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Shining a LAMP on the applications of isothermal amplification for monitoring environmental biosecurity

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Abstract

Environmental biosecurity risks associated with the transnational wildlife trade include the loss of biodiversity, threats to public health, and the proliferation of invasive alien species. To assist enforcement agencies in identifying species either intentionally (trafficked) or unintentionally (stowaway) entrained in the trade-chain pathway, rapid forensic techniques are needed to enable their detection from DNA samples when physical identification is not possible. Loop Mediated Isothermal Amplification (LAMP) is an emerging technique, with recent applications in biosecurity and forensic sciences, which has potential to function as a field-based detection tool. Here we provide an overview of current research that applies LAMP to environmental biosecurity, 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 trade 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 at biosecurity checkpoints. Based on our assessment of the literature we recommend broader interest, research uptake, and investment in LAMP as an appropriate field-based species detection method for a wide range of environmental biosecurity scenarios.

Keywords

Environmental biosecurity, invasive species, loop mediated isothermal amplification (LAMP), wildlife forensics

Introduction

A primary biosecurity concern is the early detection and accurate identification of novel invasive species (Early et al. 2016) 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 (Cuthbert et al. 2022). Yet proactive prevention measures accounted for only *c*. 3% of the management cost (Cuthbert et al. 2022). This indicated a strong priority in most countries for spending on post-establishment control and eradication, despite the obvious benefit of preventative management, including effective interception and detection measures (Cuthbert et al. 2022). As such, strong and effective environmental biosecurity measures are required, here defined as the protection of the environmental and social amenity from the negative impacts of invasive species (DCCEEW 2022). Environmental biosecurity spans the whole biosecurity continuum, which includes pre-border preparedness, border protection and post border management and control.

The implications of poor biosecurity management extend to biodiversity loss, which is well documented for source populations when species are subject to illicit trade (Morton et al. 2021). Wildlife trade can additionally incur a biosecurity threat when species with high invasion potential are introduced and establish in novel ecosystems resulting in a loss of ecosystem services (Charles and Dukes 2007). The intersection of biodiversity loss and biosecurity is often realised as a consequence of ongoing globalisation and transnational trade in live animals (stowaways (Hulme 2021) or pets (Lockwood et al. 2019) and wildlife products (food, medicines and ornaments (Ege et al. 2020)). Limiting the impacts of biodiversity loss is a key biosecurity goal which spans all points along the biosecurity continuum (Outhwaite 2010).

Within environmental biosecurity several molecular biomonitoring techniques have gained prominence. These techniques include DNA barcoding (Armstrong and Ball 2005), with recent advancements including DNA barcode sequencing at border checkpoints (Abeynayake et al. 2021), species-specific TaqMan assays leveraging portable thermocycling technologies (Trujillo-González et al. 2022), and metabarcoding approaches in ports (Borrell et al. 2017; Grey et al. 2018). To achieve reliable biosecurity intervention, low-cost, low-resource, rapid forms of species detection and identification are required. 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 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 rapid species detection (Masters et al. 2019; Smith et al. 2019). Biosecurity is one such field that has recently embraced the advent of new technologies including portable thermocyclers and recent isothermal amplification to tackle rapid onsite (defined as the point of interception) detection of emerging threats.

The application of isothermal amplification methods for onsite monitoring of non-native species crossing transnational borders has been explored (Kyei-Poku et al. 2020; Vythalingam et al. 2021); as they offer an operational tool well suited for highly sensi-

tive and specific field-based detection (Figure 1). Here, we critically examine the 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) for biosecurity detection of invasive alien species, with a particular focus on animals. We highlight 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, when applied to field-based detection. Notably, we recommend broader interest, greater research uptake, and further investment in LAMP as an appropriate field-based species detection method for a wide range of environmental biosecurity scenarios.

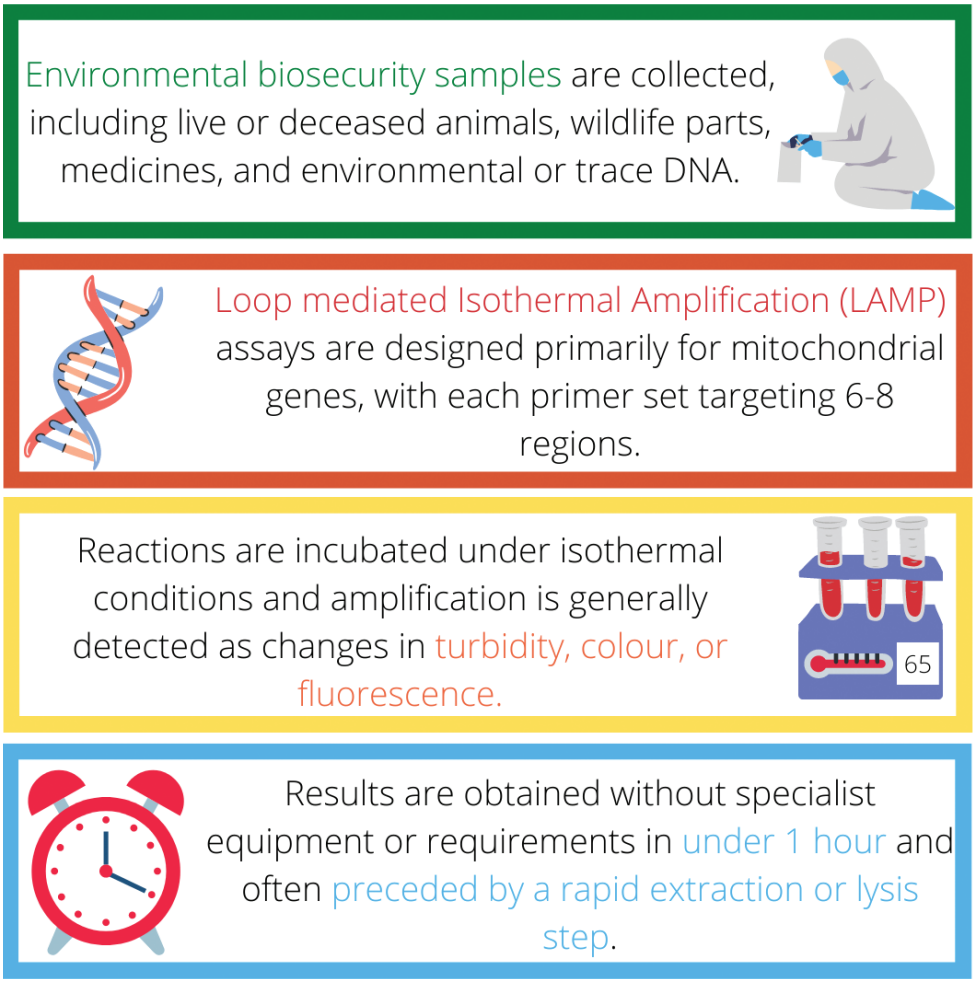

Figure 1. Workflow indicating the integration of Loop Mediated Isothermal Amplification (LAMP) into an environmental biosecurity scenario. This generally requires appropriate sample collection and storage, *in silico* primer design and validation, isothermal incubation conditions with detection facilitated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

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 (Notomi et al. 2000; Nagamine et al. 2002). 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). In addition to target specificity, primary advantages include the speed and simplicity of the reaction, which is conducted at a single (isothermal) 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).

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 (Scott et al. 2020; Layne et al. 2021; 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).

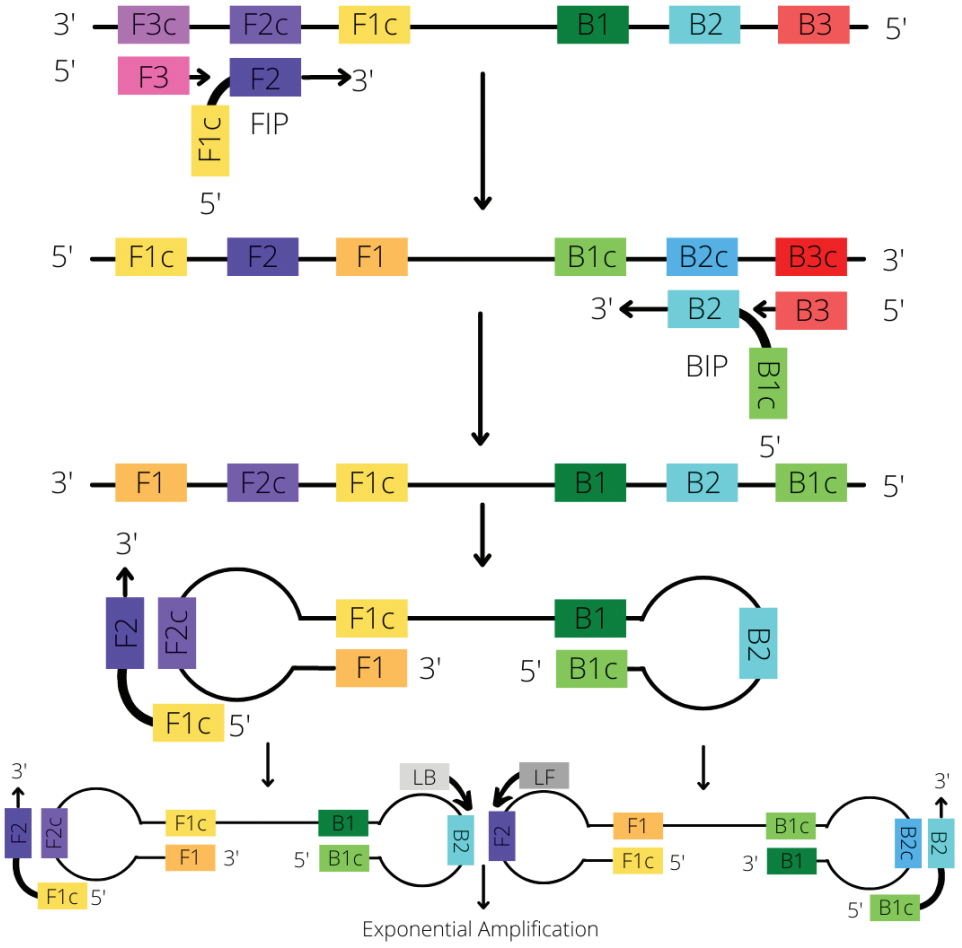

Figure 2. Loop mediated amplification mechanism. Two inner primers consisting of the F3 and forward inner primer (FIP) and two backward primers, the B3 and backward inner primer (BIP) are used to target 6 regions. Additionally, loop primers are often used to accelerate the reaction, denoted here as LF (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.

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 (Liu et al. 2017b; Hardinge and Murray 2019). 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 target sequences utilising a fluorometer to detect as few as 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).

Table 1 lists the advantages and disadvantages of LAMP in contrast to PCR, which is the most generally applicable amplification method in environmental biosecurity (McGraw et al. 2011). However, sample type, equipment availability, DNA isolation and handling, downstream analysis and intended application will influence suitability. PCR methods, as applied to environmental biosecurity, are also rapidly developing to incorporate field detection using compact and mobile thermocyclers with integrated molecular probes, exemplified by comparisons conducted for TaqMan assays and LAMP assays (Trujillo-González et al. 2022). Perhaps the main advantage of LAMP in contrast to advancements in PCR, is the ease of integration due to the lack of thermocycling requirements. Denaturation is facilitated by a strand displacing polymerase, and amplification is conducted at a constant (*c.* 65 °C) incubation temperature (Nagamine et al. 2001). These key features drastically lower the equipment requirements to a simple heat block, pipettes and an appropriate sample handling space that facilitate the integration of this technique into onsite contexts without large financial burdens to the end-user.

Thus far the application of LAMP has primarily focused on cases in which high quality DNA is available from sample types such as tissue and whole specimens. However, many environmental biosecurity samples include degraded DNA (e.g. animal material that has been cooked, processed or treated with chemicals) or environmental DNA (e.g. faecal samples, swabs of empty containers, water, soil or air samples). LAMP could function sub-optimally in scenarios which commonly involve highly degraded template DNA due to the need for more than two primers and environmental samples that contain DNA from multiple sources. As such we recommend further research into LAMP suitability for a greater range of taxa and sample types including degraded DNA and environmental DNA subject to complex and varied environmental conditions as this influences DNA decay rates (Andruszkiewicz Allan et al. 2021).

Table 1. Advantages and disadvantages of LAMP compared to the most common amplification method in environmental biosecurity, the Polymerase Chain Reaction (PCR).

Method	Advantage	Disadvantage
LAMP	High specificity (4–6 primers), with probe capacity.	Challenging design parameters.
	No heat denaturation step required.	Degraded DNA may prevent primer annealing.
	Isothermal incubation, with low resource requirements.	Limited downstream applications for amplicons.
	Colorimetric, turbidimetric, fluorogenic real-time and endpoint detection capacity.	
	Speed of reaction (generally <60 minutes).	
	High tolerance to inhibitors commonly encountered in field samples.	
PCR	Widely available	Thermocycling required, including high temperature for separation of strands, to facilitate primer binding
	Downstream capacity, including sequencing.	Expensive thermocycling equipment required, often restricted to a dedicated laboratory space.
	Low cost of reagents and primer synthesis.	Lower specificity due to only two primers, however probes can be integrated but at much greater cost to the end-user.
		Visualisation of results requires fluorometer or gel electrophoresis.

LAMP research for environmental biosecurity applications

The most common application of DNA based detection in wildlife forensic science investigations is species detection and identification (Linacre 2021). As a result, LAMP has been applied to environmental biosecurity detection cases relating to: (1) identification of adulterated animal products (Cho et al. 2014; Liu et al. 2019; Nikunj and Vivek 2019; Sul et al. 2019); (2) detection of conservation significant species and disruption of illegal wildlife trade (Yu et al. 2019; But et al. 2020; Wimbles et al. 2021); and (3) biosecurity screening (Blaser et al. 2018b; Kyei-Poku et al. 2020), and detection of invasive species (Williams et al. 2017; Rizzo et al. 2021; Vythalingam et al. 2021); including disease monitoring (Sahoo et al. 2016). Additionally, LAMP has a strong presence in bacterial and viral point-of-care detection methods research (Nguyen et al. 2019b; Kashir and Yaqinuddin 2020).

(1) 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 environmental biosecurity concerns of illicit harvesting and subsequent commercial fraud. The assay is tolerant to inhibitors such as pigments, dyes, or other fur components, with the authors highlighting its potential role as an on-site species identity test, without costly requirements or specialist equipment (Yu et al. 2019). 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, tested on meat samples with a detection limit of 10 pg of DNA (Kumari et al. 2019). The underlying drivers behind this research interest are varied and include 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 from tissue samples, 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 from tissue samples, 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 for identification of tissue samples 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 trade in bushmeat is 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.

(2) Illegal wildlife trade and conservation monitoring

LAMP has been showcased for field-based detection of illegal trade in shark fin products, which can be directly applied to enforcing environmental biosecurity regulations and CITES obligations; as rapid LAMP detection has been developed for twelve CITES-listed shark species (But et al. 2020). The assays include primers that target the *CO1* and *NADH2* sequences and can detect all twelve species individually from tissue sample within an hour at constant temperatures (But et al. 2020). The cost of each LAMP reaction was *c.* US$0.6 compared with *c.* 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 that 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 and the prevention of wildlife crime have 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); the consequent nefarious trade has received widespread 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 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 (Kumar et al. 2012; Ghosh et al. 2019) 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, particularly when indistinguishable tissue samples are the only form of remaining evidence.

(3) 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 (Williams et al. 2017; Vythalingam et al. 2021). The emphasis on monitoring primarily concerns invertebrate pests, largely when all or part of the organism is available, as demonstrated by the development of LAMP assays for point of entry detection (Blaser et al. 2018a).

Border surveillance of emerging insect incursions

A range of LAMP assays have been developed for multiple insect species commonly of environmental biosecurity concern (Table 2). 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, studies have tested detection in mixed samples, including 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). Another horticultural focus is the detection of fruit fly species (Huang et al. 2009; Blaser et al. 2018a; Blaser et al. 2018b; 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, indicated 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 contrasts 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 (c. 10 mins to result) (Agarwal et al. 2022).

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

Table 2. Summarised LAMP assays as applied to environmental biosecurity of high-risk insects. Includes the species name, the gene that the LAMP primers target, the tested sample types, 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	Tested sample types	Limit of detection	Time to detection	Source
Emerald ash borer	*CO1*	Adults, larvae, eggs, larval frass	0.1 ng	30 min	(Kyei-Poku et al. 2020)
Red fire ant	*CO1*	Whole specimen	N/A	90 min	(Nakajima et al. 2019)
Species belong to genera *Bactrocera* and *Zeugodacus* and *Bemisia tabaci* and *Thrips palmi*	*CO1*	Adults, larvae, and 1 mm^3 of larval tissue	N/A	60 min	(Blaser et al. 2018a)
Aedes mosquito species	*ITS1* and *ITS2*	Adult or larval stage specimen and eggs	N/A	60 min	(Schenkel et al. 2019)
Walnut twig beetle	28S rRNA	Adults and frass	1.3 pg and 6.4 pg for adults and frass, respectively	<30 min	(Rizzo et al. 2021)
Fall army worm	*CO1*	Adult and larval specimen	2.4 pg	<20 min	(Agarwal et al. 2022)
Fall army worm	*CO1*	Larvae	24 pg	<30 min	(Congdon et al. 2021)
Fall army worm	*tRNA* coding region between *ND3,* and *ND5*	Larval tissue	10 pg	90 min	(Kim et al. 2021)
Khapra Beetle	18s rRNA	Adults and larvae	1 fg	<25 min	(Rako et al. 2021)
New Guinea fruit fly	*CO1, EIF3L*	Tissue (3 fly legs)	10 copies for COI and 1000 copies for BtrivEIF3L	<25 min	(Starkie et al. 2022)

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 is the role that 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 (Liang et al. 2006; Lockwood et al. 2019), with invasiveness positively associated with commercial success of pets in trade (Gippet and Bertelsmeier 2021). 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 that 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 local aquariums and pet shops for the purpose of LAMP development. DNA was extracted from caudal fin cuttings, with dilutions used to assess limits of detection. The resulting highly sensitive assays utilised 5 separate species-specific primer sets with a detection limit of between 0.02 pg and 2×10^{-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 a 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 (Williams et al. 2017; Carvalho et al. 2021). 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 0.0001 pg. A further two *CO1* assays targetted quagga and zebra mussels seperately, with a sensitivity of 0.001 pg and 0.01 pg respectively (Williams et al. 2017). Sample types included grab and concentrated surface water samples, containing both free DNA as well as larger cells and particulates, such as veligers, eggs, or seeds (Williams et al. 2017). These sample types were subject to direct amplification without DNA extraction, illustrating the tolerance of LAMP to environmental inhibitors (Williams et al. 2017). A subsequent novel zebra mussel assay targetted the *CO1* gene with a detection limit of 1.12 pg when tested against meat and water samples, 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 European rivers, protecting consumers against mislabelled eel consumables and could serve a role in ecological studies (Spiel-

mann 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 specificity; both assays had a limit of detection of 500 pg (Spielmann et al. 2019). Sample types included muscle tissue of smoked processed fish and single eggs from *A. aguilla* as a proxy for on-site detection (Spielmann et al. 2019).

Current conventional aquatic eDNA monitoring methods are rapidly developing, including integration of qPCR assays with specialised eDNA sampling methods and miniaturised infield thermocyclers (Thomas et al. 2020). LAMP assays can function either in parallel to current best practise qPCR methods or in low resource contexts where isothermal end-point reaction conditions are most suitable. LAMP assays developed to target Quagga and Zebra mussels, are some of the few assays which have been tested against samples subject to environmental conditions and indicated tolerance and suitability within their respective contexts (Williams et al. 2017; Carvalho et al. 2021). The use of LAMP as an emerging surveillance technique for biosecurity officers and wildlife managers could thus be instrumental for conducting routine monitoring programs and detecting high risk environmental biosecurity threats.

Health and disease: detection and prevention

An often-overlooked component of environmental biosecurity 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 in the emergence of zoonotic diseases (Hilderink and de Winter 2021). Consequently, LAMP-based detection could be highly suitable to the detection of domestic and wildlife related introductions of novel diseases, strengthening environmental biosecurity. There are, in fact, a myriad of studies that explore LAMP based detection of COVID-19 to address on-site testing capacity of this global health concern (Augustine et al. 2020; Kashir and Yaqinuddin 2020; Dewhurst et al. 2022). Extending this research to a broader range of emerging pathogens and hosts 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 Illegal Wildlife Trade (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 indicated 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 that 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 °C (Chen et al. 2016). Storage and shelf life are additional considerations that are often omitted from publications concerning field-ready LAMP. These are, however, conditions that 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 that 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 57–67 °C 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 that outlined the assays development highlighted the superior 10-fold sensitivity of LAMP when contrasted 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 stated 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 incursion detection tool at transnational points of entry, particular for live wildlife and domestic animal trade. This has been exemplified with emerging concerns of Foot and Mouth Disease (FMD) incursions globally, and several LAMP assays (Dukes et al. 2006; Bath et al. 2020) developed to address rapid screening. Circumventing resource and time inten-

sive identification methods by utilising LAMP as a point of care diagnostic system could additionally reduce the biosecurity risk posed by potential disease carrier incursions, particularly by reducing the time to outcome and required resources.

Conclusion: LAMP Integration for biosecurity monitoring and surveillance

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 (Zhou et al. 2014; Nguyen et al. 2019b; Wan et al. 2019). The ability to conduct LAMP in resource constrained environments where traditional PCR-based technologies may not work, has shown to be highly advantageous in a low resource field-based environment (Raele et al. 2019; Wimbles et al. 2021). 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.

Despite the substantial body of literature, LAMP is yet to receive widespread uptake in research and applied environmental biosecurity monitoring, detections, and enforcement. In the face of globalisation, applying these techniques to DNA-based monitoring in environmental biosecurity contexts is well suited. LAMP as a point of care technology presents great potential for the onsite detection of trace DNA relating to intentional (trafficking) or unintentional (stowaway) transport of live animals, wildlife parts, medicines, and ornamental derivatives. The capacity for LAMP to bridge gaps relating to on-site biosecurity practices, 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) allow 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 function in parallel to PCR, providing widespread molecular diagnostic capacity for biosecurity scenarios.

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