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Shining a LAMP on the applications of isothermal amplification to monitoring illegal wildlife trade

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Abstract

Environmental biosecurity risks associated with the Illegal Wildlife Trade (IWT) include the loss of biodiversity, threats to public health, and the proliferation of invasive alien species. To assist enforcement agencies in identifying trafficked species, rapid forensic techniques enable the detection of trace Environmental DNA (eDNA) where physical identification is not possible. Loop Mediated Isothermal Amplification (LAMP) is an emerging technique with recent applications in biosecurity and forensic sciences, and with potential to function as a field-based detection tool. Here we provide an overview of current research that applies LAMP to human and wildlife forensic science, including identification of ornamental wildlife parts, consumer products, and invasive species monitoring and biosecurity detection. We discuss the current scope of LAMP as applied to various wildlife crime scenarios and biosecurity checkpoint monitoring, highlight the specificity, sensitivity, and robustness for these applications, and review the potential utility of LAMP for rapid field-based detection within the IWT. Based on our assessment of the literature we recommend broader interest, research, and investment in LAMP as an appropriate field-based species detection method for a wide range of environmental biosecurity scenarios.

Keywords: Biosecurity; Illegal Wildlife Trade; Invasive Species; Loop Mediated Isothermal Amplification; Wildlife Forensics

Introduction

Approximately one-quarter of all terrestrial vertebrates (birds, mammals, amphibians, and squamate reptiles) are traded globally (Scheffers et al. 2019). Correctly identifying these animals and their bio-products in the Illegal Wildlife Trade (IWT) is challenging, due to corruption, falsified documents, and imprecise species or wildlife product knowledge (Zain 2020). Ultimately, this results in less than 30% of wildlife crimes leading to successful prosecution (Gouda et al. 2020). To address this issue, wildlife forensic science techniques have been developed to identify the species common in the IWT, including derivative products such as rhino horn and elephant ivory (Conte et al. 2019; Ewart et al. 2018a). Molecular species identification methods generally follow a standardised approach which includes: 1) DNA extraction; 2) extract quantification; 3) Polymerase Chain Reaction (PCR) amplification of mitochondrial DNA section; 4) confirmation of PCR product generation; 5) amplicon purification; 6) bi-directional DNA sequencing; and 7) comparison to a reference dataset (Linacre 2021). The problem with this approach is its limited application to the field-based detection of IWT, due to significant resources, time, expertise, and facilities required to conduct such intensive molecular protocols. Lengthy analysis can result in delayed legal action with substantial resource-based costs, including long turnaround times (Masters et al. 2019). Within the literature there is an ever-increasing emphasis on the benefits of cross disciplinary collaboration, and research to aid in the development of field-ready technologies to address these limitations and increase detection of illegal activities (Masters et al. 2019; Smith et al. 2019).

A primary concern relating to environmental biosecurity is the role of IWT in the spread of novel invasive species (García-Díaz et al. 2017; Gore et al. 2019), and diseases (Bezerra-Santos et al. 2021). The costs of managing invasive species globally since 1960 are at least $95 billion (Cuthbert et al. 2022), with the damages and losses caused being at least a magnitude greater; amounting to at least $1131 billion (Cuthbert et al. 2022). Yet proactive prevention measures accounted for only $3 billion of the $95 billion management cost (Cuthbert et al. 2022). This indicates a strong priority in most countries for post-invasion spending on control and eradication, despite the obvious role of proactive management, including strong onsite biosecurity

detection measures, being capable of reducing future costs in the order of trillions of dollars (Cuthbert et al. 2022).

The need for onsite detection methods in biosecurity has led to a requirement for low-cost, low-resource, rapid forms of molecular detection. To bridge this gap, recent research has focussed on novel applications of isothermal amplification methods such as Loop Mediated Isothermal Amplification (LAMP) (Yu et al. 2019) and Recombinase Polymerase Amplification (RPA) (Hsu et al. 2021). LAMP effectively eliminates the operational constraints associated with PCR by processing the reaction at a constant temperature using an enzyme with strand displacement affinity (Tomita et al. 2008), commonly *Bst* polymerase (Hafner et al. 2001). This technique first emerged in the early 2000's, with a primary focus on clinical medicine (Notomi et al. 2000). Soon after LAMP's inception, the technique was advanced by the addition of loop primers, which significantly accelerated the reaction (Nagamine et al. 2002).

In forensic science, LAMP has been explored as an inexpensive, rapid discriminatory testing tool to identify the presence of human DNA (Watthanapanpituck et al. 2014). This assay targeted the *cytochrome b* region for trace human DNA and was tested against 11 non-target animal species, including closely related species such as orangutans and chimpanzees (Watthanapanpituck et al. 2014). Resulting sensitivity was exceptional with a detection limit of as low as 718 fg of genomic DNA (Watthanapanpituck et al. 2014). Applications of LAMP extend to detection of human male DNA from sperm (Scott et al. 2019) and differentiation of unknown body fluids, including venous blood, semen, and saliva, based on colorimetric responses (Jackson et al. 2020; Layne et al. 2021); with promising results for forensic science casework protocols. Similarly, the application of isothermal amplification methods for onsite monitoring of invasive species crossing transnational borders has been explored (Kyei-Poku et al. 2020; Vythalingam et al. 2021); as it offers an operational tool well suited for highly sensitive and specific field-based detection (Figure 1).

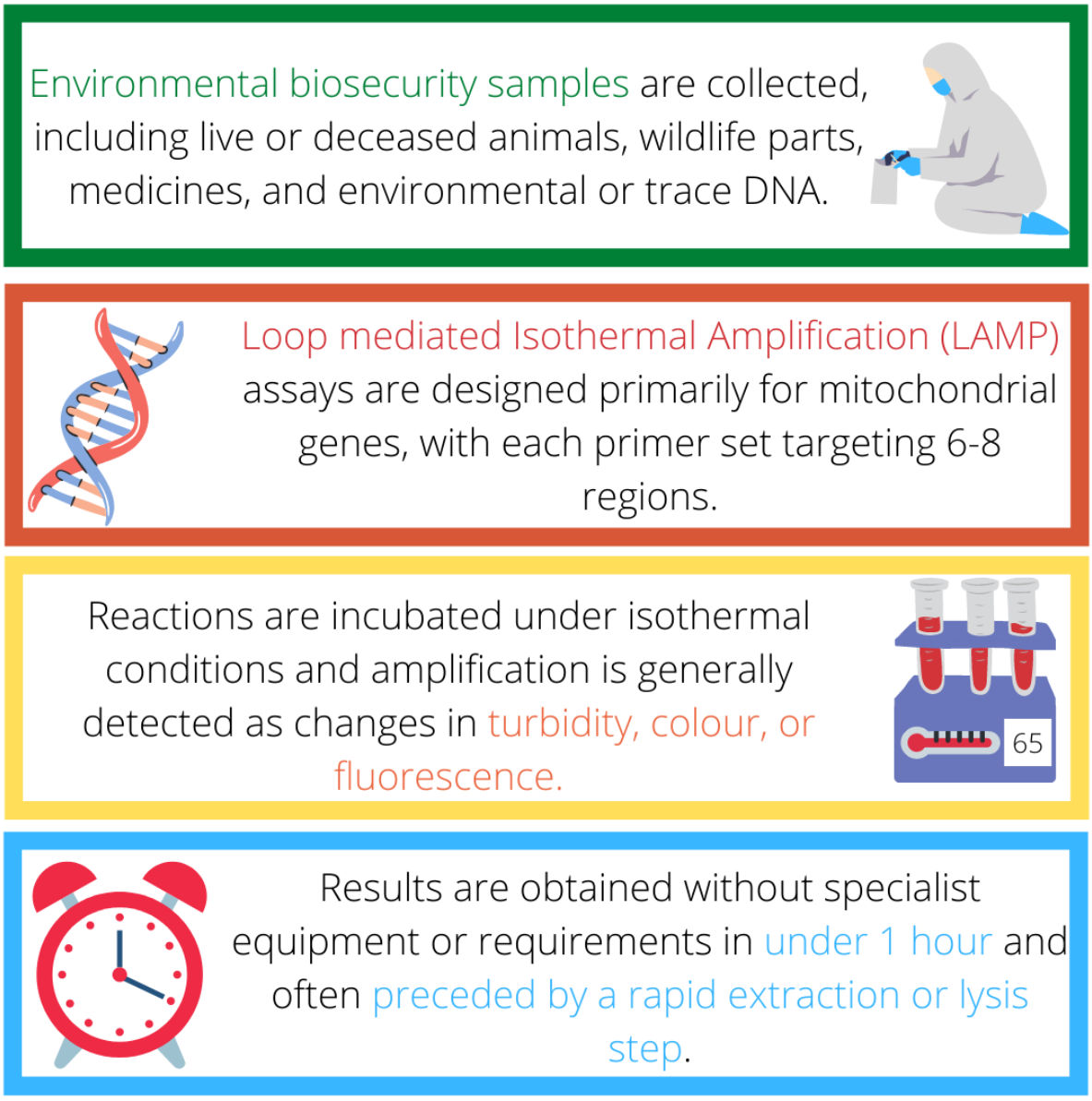

Figure 1. Workflow indicating the integration of Loop Mediated Isothermal Amplification (LAMP) into an environmental biosecurity scenario. This generally requires appropriate sample collection and storage, *in silico* primer design and validation, isothermal incubation conditions with detection facilitated by changes in turbidity, colour or fluorescence. LAMP reactions often lead to positive detection in under 1 hour without requiring specialist equipment.

LAMP shows great potential for compliance checks, species level presumptive detection, and situations lacking physical evidence, which rely on remnant trace DNA on surfaces or from tissue samples (Raele et al. 2019). Here, we present an overview of the LAMP technique for combatting the IWT, highlighting the benefits for onsite detection and discuss research that has explored this tool for wildlife forensic science, biosecurity, and interrelated fields. We discuss emerging technologies and the future

direction of LAMP applied to field-based detection to address IWT. We recommend broader interest, research, and investment in LAMP as an appropriate field-based species detection method for environmental biosecurity scenarios.

Loop Mediated Isothermal Amplification (LAMP)

LAMP is a nucleotide amplification method that functions by auto-cycling strand displacement DNA synthesis, performed by a DNA polymerase with high strand displacement affinity (Nagamine et al. 2002; Notomi et al. 2000). This method combines rapid, simple, and highly specific target sequence amplification (Notomi et al. 2015). LAMP utilises two inner and two outer primers with the option of additional loop primers that together recognise six to eight distinct regions on the target DNA, facilitating high specificity (Nagamine et al. 2002; Tomita et al. 2008). The LAMP technique can amplify a few copies of DNA exponentially in less than one hour. The reaction process consists of two forms of elongation occurring via a loop region. This includes template self-elongation starting at the stem loop formed at the 3’-terminal end and subsequent binding and elongation of new primers to the loop region (Figure 2) (Notomi et al. 2015). The primary advantages pertain to the speed of the reaction, which is conducted at a single reaction temperature (Francois et al. 2011). This reduces the need for sequential thermocycling stages and the associated expensive and specialised thermocycling equipment, most often restricted to a dedicated laboratory (Francois et al. 2011). LAMP has additionally shown tolerance to PCR inhibitors, pH and temperature variability (Francois et al. 2011).

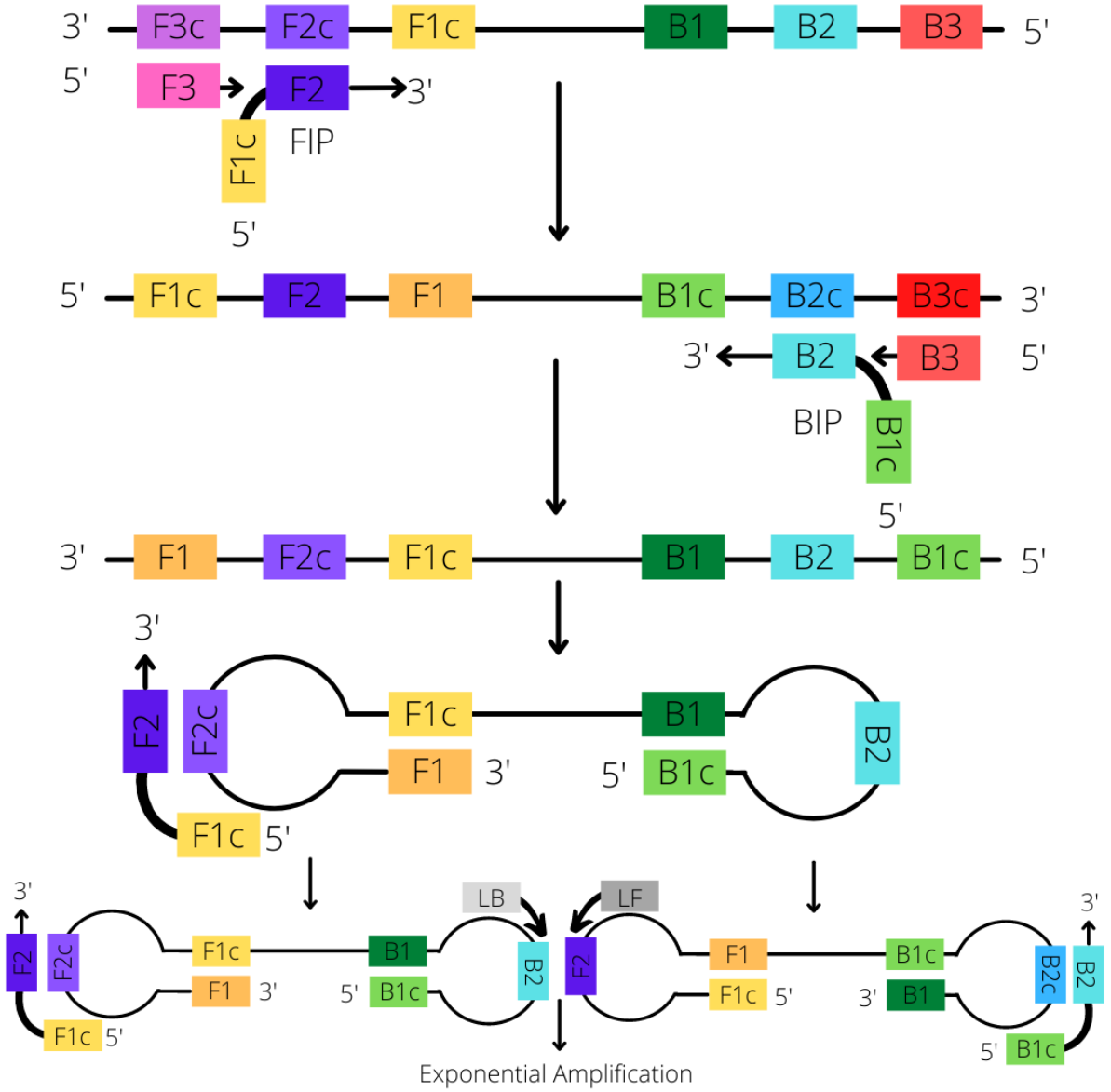

Figure 2. Loop mediated amplification mechanism. Two inner primers consisting of the F3 and forward inner primer (FIP) and two backward primers, the B3 and backward inner primer (BIP) are used to target 6 regions. Additionally, loop primers are often used to accelerate the reaction, denoted here as LF (loop forward) and LB (loop backward) targeting two additional distinct regions. The *Bst* polymerase displaces each of the DNA strands and initiates synthesis, this leads to the formation of loop structures which facilitate subsequent rounds of amplification.

LAMP is versatile, as detection methods can be divided into three primary categories including turbidity, fluorescence, or colorimetric. Initially detection was measured as a change in turbidity visible due to white by-product precipitation of magnesium pyrophosphate in the reaction mixture (Mori et al. 2001). This is possible as both an endpoint and real-time measurement, as the production of precipitate correlates with

the amount of DNA synthesised (Mori et al. 2004). In terms of fluorescence detection several studies indicated the use of intercalating fluorescent dyes, including SYBR green I (Kumari et al. 2019) and melting and annealing curve analysis post real-time monitoring (Cho et al. 2014). Additionally, results of the LAMP reaction are often visualised as a unique banding pattern by gel electrophoresis (Chen et al. 2013), which may also serve as a confirmatory indicator of LAMP reaction success (Jackson et al. 2020). The use of colorimetric methods, particularly by use of additives such as hydroxy naphthol blue, phenol red, calcein, leuco crystal violet, and malachite green (Goto et al. 2009; Scott et al. 2020), are common and widespread in several applications and often depend on pH (Tanner et al. 2018). All three forms of detection can be monitored by eye at the endpoint of the reaction. However, innate subjectivity remains an issue, and as such, turbidimeters and fluorometers are often used to facilitate quantitative measures of the LAMP reaction (Zhang et al. 2014). Concerning colorimetric methods, LAMP detection is often accompanied by optimised imaging procedures (Rodriguez-Manzano et al. 2016) or software to eliminate innate colour subjectivity. In some cases, open source (e.g., ImageJ (Schneider et al. 2012)) plugins have been developed to distinguish between negative and positive reactions based on colour components such as hue (Layne et al. 2021; Scott et al. 2020; Woolf et al. 2021). Additionally, the properties of colorimetric reactions can allow for conformation assessments by use of the UV-vis spectrum to observe the transition of colour altered peak intensities between positive and negative reactions (Nguyen et al. 2019a).

Until recently, the primary role of LAMP was to detect single targets with reasonably high specificity. The use of turbidimetric, colorimetric and fluorometric detection is often considered a form of indirect evaluation functioning in a similar way to SYBR green qPCR assays (Liu et al. 2017b). The integration of molecular probes or beacons in LAMP research emerged as a means of reducing false positives due to non-specific amplification (Hardinge and Murray 2019; Liu et al. 2017b). One of the initial studies incorporated a quencher-fluorophore duplex region on LAMP primers aimed at expanding detection to multiple targets (Tanner et al. 2012). When primers anneal to the desired target the fluorophore is released and a gain of fluorescent signal can be observed. This has been showcased for real time detection of 1-4 targets utilising a fluorometer, with a detection limit of 100 copies of human genomic DNA (Tanner et al.

2012). The molecular probe-based approach has facilitated greater specificity and unlocked multiplexing capacity. These methods have also diversified to include assimilating probes (Kubota et al. 2011), TaqMan coupled LAMP (Yu et al. 2021), fluorogenic bidirectional displacement probe-based real-time LAMP (Ding et al. 2016), locked nucleic acid molecular beacons (Bakthavathsalam et al. 2018) and self-quenching/de-quenching probes (Gadkar et al. 2018). Additionally, the role of primer dimer and self-amplifying hairpins on reverse transcription LAMP when detecting viral RNA has also been explored (Meagher et al. 2018). Minor displacements of primers to regions of self-complementarity away from the 3' end of the primer dramatically reduced the occurrence of secondary structures and improved speed and in some cases sensitivity (Meagher et al. 2018). Furthermore, mathematical models to identify non-specific amplification, distinguishing between target and non-target amplification based on microchip electrophoresis have also been developed (Schneider et al. 2019). Stoichiometric and pseudo kinetic modelling has also been conducted to classify LAMP products into uniquely identifiable categories, aimed at aiding robust probe-based detection strategies enhancing specificity (Kaur et al. 2020).

LAMP research applied to environmental biosecurity

The most common application of DNA based detection in environmental biosecurity investigations is species identification (Linacre 2021). As a result, the use of LAMP to combat IWT extends to adulterated meat products (Cho et al. 2014; Liu et al. 2019; Nikunj and Vivek 2019; Sul et al. 2019), detection of conservation significant species (But et al. 2020; Wimbles et al. 2021; Yu et al. 2019), biosecurity screening (Blaser et al. 2018b; Kyei-Poku et al. 2020), invasive species detection in novel ecosystems (Rizzo et al. 2021; Vythalingam et al. 2021; Williams et al. 2017), and disease monitoring (Sahoo et al. 2016). Additionally, LAMP has a strong presence in bacterial and viral point-of-care detection methods research (Kashir and Yaqinuddin 2020; Nguyen et al. 2019b).

Falsified consumer items and product authenticity

Detection of falsified fur products has been explored using a highly specific fluorescence based LAMP assay targeting the *cytochrome oxidase subunit (CO1)* gene for both fox and cat fur (Yu et al. 2019). This assay was developed in response

to commercial fraud and wildlife crimes and is tolerant to PCR inhibitors such as pigments, dyes, or other fur components (Yu et al. 2019). The authors highlighted the role of the assay as an on-site species identity test, without costly requirements or specialist equipment. Sensitivity is similar to PCR, detecting down to 10 and 1 pg of DNA for cats and foxes respectively (Yu et al. 2019).

The detection of food products which have been mislabelled, tampered, or contain mixed species material is of particular interest. Assays targeting the *16s rRNA* region have been developed to detect chicken from processed meat samples, in under 30 minutes, with a detection limit of 10 fg (Sul et al. 2019). Similarly, targeting the *cytochrome b* region, ostrich meat can be detected in mixtures constituting only 0.01% in as little as 15-20 minutes (Abdulmawjood et al. 2014), and pork with a detection limit of 1 pg without cross reactivity (Yang et al. 2014). Additionally, a LAMP assay targetting the mitochondrial D-loop region has been developed for cattle, with a detection limit of 10 pg of DNA (Kumari et al. 2019). The underlying drivers behind this research interest are varied and includes religious certification, and concerns relating to allergens (Mao et al. 2020; Sheu et al. 2018), fraud (Kumari et al. 2019), disease (Pang et al. 2018; Zhao et al. 2010) and identifying species of conservation significance (But et al. 2020).

LAMP assay development also extends to the seafood industry, including detection of jumbo flying squid, with a LAMP assay targeting *CO1* with a detection limit of 10 pg of DNA per reaction (Ye et al. 2017). Several studies focussed on the detection of mislabelled or falsified seafood products have integrated molecular beacons into LAMP assays, facilitating increased specificity. Two such studies utilise self-quenching fluorogenic probes targeting skipjack tuna (Xu et al. 2021) and Atlantic salmon (Li et al. 2022). An initial skipjack tuna LAMP assay utilised non-specific fluorescent dyes targeting the *cytochrome b* region relying primarily on the specificity of primer annealing for species-specific sequences (Xiong et al. 2021b). The integration of a self-quenching fluorogenic probe, attached to the FIP primer, facilitated skipjack tuna authentication, and decreased the likelihood of false-positive signals when assessing six commercial tuna products (Xu et al. 2021). This assay displayed

exceptional sensitivity detecting as little as 5 fg of skipjack tuna DNA (Xu et al. 2021). Similarly, an initial non-specific fluorescence based LAMP assay was developed for Atlantic salmon targeting a section of the *cytochrome b* (Xiong et al. 2021a), prior to integrating a self-quenching fluorogenic probe attached to the backward loop primer with a detection limit of 5 pg (Li et al. 2022).

Highly specific, sensitive, and rapid detection of bushmeat samples is of considerable interest to conservation scientists and environmental biosecurity enforcement bodies, as these crimes are directly linked to biodiversity loss (Ripple et al. 2016) and emerging zoonotic disease (Hilderink and de Winter 2021). Therefore, research presented here could have similar implications for the detection of bushmeat related wildlife crimes. Providing point-of-entry detection could facilitate greater biosecurity preparedness and decrease transnational incursions through wildlife crime interception. Genetic reference frameworks for African forest bushmeat have already been established (Gaubert et al. 2015) and could form the basis for LAMP onsite detection of transnational trafficking. This is particularly true when identifying bushmeat for species covered by national or international protections as conducted for the Cameroonian bushmeat trade, where >50% of bushmeat species traded were nationally protected (Din Dipita et al. 2022). Nearly half of all samples collected from the Cameroonian bushmeat trade, subject to morphological identification, were corrected when subject to DNA based analysis, with additional high rates of incorrect identification at Parisian customs (Din Dipita et al. 2022). This further illustrates the need for highly specific, rapid forms of species identification based on LAMP, operationalised for a field environment.

Biodiversity and wildlife crime

The intersection of conservation goals and wildlife forensic science objectives is an under explored application of LAMP. This includes the bilateral benefits of optimal onsite detection capacity for live animal, wildlife part, medicinal or consumable trade concerning protected species. PCR based techniques, which are more routinely used for these types of compliance checks, are time consuming and require facilities and expertise which can hinder biosecurity practices. LAMP has been showcased for field-

based detection of illegal trade in shark fin products, which can be directly applied to enforcing CITES obligations; as rapid LAMP detection has been developed for all twelve CITES-listed shark species (But et al. 2020). The assays include primers which target the *CO1* and *NADH2* sequences and can detect all twelve species individually within an hour at constant temperatures (But et al. 2020). The cost of each LAMP reaction was c. US$0.6 compared with US$0.25 for a comparable PCR workflow, with the advantages of LAMP primarily spanning field applicability and high specificity (But et al. 2020). This study presented a novel application of LAMP onsite checkpoint monitoring for species with high wildlife crime concern. Similar methods could be explored for the rapid identification of other endangered species including those common in the illegal pet trades. This is also true for current wildlife forensic methods which employ PCR, as they could benefit from LAMP based presumptive testing prior to laboratory validation reducing the number of samples requiring exhaustive laboratory-based testing.

The mutual benefits of field-based LAMP monitoring for conservation, biosecurity and the prevention of wildlife crime has already been realised for combatting cases of wildlife poaching, specifically for the white rhinoceros (Wimbles et al. 2021). Rhinoceros horn is a commodity common in illegal transnational marketplaces (Hübschle 2016), consequently nefarious trade has received wildlife forensic attention (Ewart et al. 2018a; Ewart et al. 2018b). The internationally standardised rhinoceros horn identification test is PCR based and as such has limited applicability to onsite detection outside of a laboratory setting. Wimbles et al. (2021) presented a white rhinoceros specific LAMP assay, targeting the *cytochrome b* region, integrated into a microfluidic device capable of field-based detection in 30 minutes from dung samples, including field testing carried out at the Knowsley Safari, the approach could similarly play a role in the detection of wildlife crimes. The microfluidic device presented by Wimbles et al. 2021 included DNA extraction followed by three wash chambers prior to LAMP, with positive and negative control chambers adjacent to the field sample chamber for confirmation of positive detection. This study highlighted the possibility of LAMP microfluidic devices to operate in a myriad of wildlife crime situations, offering rapid, cost-effective, portable presumptive genetic testing.

Other forms of wildlife crime, including additional cases of poaching (Ghosh et al. 2019; Kumar et al. 2012) and trafficking of wildlife parts (Gupta 2018), could also benefit from on-site presumptive detection. This is particularly true for situations in which the sample itself bears insufficient physical characteristics or on-site detection to species level is time sensitive. Onsite identification has been showcased for a species susceptible to illegal hunting, the Formosan Reeves' Muntjac (Hsu et al. 2021). An RPA assay has been developed for the isothermal detection of bush meat in combination with a lateral flow strip. The described assay targeted the *cytochrome b* gene region and detected the target species from extraction to result in around 30 minutes. As such, the application of isothermal amplification methods to the detection of a range of wildlife crimes seems well suited.

Invasive species monitoring

Monitoring and related control programs have recently focussed on the role of eDNA in invasive species detection (Hunter et al. 2015; Morisette et al. 2021), with several studies focusing on LAMP as a potential eDNA monitoring tool (Vythalingam et al. 2021; Williams et al. 2017). The emphasis on monitoring primarily concerns invertebrate pests, as demonstrated by the development of LAMP assays as a means of point of entry detection or field-based detection.

Border surveillance of emerging insect incursions

A range of LAMP assays have been developed for multiple insect species commonly of environmental biosecurity concern (Table 1). This primarily concerns stowaways, with some assays developed as early warning tools for incursion events (Kyei-Poku et al. 2020). In addition to early detection some have tested detection in mixed samples including for red fire ants (Nakajima et al. 2019). Red fire ants are classed as a super pest with introductions as stowaways linked to early global trade routes (Gotzek et al. 2015), continued interest in their further spread throughout Australia and Asia demands robust biosecurity testing (Wylie et al. 2020). A large focus area is the detection of fruit fly species (Blaser et al. 2018a; Blaser et al. 2018b; Huang et al. 2009; Sabahi et al. 2018). One study focussed on the detection of several regulated quarantine insects at Swiss borders which included fruit fly

genera *Bactrocera* and *Zeugodacus* (Blaser et al. 2018a). Several primer sets targeting *CO1* were used to detect fruit fly and *Bemisia tabaci*, *Thrips palmi*, which are two additional species of biosecurity concern (Blaser et al. 2018a). Laboratory evaluations of the developed assays for 282 specimens suspected to be invasive, indicate a 99% test efficiency in under 1 hour (Blaser et al. 2018a). Several studies have focused on the detection of fall armyworm (Agarwal et al. 2022; Congdon et al. 2021; Kim et al. 2021). The most recent is based on the *CO1* gene, with high specificity and sensitivity down to 2.4 pg of DNA (Agarwal et al. 2022). Furthermore, the study contrast previous work (Kim et al. 2021) conducted for a *tRNA* based LAMP assay indicating the time based advantage of added loop primers for the described *CO1* assay (approx. 10 mins to result) (Agarwal et al. 2022). These examples illustrate the role LAMP methods can play in an environmental biosecurity setting and provide exemplars for the detection of key emerging incursion species yet to establish in novel ecosystems.

Table 1. Summarised LAMP assays as applied to environmental biosecurity of high-risk insects. Includes the species name the gene which the LAMP primers target, the detection limit tested in the described study, time to detection and source. Fields containing 'not applicable' (N/A) are those for which detection limit wasn't tested directly or a different measure of sensitivity was used.

Species	**Target**	**Limit of detection**	**Time to detection**	**Source**
Emerald ash borer	*CO1*	0.1 ng	30 min	(Kyei-Poku et al. 2020)
Red fire ant	*CO1*	N/A	90 min	(Nakajima et al. 2019)
Species belong to genera *Bactrocera and Zeugodacus* and *Bemisia tabaci and Thrips palmi*	*CO1*	N/A	60 min	(Blaser et al. 2018a)

Aedes mosquito species	ITS1 and ITS2	N/A	60 min	(Schenkel et al. 2019)
Walnut twig beetle	*28S rRNA*	1.28 pg and 6.4 pg for adults and frass, respectively	<30 min	(Rizzo et al. 2021)
Fall army worm	*CO1*	2.4 pg	<20 min	(Agarwal et al. 2022)
Fall army worm	*CO1*	24 pg	<30 min	(Congdon et al. 2021)
Fall army worm	Transfer RNA coding region between *ND3,* and *ND5*	10 pg	90 min	(Kim et al. 2021)
Khapra Beetle	*18s rRNA*	1.02 fg	<25 min	(Rako et al. 2021)

Additionally, a LAMP assay has been developed for Khapra beetle targeting the *16s rRNA* region with an additional LAMP assay targeting the *18s rRNA* region used to detect the presence of interspecific beetle DNA (Rako et al. 2021). The Khapra LAMP assay had a limit of detection comparable to the Khapra real-time PCR test with a detection limit of 1.02 fg (Rako et al. 2021). This assay was assessed for extracts from Khapra beetle tissue samples using both laboratory-based, destructive, and crude extraction methods. A subsequent comparative study assessed the utility of this Khapra LAMP assay against two Khapra beetle specific TaqMan PCR assays for onsite biosecurity for samples collected from airborne and floor dust (Trujillo-González et al. 2022). Notably, extracted Khapra beetle eDNA from dust samples was amplified by qPCR but not using the LAMP assay (Trujillo-González et al. 2022). A potential reason for the discrepancy between amplification methods could be the use of six primers which may not all anneal to desired template DNA in situations with degraded DNA (Trujillo-González et al. 2022). These results highlighted an important consideration for LAMP application to environmental biosecurity, primarily sample

types and end user application prior and throughout the assay development. LAMP assays may thus function best in environmental biosecurity scenarios from which high-quality DNA can be acquired, offering rapid presumptive species level testing.

Invasive aquatic species detection

A primary issue concerning biosecurity and IWT is the role transnational trade in exotic pets can play as a source of invasive species, documented by the pet release pathway (Sinclair et al. 2020). Pet releases are often a driver of invasive species introductions (Lockwood et al. 2019). Additionally, the import and export of pets is often highly regulated or strictly banned under national jurisdictional law (Ege et al. 2020). This has led to the development of detection methods for common aquatic pet species which double as invasive species in Malaysian waterways (Vythalingam et al. 2021). The focus species included guppies, goldfish, siamese fighting fish, Amazon sailfin catfish, koi and African sharptooth catfish which were collected from pet shops and local aquariums for the purpose of LAMP development. The resulting highly sensitive assays utilised 5 separate species-specific primer sets with a detection limit of between 0.02 pg and 2 x 10^{-12} pg for all 5 species (Vythalingam et al. 2021). The aim of the developed assay is to aid authorities in handling monitoring programs by providing rapid identification of non-native fish in ecosystems. Coupling this technique with optimal environmental DNA sampling has great potential for onsite monitoring. As such, programs tackling ecosystem monitoring for invasive species could benefit from assays targeting wider range of invasive species. It is generally agreed that prevention is preferable to control of an established pest (Leung et al. 2002), as such investment in appropriate on-site LAMP detection could be paramount in preventing novel introductions.

Confirming the presence of aquatic pest species has been explored through the development of LAMP based assays for monitoring quagga and zebra mussels in river basins (Carvalho et al. 2021; Williams et al. 2017). The first study addressing LAMP development aimed at streamlining the eDNA detection of quagga and zebra mussels in Michigan lakes (Williams et al. 2017). This included the development of three LAMP assays, one targetting the *18s rRNA* gene, amplifiying both target species DNA with a

detection limit of 0.1 fg. A further two *CO1* assays targetting quagga and zebra mussels seperately, with a sensitivity of 0.001 pg and 0.01 pg respectively (Williams et al. 2017). A subsequent novel zebra mussel assay targetted the *CO1* gene with a detection limit of 1.12 pg, which was also developed and field tested for a range of sample types collected from Portuguese, Spanish and French sources (Carvalho et al. 2021). An additional application has been the delimitation of eels in the genus *Anguilla,* with a focus on *Anguilla anguilla*, a critically endangered species (Spielmann et al. 2019). This assay was developed as a detection method for introduced foreign eel species in Europeam rivers, protecting consumers against mislabelled eel consumables and could serve a role in ecological studies (Spielmann et al. 2019). One LAMP assay was developed to detect all *Anguilla* species targetting the *C-type lectin* gene, while another targetted the mitochodrial D-loop region of *A. anguilla* with high specificty, both assays had a limit of detection of 500 pg (Spielmann et al. 2019).

Border surveillance using invasive species-specific LAMP assays with the capacity for field-based community or ecosystem wide surveillance show potential. The use of LAMP as an emerging surveillance technique for biosecurity officers and wildlife managers could thus be instrumental for conducting routine monitoring programs.

Health and disease: detection and prevention

An often-overlooked component of transnational wildlife crime is the potential introduction of foreign or novel wildlife diseases or zoonoses (Smith et al. 2012). An influx in zoonotic disease research brought on by the COVID-19 pandemic has highlighted the role of wildlife trade and conservation initiatives in the emergence of zoonotic diseases (Hilderink and de Winter 2021). Consequently, LAMP based detection could be highly suitable to the detection of wildlife related introductions of novel diseases. There are in fact a myriad of studies which explore LAMP based detection of COVID-19 to address on-site testing capacity of this global health concern (Augustine et al. 2020; Dewhurst et al. 2022; Kashir and Yaqinuddin 2020). Extending this research to a broader range of emerging pathogens and hosts common in the IWT could prevent future outbreaks and curb pandemics.

He et al. (2022) has presented multiple threatening pathogens, which are hosted by wild animals prized as delicacies in the Chinese IWT. When 1941 animals from five mammalian orders were surveyed, 102 mammalian infecting viruses were discovered with 21 of those posing potential risk to humans (He et al. 2022). Among the species that had their virome characterised was the Raccoon dog, this species was identified as carrying a range of novel pathogens (He et al. 2022), including previous detections of close relatives of SARS-CoV and SARS-CoV-2 (Guan et al. 2003) and *Rotavirus A* (Abe et al. 2010). Raccoon dog meat is often used as a subsidiary component in meat mixtures, with reports of health deterioration in some consumers (Liu et al. 2017a). Consequently, a LAMP assay targeting *cytochrome b* has been developed to detect Raccoon dog in processed meat, indicating no cross reactivity with seven non-target species and target DNA detection limits of 0.2 pg (Liu et al. 2017a). These results indicate a demand for the detection of species common in the IWT and present an opportunity for multiplex LAMP assays targeting both pathogens and hosts in tandem.

Detection of other zoonotic diseases has also gained some traction with the development of a LAMP assay for *Leptospira* (Chen et al. 2016). Leptospirosis is one of the most widespread zoonosis and is caused by a pathogen which colonises the renal tubules of hosts such as dogs, rats, and cattle (Chen et al. 2016). The *Leptospira* LAMP assay, targeting the *lipL32* and *lipL41* genes, offers exceptional sensitivity with a detection limit of 12 DNA copies. Additionally, LAMP reagents were lyophilised and stored, remaining stable for as long as 3 months at 4^{o}C (Chen et al. 2016). Storage and shelf life are additional considerations which are often omitted from publications concerning field-ready LAMP. These are, however, conditions which will have major impacts on field suitability and should thus be assessed.

Salmonella is considered a major food borne pathogen globally, which is responsible for food contamination leading to food poisoning (Zhao et al. 2010). As such, a myriad of LAMP assays and related methodologies have been devised for rapid point-of-care detection (Zhao et al. 2010). Initial studies developed assays targeting the genus specific *InvA* target which could detect 214 strains in 45 minutes with a detection limit of 1 pg of DNA (Zhao et al. 2010). Assessment of LAMP robustness has also been conducted for *Salmonella enterica* serovar Typhi, indicating consistency across two

pH units (7.3-9.3) and temperatures of 57-67ºC with maintained specificity (Francois et al. 2011). This has since progressed with the integration of molecular probes (Mashooq et al. 2016), development of a related handheld device for the detection of *Salmonella enterica* (Jenkins et al. 2011) and integration of disk-based compact micro-reactors for detection of *Salmonella* spp. (Santiago-Felipe et al. 2016).

Detection of *Haemonchus contortus,* a biosecurity risk parasite for ruminants, has also successfully been showcased (Melville et al. 2014), with an additional study contrasting LAMP to several other detection methods including (a) McMaster egg counting; (b) counts post staining with peanut agglutinin (PNA); and (c) quantitative polymerase chain reaction (qPCR) (Ljungström et al. 2018). The LAMP assay used in both studies targets the first internal transcribed spacer (ITS-1) with detection in under 1 hour. The initial study which outlined the assays development, highlighted the superior 10-fold sensitivity of LAMP when contrast with conventional PCR, detecting 10 fg and 100 fg of DNA, respectively (Melville et al. 2014). The comparative study indicated that an adapted LAMP assay was second to qPCR but with similar sensitivity results (Ljungström et al. 2018). The authors state that LAMP is a particularly viable method as it can be applied in resource constrained small diagnostic laboratories, generating sensitive and reliable results in under 1 hour (Ljungström et al. 2018).

The role of LAMP in detecting diseases in tandem to species identification for samples of biosecurity concern could function as an appropriate risk assessment tool at transnational points of entry. Circumventing resource and time intensive identification methods by utilising LAMP as a point of care diagnostic system could additionally reduce the biosecurity risk posed by unknown biosecurity samples, particularly by reducing the time to outcome and required resources.

Conclusion: LAMP Integration for biosecurity monitoring and surveillance

Market research has highlighted the demand for integrated field testing for tackling wildlife crime with the primary concerns of end users spanning contamination risks and reductions in quality assurance (Masters et al. 2019). Recent advances in molecular detection methods have led to the development of simple and cheap devices for the

ultrasensitive detection of nucleic acids for clinical diagnosis, food adulteration detection and environmental monitoring (Zhang et al. 2019). This has largely been due to a growing demand for monitoring and detection of nucleic acid biomarkers and the ever-increasing demand for more stringent sensitivity, specificity, and robustness of biomonitoring technologies (Zhang et al. 2019). LAMP methods have emerged as a promising alternative to PCR based systems due to simplicity and point-of-care capabilities (Nguyen et al. 2019b; Wan et al. 2019; Zhou et al. 2014). The ability of LAMP based devices to operate in resource constrained environments where traditional PCR-based technologies may not, has shown to be highly advantageous in a low resource field-based environment (Raele et al. 2019; Wimbles et al. 2021). The primary case for the development of microfluidic devices encompassing the LAMP reaction components is the reduced risk of sample contamination and minimal required reaction volume (Zhang et al. 2019). Several platforms exploiting isothermal nucleic acid amplification methods have recently become commercially available, widespread, and diverse, including OptiGene (http://www.optigene.co.uk/) Genie systems and the Biomeme (https://shop.biomeme.com/) Franklin.

Despite the substantial body of literature, LAMP and related microfluidic devices are yet to receive widespread uptake in research or applied wildlife crime monitoring detections and enforcement. Limited attention has been directed toward illegal wildlife trafficking of live animals. In the face of globalisation, applying these techniques to DNA-based monitoring of highly elusive IWT is well suited. The overwhelming risks presented by IWT (Cardoso et al. 2021) demands specialist point-of-care capacity. LAMP as a point of care technology presents great potential for the onsite detection of trace DNA relating to suspected trafficking of live animals, wildlife parts, medicines, and ornamental derivatives. The capacity for LAMP to bridge gaps relating to biosecurity and biodiversity on-site detection, makes it an excellent tool for a range of field-based applications. Furthermore, the low financial, time and resource-based costs render isothermal amplification methods well suited for point of entry detection. Specificity, sensitivity, and robustness comparable to current best practise methods (Francois et al. 2011) allows the integration of these methods into the wildlife forensic science arsenal without compromise (Masters et al. 2019). The ever-increasing interest in LAMP as a point of entry detection method suggests that it may soon

533 function in parallel to PCR, providing widespread molecular diagnostic capacity for
534 biosecurity scenarios.

5.0 References

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