using 10 fold serial dilutions of gDNA from purified ORFV (Lane 1-9: 125 ng to 1.25 fg level of gDNA) and Lane 10: NTC; (**B**) HNB and (**C**) calcein dye based closed tube visible detection showing the analytical sensitivity up to 12.5 fg (Tube 8).

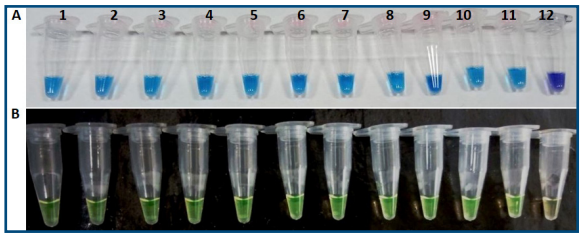

Figure 4. *Screening of ORFV isolates by developed closed tube LAMP assay using HNB (***A***) and Calcein (***B***) dyes showing positive signal in tested ORFV isolates (Tube 1: rB2L plasmid; Tubes 2-11: Muk 59/05-P10, Muk 59/05-P50; Muk/09, Izatnagar 79/04, Shajahanpur 83/04, Assam/10, Muk/18, Sundhalkal/18, Muk 66/04; Assam/10, Bareilly/BB (Lane 1-10) and no such signal in negative control (Tube 12).*

diagnosis in the field conditions (Tsai *et al.* 2009, Li *et al.* 2013, Wang *et al.* 2016). Schematic of the main difference between open tube and closed tube LAMP assay is shown as Supplementary Figure 1. To overcome major drawback, the closed tube format for LAMP assays have been attempted for rapid and safe detection of pathogens using different strategy (Quoc *et al.* 2018, Karthik *et al.* 2014).

In this study, a closed tube LAMP test in simplified way using HNB/calcein containing master mix has been optimized. Colorimetric dye based on visual detection will be of cost and time effective to suit remote/onsite field conditions as diagnostic kit (Parida *et al.* 2008, Gota *et al.* 2009, Venkatesan *et al.* 2015, Mansour *et al.* 2015). The assay targeted the highly conserved *B2L* gene of ORFV for rapid visual detection in clinical samples. In general, closed tube format for ORFV here has shown to be rapid, safe and simple without compromising diagnostic sensitivity and specificity in line with open tube system developed earlier. However, the use of SYBR green in agar gel capsule kept in lid of reaction tube may not be affordable in field settings as compared to HNB dye. Similarly, closed tube LAMP assays using commercial master mix along with real-time fluorimeter or any other costlier equipments may not be cost effective to use in resource limited diagnostic centers of developing countries. In our study on developing closed tube format, HNB was preferred over SYBR green as the later is costlier and not adaptable in pre-addition LAMP mix. But HNB used in this study was adapted in close tube system as a 'ready to use master mix' that makes this new improved LAMP as an easily applicable point of care test. Further, the simplified DNA extraction techniques used in this study will be useful in resource limited field diagnostic settings to process the samples in combination with this closed tube LAMP assay. Comparable sensitivity and specificity of this closed tube LAMP assay to open tube system for ORFV detection have shown potential advantages: [i] the assay did not require high cost equipments (PCR cycler/Gel apparatus/real-time fluorimeter) [ii] this close tube LAMP require only a simple water bath and mini centrifuge, [iii] it avoids the cross contamination completely as the use of HNB master mix prevents the opening of reaction tube and it can be operated in a simple and rapid way to detect ORFV.

As future directions, stability of LAMP master mix at different temperature conditions can be tested and a simple method to process the field tissue samples will strengthen this viable technology. Compared to open tube system, this *B2L* gene based closed tube system showed equal diagnostic potential with safe and contamination free detection of ORFV DNA in clinical samples. It may have potential in the rapid and specific clinical diagnosis and surveillance of ORFV in susceptible species including sheep and goats especially in the developing countries.

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Annex 1

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Supplementary Figure 1. *Schematic representation of differences between closed tube and open tube LAMP assays.*