Isothermal amplification techniques for FBP detection. A focus on LAMP application for the detection of *Toxoplasma gondii* in different food matrices.

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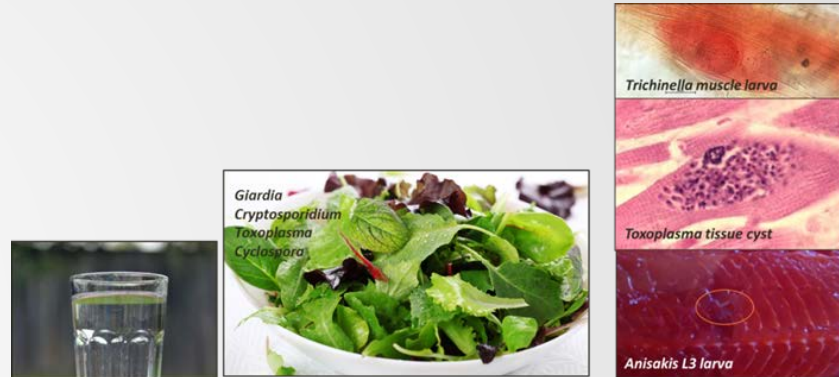
Detection of parasite in food: technical and conceptual challenges

➢A major shortcoming in food parasitology is the **lack of well standardized protocols to recover, detect, and identify sparsely distributed parasites in food and in the entire food chain**

- Toxoplasma tissue cysts in meat
- Anisakis larvae in fish flesh
- Protozoan (oo)cysts in water and vegetable



➢Parasites load in foodstuff and water is different from the load in clinical samples (i.e. Cryptosporidium oocyst in stool vs vegetables)

➢Large number of **different matrices to analyze**

➢Unlike most bacteria, parasites cannot be enriched by culturing; therefore, **an optimal recovery process for parasite from suspected foods is critical**.

➢**Diagnostics are still based on macro- and microscopic inspection, and require highly qualified personnel.**

➢**Nucleic acid based test improve sensitivity and specificity while also reduce detection time**

➢Necessity to **detect very low number of organisms**

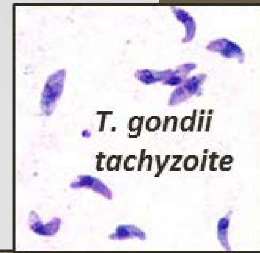
Diagnostic solutions: qPCR, PCR, Microarray, Loop-mediated isothermal amplification (LAMP)

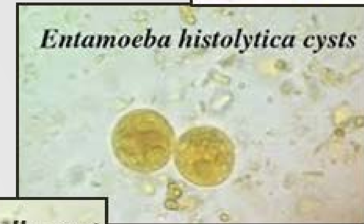
➢Necessity to **distinguish between human and non-human pathogens** and/or trace the source of infection **(outbreak investigations)**

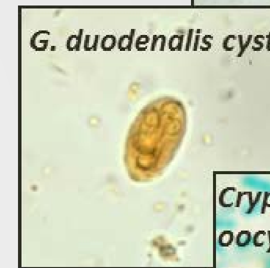
Diagnostic solutions: PCR (polymorphic loci), qPCR, Microarray, Single-strand conformation polymorphism (SSCP), Nucleic acid sequence based amplification (NASBA)

➢Necessity to **distinguish between viable (infectious) and non-viable organisms**

Diagnostic solutions: RT-PCR, RT-LAMP, Nucleic acid sequence based amplification (NASBA), FISH, but also the use of vital dyes and bioassays







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Commonly used nucleic acid-based assays

➢**Polymerase Chain Reaction (PCR)**

- Single-Step PCR
- Nested-PCR
- Multiplex PCR
- PCR-RFLP (Restriction fragment length polymorphism)
- Quantitative PCR (Real-time PCR or qPCR)
- Capillary Electrophoresis-Single Strand Conformation polymorphism (CE-SSCP)
- Random Amplified Polymorphic DNA (RAPD)-PCR

Requirements:

➢thermal cycler (or more sophisticated instruments with detector)

➢high quality nucleic acid preparation

➢technical expertise of personnel

➢cold chain to preserve heat-labile reagents

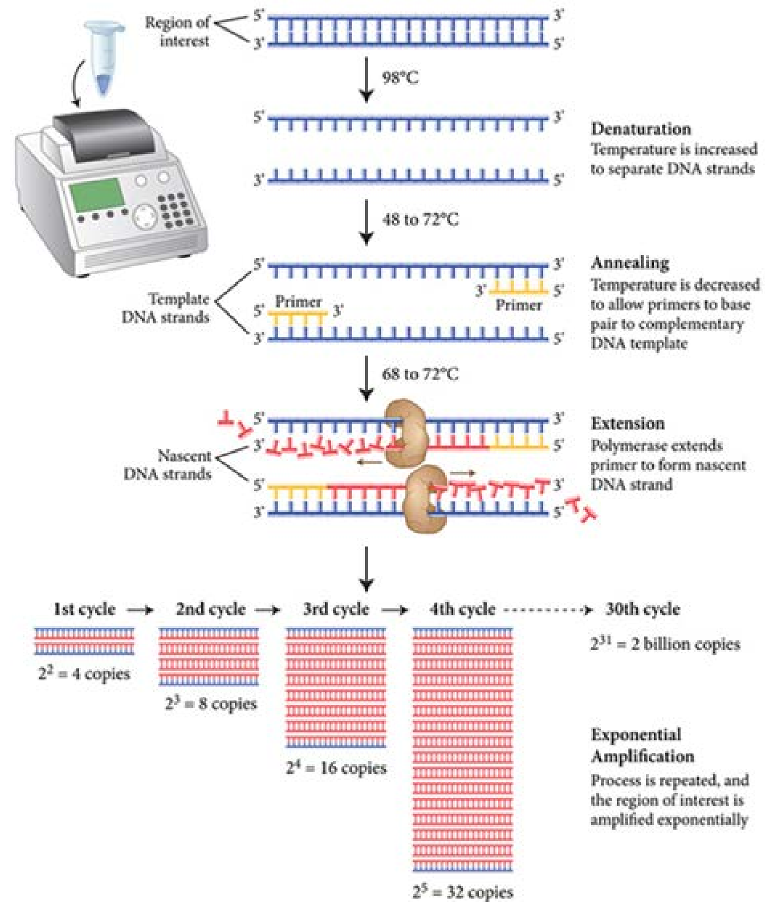
Drawbacks:

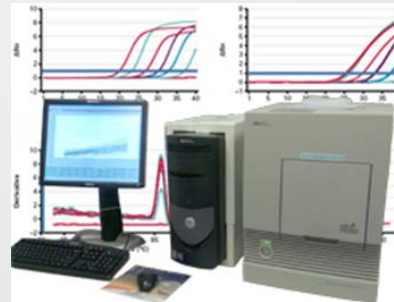
➢time consuming

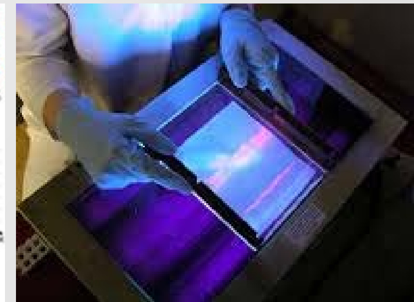
➢ high cost

➢easy contamination

➢susceptibility to false amplification







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Non-PCR nucleic acid-based assays

- **Loop-mediated isothermal Amplification (LAMP)**
- **Nucleic Acid Sequence-Based Amplification (NASBA)**
- **Recombinase polymerase amplification (RPA)**
- **Strand displacement amplification (SDA)**
- **Rolling circle amplification (RCA)**
- **Helicase-dependent amplification (HDA)**

Isothermal amplification techniques have several advantageous qualities:

- short reaction times
- high sensitivity
- not readily affected by inhibitory substances that are often abundant in complex matrices (highly pure nucleic acids not required)
- simple heating device is sufficient to run the reactions (not need of expensive instruments)
- are well suited for incorporation into lab-on-a-chip devices
- amplification feasible in low-resource settings or at the point-of-need

Isothermal technique	Target	Primers needed	Initial heating	Incubation temperature (°C)	Amplification time (min)	Limit of detection (copies)	Multiplexing	Lyophilised reagents	FDA approved tests
NASBA	RNA	2	No	41	60–180	1	Yes	Yes	Yes
SDA	DNA	4	Yes	30–55	60–120	10	Yes	No	Yes
RCA	DNA/RNA	1	Yes	30–65	60–240	10	No	No	No
LAMP	DNA	4–6	Yes	60–65	60	≈ 5	Yes	No	Yes
HDA	DNA	2	No	65	30–120	1	Yes	No	Yes
RPA	DNA/RNA	2	No	37–42	20–40	1	Yes	Yes	No

Lobato and O'Sullivan, Trends in Analytical Chemistry, 2018

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European Union Reference Laboratory for Parasites

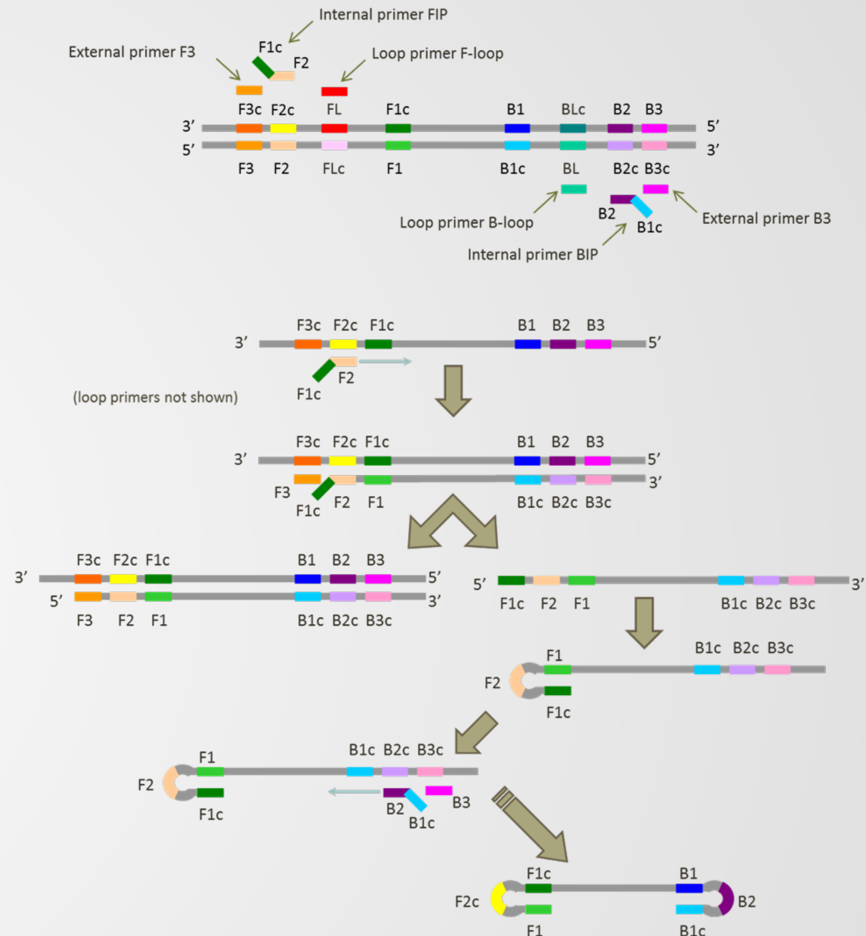
Loop-Mediated Isothermal Amplification (LAMP)

- To increase the sensitivity and specificity, amplification is performed under isothermal conditions employing 4-6 primers spanning 6-8 distinct sequences on the target DNA.

- It uses a robust polymerase (BST), originally from *Geobacillus stearothermophilus* to amplify target DNA (or RNA by inclusion of reverse transcriptase) proceeding to an autocycling strand displacement mechanism, at a **constant temperature (61-65°C)**

- Producing detectable product in approximately 30-60min (amplify from a single copy to 10^9).

- Production of magnesium pyrophosphate during the reaction increases turbidity and allows naked eyes detection of end product (with or without the addition of fluorescent dye)



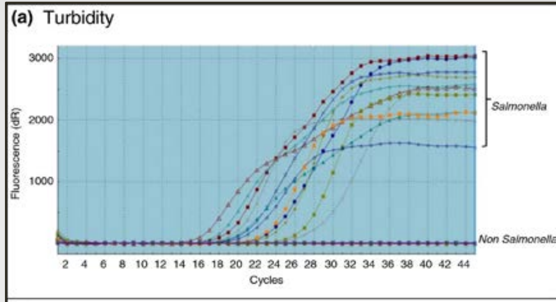
Notomi et al. 2000. Nucleic Acids Research 28:e63.

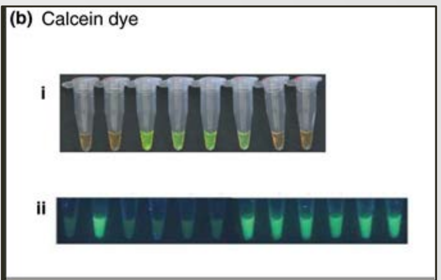
Loop-Mediated Isothermal Amplification (LAMP)

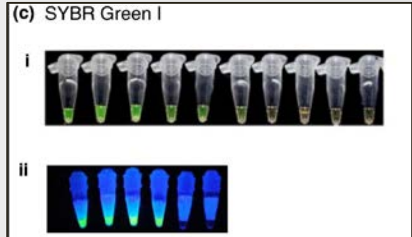
Advantages

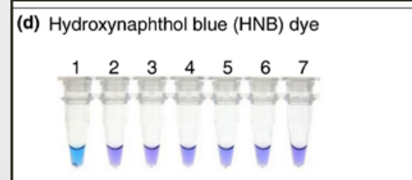
- Bst polymerase: 5'→3' DNA polymerase activity and strand-displacement activity. Not affected by inhibition due to the secondary structure of DNA
- Amplification may be performed simply in a heating block or water bath.
- Very rapid amplification (30-60 minutes)
- High specificity
- **Tolerance of polymerase inhibitors** can be applied to nucleic acid extracts of unpurified samples or even to samples without nucleic acid extraction
- Suitable for synthesis of DNA strands having high GC content.
- Amplification and detection may be carried out in one single tube.
- Can amplify RNA sequences through combination with reverse transcription.
- Versatile detection (turbidimer, fluorescent dye, gel electrophoresis, flow-dipstick).

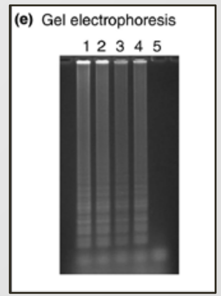
LAMP detection using Loopamp Realtime Turbidimeter LA-500. Increase in graph indicate positive LAMP amplification (indicated with *Salmonella*) whereas no amplification curve indicates negative LAMP amplification (indicated with non *Salmonella*) (image adapted from Mashooq *et al.*, 2016)
Color change using DNA intercalating dye, Calcein. Color changes from orange to green indicate positive LAMP reaction (i.) (image adapted from Zhou *et al.*, 2014). The reaction between free calcein and magnesium ions intensified the fluorescence of LAMP product (ii.) (image adapted from Chen *et al.*, 2014).
Naked eye observation using DNA fluorescence SYBR Green I dye. Notable color changes from orange to green, indicate positive reaction (i., image adapted from Chen *et al.*, 2017). The LAMP positive tubes show bright green fluorescence, negative tubes remain dark (ii., image adapted from Tao *et al.*, 2011).
LAMP end-point detection using HNB dye as visual indicator. Positive LAMP amplification changes the violet (tube labeled 2–7) to sky blueacolor (tube labeled 1), image adapted from Luo *et al.*, 2014.
Gel electrophoresis with typical ladder like pattern (positive indicated by 1-4, negative indicated 5)











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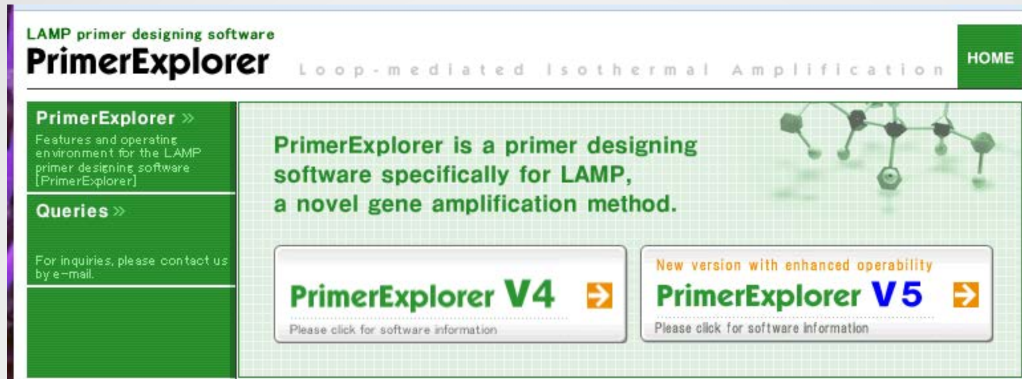
❑ Disadvantages

- Primer design quite complicated, a software is needed.



- Inadequate for the detection of unknown or unsequenced targets
- Challenges to using LAMP for multiplex assays in a single sample
- Challenges in quantitation of target DNA.
- High risk of carryover contamination which often leads to false-positive results in negative controls. Procedure for in-tube detection of DNA amplification are necessary.
- In case contamination, perform LAMP in a laminar flow hood and use separate pipettes and filtered tips.
- For procedures that involved opening of the reaction tubes, carry out LAMP in an isolated room

Table 1
Overview of LAMP assays for the detection of bacterial pathogens and toxicants as well as mycotoxinogenic fungi in food.

Target species		DNA target	Assay type	Food sources	Detection limit	Reference
Gram negative bacterial pathogens						
Salmonella	spp.	*invA* gene	LAMP[a]	Cilantro (coriander leaves), lettuce, parsley, spinach, tomato, jalapeno pepper	2 cfu/25 g	Zhang et al., 2011
	spp.		LAMP	ND[b]	100 fg DNA/reaction	Wang et al., 2008b
	spp.		LAMP	Milk	10^2 cfu/ml	Zhu et al., 2008
	spp.		LAMP	Raw milk	10^8 cfu/ml	Wang et al., 2008a
	spp.		LAMP kit[c]	Egg, milk, yogurt, omelet, hamburger, raw pork, beef, chicken, roast beef, lettuce, vegetable salad, watermelon, apple juice, spinach sauté, cake, blood and human stool of healthy human	10^2 cfu/ml	Ueda and Kuwabara, 2009
	spp.		EMA[d] LAMP	ND	100 fg DNA/tube	Lu et al., 2009
	spp.		PMA[e] LAMP	Cantaloupe, spinach, tomato	6.1×10^3–6.1×10^4 cfu/g	Chen et al., 2011a
	spp.		*in situ* LAMP	Eggshell	1 cfu/cm^2	Ye et al., 2009, 2011
	spp.		LAMP kit	Poultry, livestock, other raw meat, dairy products	10^2 cfu/ml	He et al., 2010
	enterica		LAMP	Liquid egg	2.2 cfu/test tube	Hara-Kudo et al., 2005
	enterica serovar *Enteritidis*		Reverse-transcription LAMP[f]	Liquid whole eggs	10^7 cfu/25 ml; 10^6 cfu/25 ml. after enrichment	Techathuvanan and D'Souza, 2012
	Typhimurium		Reverse-transcription LAMP	Pork products	10^6 cfu/25 g; 10^2 cfu/25 g after enrichment	Techathuvanan et al., 2010
	Typhimurium		Real-time reverse transcription LAMP	Pork processing environment	10 cfu/ml	Techathuvanan et al., 2011
	Typhimurium	*hisJ* gene	LAMP	Fresh pork, whole chickens, green vegetables	16 cfu/reaction	Zhang et al., 2012
	O9 serogroup	insertion element IS200/IS1351 gene	LAMP	Chicken	10^3 cfu/ml	Okamura et al., 2008
	O9 serogroup	*phoP gene*	LAMP	Meat, milk	35 cfu/250 ml	Li et al., 2009
	serotype D	*prt* gene	LAMP	Chicken meat	10 cfu/reaction	Ravan and Yazdanparast, 2012
	serovar *Enteritidis*	sdf1 region	LAMP	ND	4 copies/µl	Yang et al., 2010
Escherichia coli		Enteroaggregative *E. coli* (EAEC)		*aggR* gene	LAMP	Radish sprouts, broccoli sprouts, ready-to-eat salads, ground pork and beef
	6.30 cfu/reaction	Yokoyama et al., 2010				
	serogroup O157	*rfbE* gene	LAMP	Raw milk	410 cfu/ml	Wang et al., 2009b
	serogroup O157, viable cells		EMA-LAMP	Raw milk	440 cfu/ml	Wang and Huo, 2012
	serotype O157:H7		LAMP	Pork meat	10 cfu/ml	Jiang et al., 2012
	serotype O157	*rfbE* gene *stx1* gene *stx2* gene	LAMP	ND	100 fg/reaction 100 fg/reaction 10 fg/reaction	Zhao et al., 2010a
	serotype O157:H7	*rfbE* gene *fliC* gene *stx2* gene	LAMP	ND	26 cfu/reaction 26 cfu/reaction 26 cfu/reaction	Zhu et al., 2009a
	serotype O157:H7	*rfbE* gene *fliC* gene	LAMP	Meat	1.8 cfu/g 1.8 cfu/g	Liu et al., 2011a
	Serogroups O157, O26 and O111	*stx1*gene *stx2* gene	Duplex[g] LAMP	Beef, radish sprouts, alfalfa sprouts	0.7 cfu/reaction	Hara-Kudo et al., 2007; Hara-Kudo et al., 2008a
	STEC serotypes		Real-time duplex LAMP	ND	10^2 cfu/reaction	Kouguchi et al., 2010
wzy gene	Shiga toxin-producing *E. coli* (STEC)	*wzx* gene	LAMP	Ground beef, beef trim, lettuce, spinach	10–20 cfu/25 g after enrichment	Wang et al., 2012a
Vibrio		*parahaemolyticus*		*gyrB* gene	LAMP	Shell fish
				87 cfu/g	Tian et al., 2011	
				tdh gene	LAMP	Oysters
				1 cell/reaction	Nemoto et al., 2009	
				tlh gene	LAMP without loop primers	ND
					10 cfu/reaction	Li et al., 2008
					LAMP	Shrimp
					5.3×10^2 cfu/g	Yamazaki et al., 2008a,b,c
				LAMP–LFD[h]	Shrimp	1.8×10^3 cfu/g
				Prompamorn et al., 2011		
				EMA-LAMP	ND	9 fg/reaction
				Wang et al., 2012b		
				tlh gene	LAMP	ND
				100 fg DNA/reaction		
	tdh gene	100 fg DNA/reaction		Zhao et al., 2010a,b		
	trh gene	1 pg DNA/reaction				
	tdh gene *trh1*gene *trh2* gene	LAMP	Shrimp, seafood samples	0.8 cfu/reaction 21.3 cfu/reaction 5.0 cfu/reaction	Yamazaki et al., 2010; Yamazaki et al., 2011	
	rpoD gene *toxR* gene	LAMP	Seafood	3.7 cfu/reaction 450 cfu/reaction	Nemoto et al., 2011	
	toxR gene	LAMP	Oyster	1.1×10^5 cfu/g	Chen and Ge, 2010	
	vulnificus	*rpoS* gene	LAMP–LFD	Oyster	11 cfu/reaction	Surasilp et al., 2011
		vcg gene	Real-time LAMP[i]	Raw oyster	2.5×10^3 cfu/g	Han et al., 2011
		vvh gene	LAMP	Oyster	10^7 cfu/g	Han and Ge, 2008
Real-time LAMP			Raw oyster	1–10 cfu/reaction	Han and Ge, 2010	
cholerae	*ct* gene	LAMP	Human stool	1.4 cfu/reaction	Yamazaki et al., 2008a,b,c	
	ompW gene	LAMP	Shrimp	2.2×10^4 cfu/g	Srisuk et al., 2010	
		LAMP	Freshwater, human stool, milk	1.6×10^3 cfu/ml 1.6×10^4 cfu/g	Ke et al., 2009	
	mdh gene	LAMP	Shrimps, crabs, oysters, meat and human diarrhea complex	32 cfu/g	Xu et al., 2010a,b	
cholerae serogroup O139	*wbfR* gene	LAMP	ND	63 cfu/reaction	Zhu et al., 2009b	
alginolyticus	*16S rRNA gene*	LAMP	Fish, shrimps, clams	10^3 cfu/ml	Xu et al., 2012	
	ompK gene	LAMP	Water	38 cfu/ml	Ding et al., 2009	
Yersinia enterocolitica		*gyrB* gene	LAMP	Pork	65 cfu/ml	Gao et al., 2009
		PhoP gene	LAMP	Pork meat, milk	2.2 cfu/100 g	Li et al., 2010b
Cronobacter sakazakii (*Enterobacter sakazakii*)		*16S-23S rRNA* gene	LAMP	Infant formula	1.2 cfu/100 g	Liu et al., 2009
			LAMP	Infant formula	1.1 cfu/g	Hu et al., 2009
Shigella spp.		*ipaH* gene	LAMP	Raw milk	6–8 cfu/ml	Wang et al., 2009c
Shewanella putrefaciens (syn. *Pseudomonas putrefaciens*)		ITS region within rRNA gene	Reverse transcription LAMP	Fish	5.4 copies/reaction	Li et al., 2012
	Campylobacter		*jejuni*	*cj0414* gene *gufA* gene	LAMP-kit	Human stool
			5.6×10^3 cfu/g 4.8×10^3 cfu/g	Yamazaki et al., 2008b		
coli	*cj0414 gene gufA gene*	Chicken meat	7.9 cfu/reaction 3.8 cfu/reaction	Yamazaki et al., 2009		
Brucella abortus		*BCSP31* gene	LAMP	Liver and spleen tissues of mice, milk	4.9×10^4 cfu/ml	Ohtsuki et al., 2008
Leptospira spp.		*lipL41* gene	Real-time LAMP	ND	100 copies/reaction	Lin et al., 2009
		16S r RNA gene	LAMP	Blood samples	10 copies/reaction	Sonthayanon et al., 2011
		16S r RNA gene	LAMP	Urine from field rats, farmed pigs, farmed buffaloes	10^3 cfu/ml	Koizumi et al., 2012
Burkholderia pseudomallei		*BPSS 1406* gene	LAMP	Sputum, urine, throat swabs, blood, pus	38 copies/reaction	Chantratita et al., 2008
Gram positive bacterial pathogens						

Table 1 (*continued*)

Target species		DNA target	Assay type	Food sources	Detection limit	Reference
Staphylococcus aureus		*femA* gene	LAMP	Raw milk	8–9 cfu/ml	Wang et al., 2009a
			LAMP	ND	8–9 cfu/ml	Li et al., 2010a
		nuc gene	LAMP	Infant formula, meat, milk	10.3 cfu/reaction	Yang et al., 2011
		*sa442*gene	LAMP	Meat samples, eggs, dairy products	42 cfu/g	Xu et al., 2010a,b
		sea gene	LAMP	Pork meat	10^3 cfu/g	Suwanampai et al., 2011
		sea gene	LAMP	ND	2.3 cfu/reaction	Goto et al., 2007
		seb gene			30.2 cfu/reaction	
		sec gene			6.9 cfu/reaction	
		sed gene			38 cfu/reaction	
		sea gene	LAMP	Street food, fermented food, bakery products, milk, milk products	100 fg/reaction	Nagarajappa et al., 2012
		seb gene			100 fg/reaction	
		sec gene			100 fg/reaction	
		sed gene			100 fg/reaction	
Listeria monocytogenes		*hlyA* gene	LAMP	Chicken	2.0 cfu/reaction	Tang et al., 2011
			LAMP	ND	100 cfu/reaction	Wang et al., 2011
			real-time LAMP	Retail food	6 cfu/reaction	Shan et al., 2012
			PMA-LAMP	Spiked chicken, pork, ground beef, milk powder	100 cfu/ml	Wan et al., 2012
		iap gene	LAMP	Raw milk	186 cfu/ml	Wang et al., 2010
		invA gene	LAMP	ND	ND	Yan et al., 2009
Bacillus cereus		*hblA* gene	FTA[j]-LAMP	Milk	57 cfu/ml	Qi et al., 2009
		16 rRNA gene	LAMP	Milk	1 cfu/ml	Liu et al., 2011b
		cesA gene	LAMP		11 cfu/ml	
Clostridium	*botulinum*	*BoNT/A* gene	Real-time LAMP	Fish, honey	1 cfu/reaction	Sakuma et al., 2009
		BoNT/B gene	Real-time LAMP		10 cfu/reaction	
	perfringens	*cpe* gene	LAMP	Meat	3 cfu/g	Kaneko et al., 2011
	Alicyclobacillus		*acidoterrestris*	*16S rRNA* gene	LAMP	Apple juice
22.5 cfu/ml	Chen et al., 2011a,b					
Mycotoxin producers and fungal spoilage organisms						
Fusarium graminearum		*gaoA* gene	LAMP	Wheat	2 pg DNA/reaction	Niessen and Vogel, 2010; Abd-Elsalam et al., 2011
Fusarium	*cerealis*	*hyd5* gene	LAMP	Barley	0.74 pg/reaction	Denschlag et al., 2012
	culmorum graminearum		Real-time LAMP	Barley	0.5% infection level	Denschlag et al., 2013
Aspergillus	*flavus*	*acl1* gene	LAMP	Peanut, Brazil nut, coffee bean	2.4 pg/reaction	Luo et al., 2012
	nomius	*amy1 gene*	LAMP	Peanut, Brazil nut, coffee bean	7.6 pg/reaction	
	parasiticus	*amy1 gene*	LAMP	Peanut, Brazil nut, coffee bean	20 pg/reaction	
	carbonarius (OTA producing strains)	*Acpks* gene	LAMP	Pure culture mycelia	50 pg/reaction	Storari et al., 2013
	niger (OTA producing strains)	*Anpks* gene	LAMP	Pure culture mycelia	50 pg/reaction	
Dekkera	*anomala bruxellensis custersiana*	ITS region within 5.8SrRNA gene	LAMP	Distilled water, wine, beer	10 cfu/ml 10 cfu/ml 10 cfu/ml	Hayashi et al., 2007
	Brettanomyces				*naardenensis*	
10 cfu/ml						

[a] LAMP – loop-mediated isothermal amplification under standard conditions as described in Notomi et al. (2000).
[b] ND – not defined.
[c] Commercially available.
[d] Ethidium monoazide.
[e] Propidium monoazide.
[f] Reverse-transcription LAMP – master mix contains reverse transcriptase to provide a cDNA target for amplification during LAMP.
[g] Duplex LAMP – two primer sets combined in one LAMP assay.
[h] LFD – lateral flow device.
[i] Real-time LAMP – amplification of DNA is monitored continuously during the LAMP reaction by measuring turbidity or fluorescence.
[j] LAMP assay using FTA (Fast Technology for Analysis of nucleic acids) membrane to bind target DNA.

Use of LAMP to detect bacteria and fungi in food

L. Niessen et al. / Food Microbiology 36 (2013) 191–206

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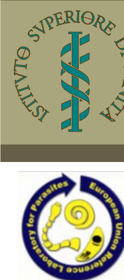


Toxoplasma detection by LAMP in food and environmental samples

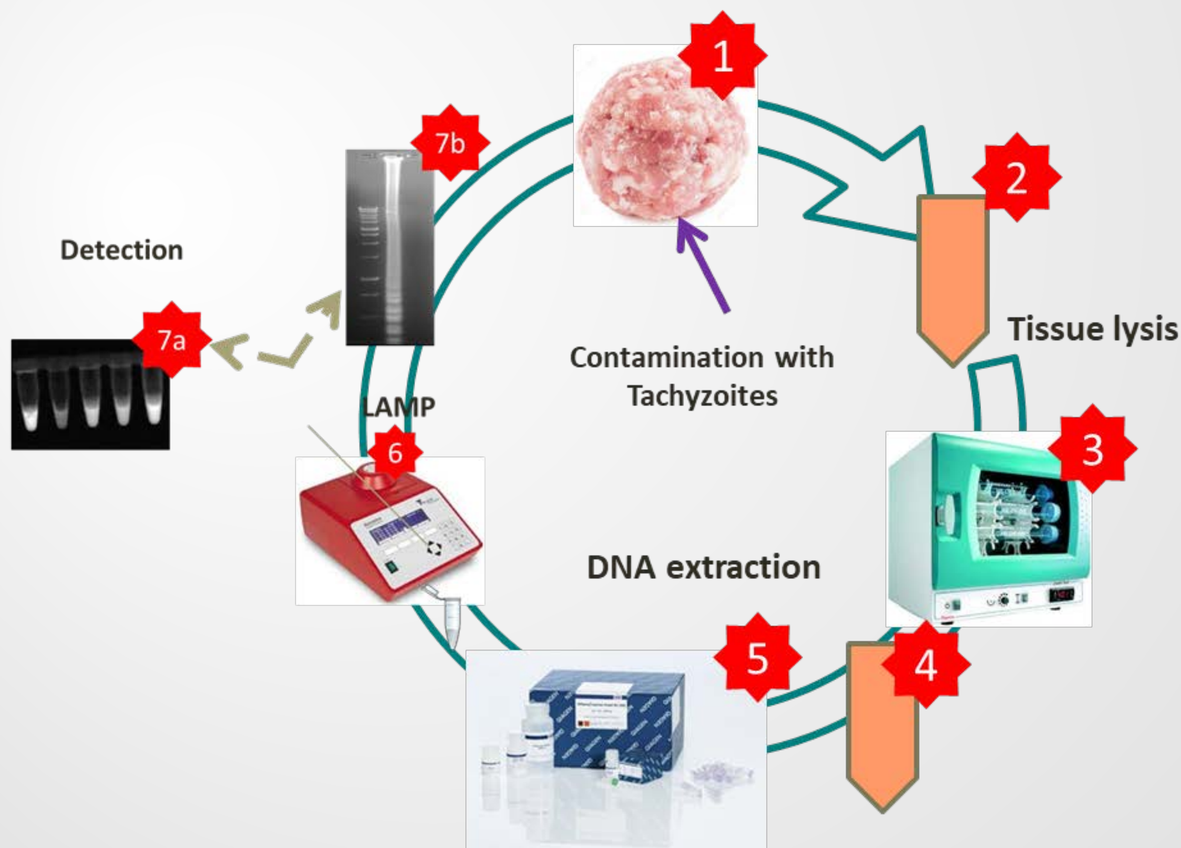
Target gene	sample type	parasite stage	sensitivity	comparison	ref.	year
529 bp repetitive region	Leafy green vegetable	oocyst	10 oocyst/50 g lettuce	LAMP=qPCR	Lalle et al., Food Microbiol. 2018 Apr;70:137-142	2018
529 bp repetitive region	formalin-fixed, paraffine embedded or frozen tissue from wild carnivores	tissue cysts			Suleman et al., J Vet Diagn Invest. 2016 Sep;28(5):536-42.	2016
SAG2 gene (single copy gene)	CNS homogenate and skeletal muscle of wild and farmed animal (25mg)	tissue cysts	10 fg (~0.1 tachyzoite)	LAMP>PCR	Trisciuoglio et al., J Vet Diagn Invest. 2015 Nov;27(6):754-7.	2015
ITS-1 (multicopy locus)	diaphragm samples obtained from pig	tissue cysts	0.9 fg	LAMP>PCR	Zhuo et al., Veterinary Parasitology (2015)	2015
SAG1	blood from experimentally infected pigs	tachyzoite (acute toxoplasmosis)	100 fg (~1 tachyzoite)	LAMP<qPCR	Wang et al., Korean J Parasitol. 2013 Oct;51(5):573-7.	2013
MIC3	soil	oocysts in the soil	5 tachyzoites in soil	LAMP>PCR	Du et al., Veterinary Parasitology 184 (2012) 141–146	2012
B1 (multicopy gene)	urine from experimentally infected mice	tachyzoite (acute toxoplasmosis)	1 pg	LAMP>PCR	Hu et al., Folia Parasitol (Praha). 2012 Feb;59(1):21-6.	2012
529 bp repetitive region	blood from experimentally infected mice	tachyzoite (acute toxoplasmosis)	0.6 fg	LAMP529>LAMP_B1	Kong et al. Parasites & Vectors 2012, 5:2	2012
529 bp repetitive region	blood samples from pigs and sheep	tachyzoite (acute toxoplasmosis)	1 fg	LAMP>PCR	Lin et al., Veterinary Parasitology 185 (2012) 296–300	2011
529 bp repetitive region	lymph nodes from pigs	tissue cysts	1 pg	LAMP>PCR	Zhang et al., Exp Parasitol. 2009 May;122(1):47-50.	2009
SAG1	infected mouse organs	tissue cysts	100 tachyzoites	LAMP>PCR	Krasteva et al., Vet Parasitol. 2009 Jun 10; 162(3-4):327-31.	2009
TgOWP and B1	concentrated water	oocysts in water	0.1 tachyzoite	LAMP=PCR	Sotiriadoua and Karanis. Diagnostic Microbiology and Infectious Disease 62 (2008) 357–365	2008

Aim

European Union Reference Laboratory for Parasites
Department of Infectious Diseases
Unit of Foodborne and Neglected Parasitic Diseases
Istituto Superiore di Sanità

IDENTIFICATION OF DNA *of Toxoplasma gondii* IN FOOD MATRICES (MEAT AND MEAT PRODUCTS) BY LAMP

- Development of LAMP based procedure to detect *T. gondii* DNA (bradyzoites) in meat and meat products
- Evaluation of sensitivity and specificity of the LAMP assay



Materials

- *Toxoplasma gondii tachyzoites* of the RH stain
- gDNA from RH stain tachyzoites
- Pork, chicken, sheep and beef minced meat were used as matrices (5gr)
- LAMP assay targeting the 529 bp repetitive region (up to 300 copy per genome) was conducted using 6 primers (Zhang et al. 2009)

Toxoplasma gondii 529 repeatitive region (GenBank: AF146527.1)

CTGCAGGGAGGAAGACGAAAGTTGTTTTTTTATTTTTTTTTCTTTTTGTTTTTCTGATTTTTGTTTTTTTTGACTCGGGCCCAGCTGCGTCTGTCGGGATGAGACCG
CGGAGCCGAAGTGCGTTTTCTTTTTTTGACTTTTTTTTGTTTTTTCACAGGCAAGCTCGCCTGTGCTTGGAGCCACAGAAGGGACAGAAGTCGAAGGGGACTACAGA
CGCGATGCCGCTCCTCCAGCCGTCTTGGAGGAGAGATATCAGGACTGTAGATGAAGGCGAGGGTGAGGATGAGGGGGTGGCGTGGTTGGGAAGCGACGAGAGTCGGA
GAGGGAGAAGATGTTTCCGGCTTGGCTGCTTTTCCTGGAGGGTGGAAAAAGAGACACCGGAATGCGATCCAGACGAGACGACGCTTTCCTCGTGGTGATGGCGGAGA
GAATTGAAGAGTGGAGAAGAGGGCGAGGGAGACAGAGTCGGAGGCTTGGACGAAGGGAGGAGGAGGGGTAGGAGAGGAATCCAGATGCACTGTGTCTGCAG

ZF3 CCACAGAAGGGACAGAAGTC
ZB3 TCCGGTGTCTCTTTTTCCAC
ZFIP TCCTCACCCTCGCCTTCATCTAGGACTACAGACGCGATGC
ZBIP TGGTTGGGAAGCGACGAGAGTTCCAGGAAAAGCAGCCAAG
ZLF TCCAAGACGGCTGGAGGAG
ZLB CGGAGAGGGAGAAGATGTTTCC

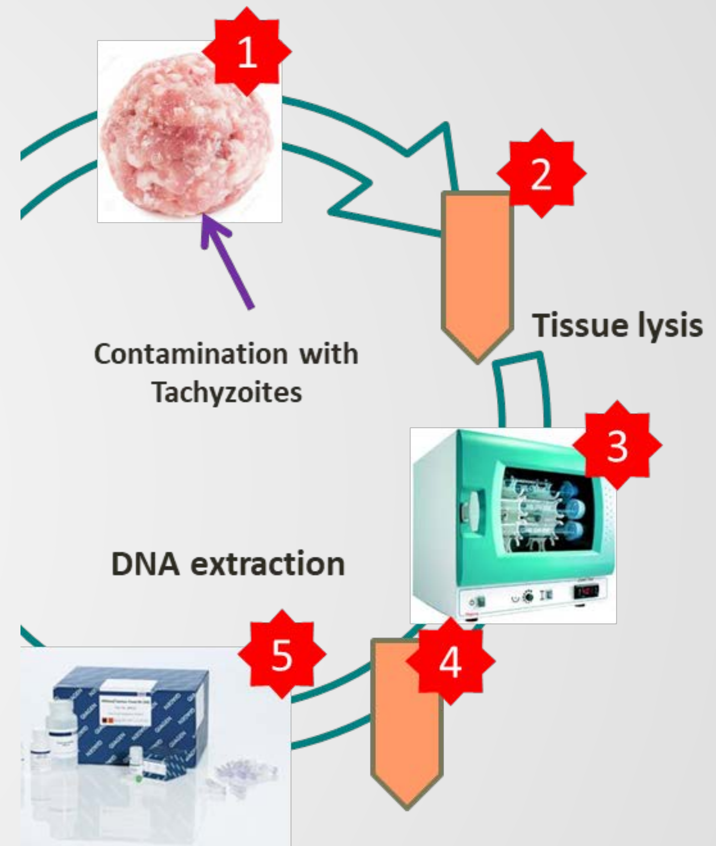
Methods

- ❖ DNA isolation from meatballs (5 gr) was performed combining homogenization with glass beads and tissue digestion with proteinase K at 55°C overnight (10 mL final volume) following Qiagen DNAeasy Tissue and blood kit DNA extraction (200 micL)

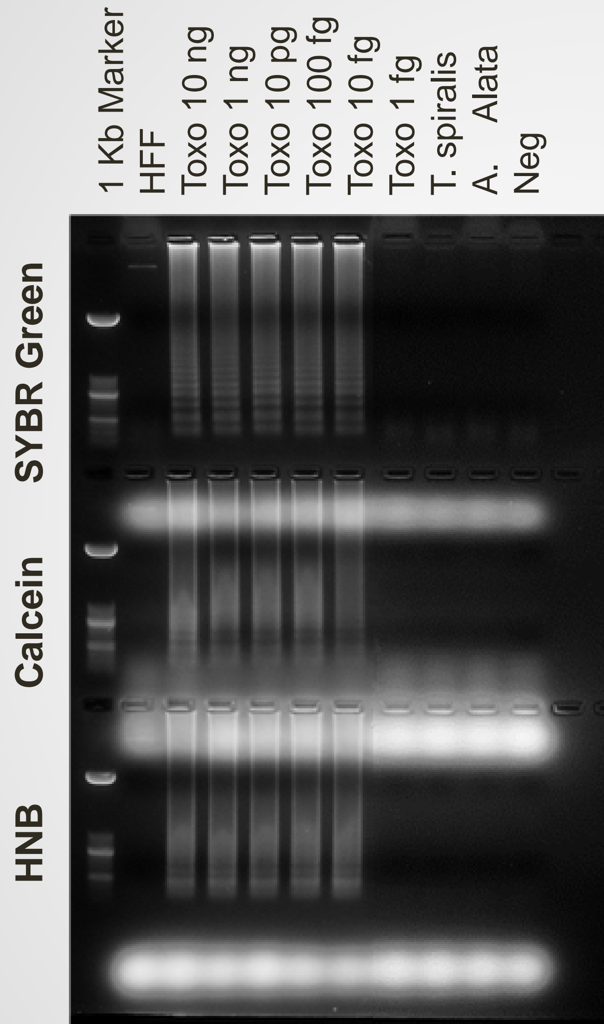
- ❖ LAMP sensitivity was determined using:

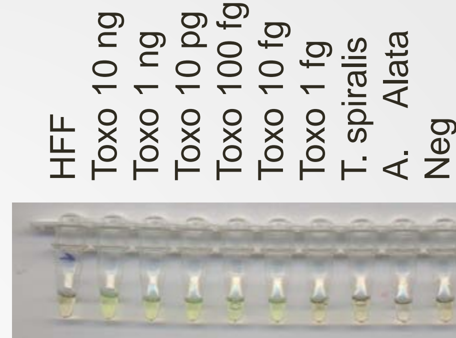
- ➢ serial dilution of *T. gondii* RH DNA

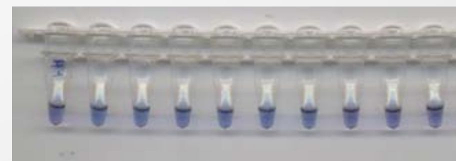
- ➢ DNA isolated from unspiked meatballs (pork, beaf, lamb or chiken) and meatballs spiked with up to 10000 tachyzoites.



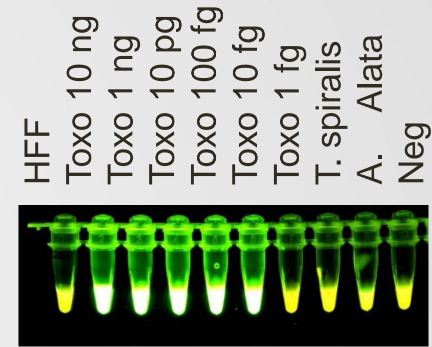
Detection of LAMP results with different visual methods

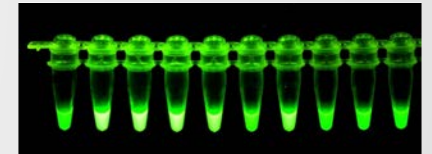






HNB (hydroxy naphthol blue)=metal ion indicator (Mg2+)





Spectrophotometric measurment at 650 nm

Specificity

No positive LAMP reaction was observed on gel electrophoresis using human, *T. spiralis or A. alata* gDNA

Sensitivity

Optimum condition for LAMP were 90 min at 63°C.

Detection limit 10 fg

Detection performance

Both gel electrophoresis and SYBR green are suitable

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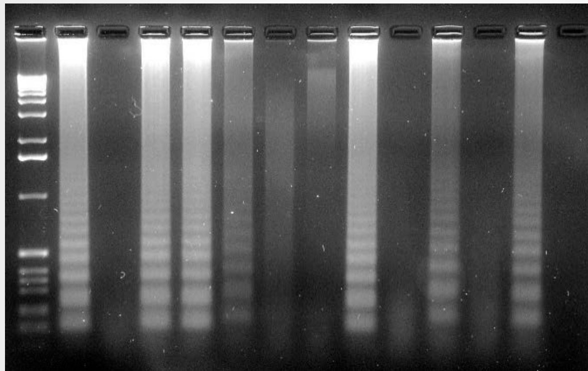
European Union Reference Laboratory for Parasites

Detection limit of tachyzoites contamination in meat by LAMP

1 Kb Marker
Toxo 10 ng
Neg
Pork + 10000 tach
Pork + 5000 tach
Pork + 1000 tach
Pork
Lamb
Lamb + 1000 tach
Chicken
Chicken + 1000 tach
Beef
Beef + 1000 tach



1 Kb Marker
HFF
Toxo 10 ng
Toxo 1 ng
Toxo 100 pg
Toxo 10 pg
Toxo 1 pg
Toxo 100 fg
Toxo 10 fg
Toxo 10 fg
Neg
Pork
Pork
Pork
Pork + 10 tach
Pork + 10 tach
Pork + 10 tach
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Pork + 100 tach
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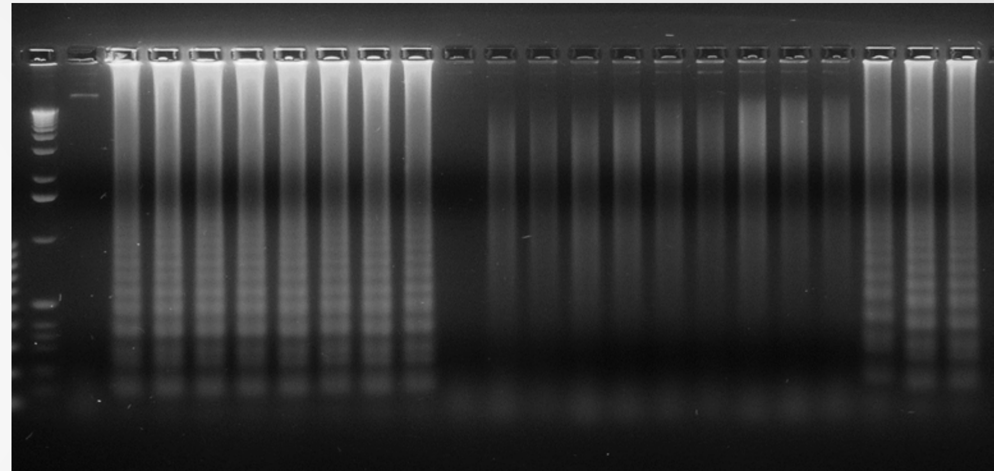
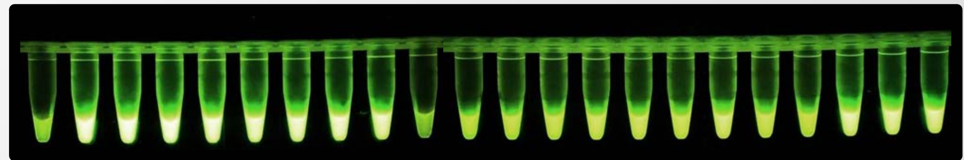
Pork + 1000 tach
Pork + 1000 tach





0,301	0,437	0,380	0,356	0,375	0,459	0,398	0,408	0,370	0,306		
0,327	0,288	0,315	0,296	0,340	0,291	0,332	0,310	0,315	0,326	0,294	0,314

Specificity
No positive LAMP reaction was observed on gel electrophoresis using as template DNA from unspiked matrices

Sensitivity
Detection limit 200 parasite/g of meat

Detection performance
SYBR green UV test was often prevented by high background fluorescence (high amount of DNA from matrix)

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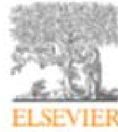
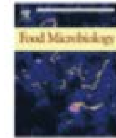
Aim





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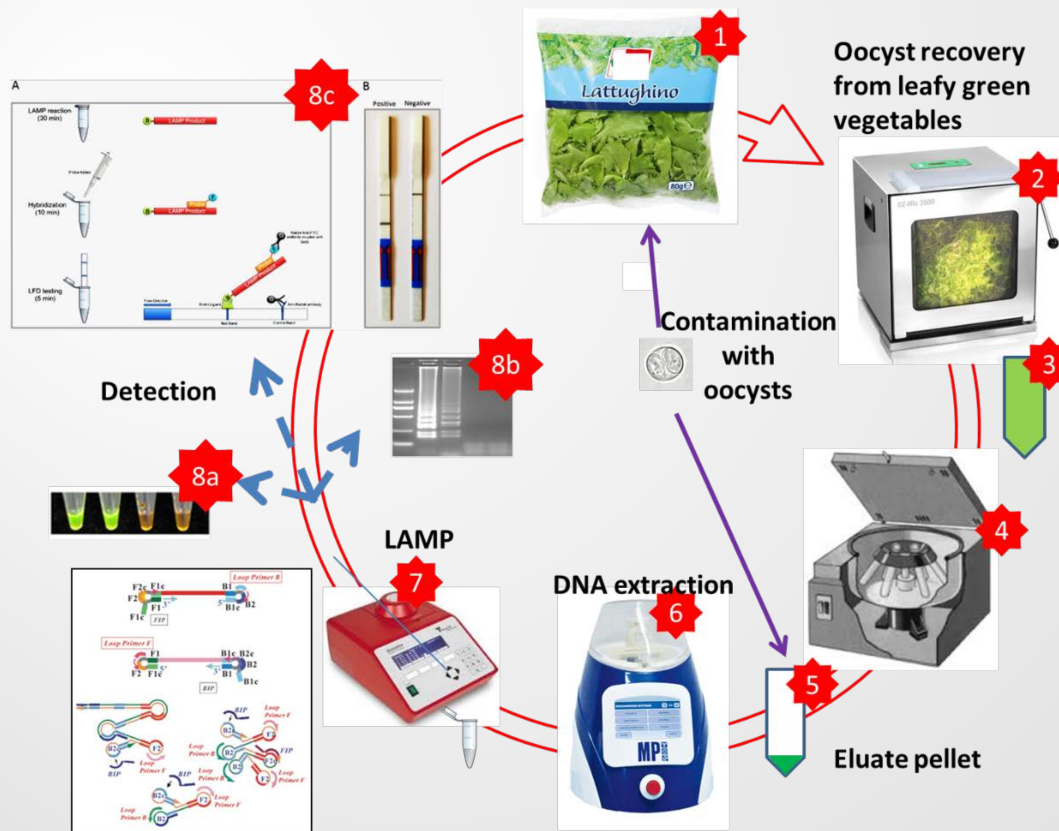
Loop-Mediated Isothermal Amplification-Lateral-Flow Dipstick (LAMP-LFD) to detect *Toxoplasma gondii* oocyst in ready-to-eat salad

Marco Lalle [a,*], Alessia Possenti [a], Jitender P. Dubey [b], Edoardo Pozio [a]

- Development of LAMP-LFD based procedure to detect *T. gondii oocysts* in ready-to-eat leafy green vegetable.
- Evaluation of sensitivity and specificity of the LAMP-LFD assay



Materials

- Toxoplasma gondii oocysts of the VEG strain
- gDNA from tachyzoites of the RH stain
- Ready-to-eat baby lettuce was used as vegetable matrix
- LAMP assay targeting the 529 bp repetitive region (up to 300 copy per genome) was conducted using 6 primers (Zhang et al. 2009)
- For BIO-LAMP the a 5'-biotinilated BIP primer was used and a specific FITC-labeled DNA probe were designed to amplify and to hybridize with, respectively, the Bio-LAMP amplicon and being visualized on a chromatographic lateral flow dipstick (LFD) (Milenia® GenLine HybriDetect, GieBen, Germany).

Toxoplasma gondii 529 repeatitive region (GenBank: AF146527.1)

```
CTGCAGGGAGGAAGACGAAAGTTGTTTTTTTATTTTTTTTTCTTTTTGTTTTTCTGATTTTTGTTTTTTTTGACTCGGGCCCAGCTGCGTCTGTCGGGATGAGACCG
CGGAGCCGAAGTGCGTTTTCTTTTTTTGACTTTTTTTTGTTTTTTCACAGGCAAGCTCGCCTGTGCTTGGAGCCACAGAAGGGACAGAAGTCGAAGGGGACTACAGA
CGCGATGCCGCTCCTCCAGCCGTCTTGGAGGAGAGATATCAGGACTGTAGATGAAGGCGAGGGTGAGGATGAGGGGGTGGCGTGGTTGGGAAGCGACGAGAGTCGGA
GAGGGAGAAGATGTTTCCGGCTTGGCTGCTTTTCCTGGAGGGTGGAAAAAGAGACACCGGAATGCGATCCAGACGAGACGACGCTTTCCTCGTGGTGATGGCGGAGA
GAATTGAAGAGTGGAGAAGAGGGCGAGGGAGACAGAGTCGGAGGCTTGGACGAAGGGAGGAGGAGGGGTAGGAGAGGAATCCAGATGCACTGTGTCTGCAG
```

```
ZF3  CCACAGAAGGGACAGAAGTC
ZB3  TCCGGTGTCTCTTTTTCCAC
ZFIP TCCTCACCCTCGCCTTCATCTAGGACTACAGACGCGATGC
ZBIP TGGTTGGGAAGCGACGAGAGTTCCAGGAAAAGCAGCCAAG
ZLF  TCCAAGACGGCTGGAGGAG
ZLB  CGGAGAGGGAGAAGATGTTTCC
```

```
LAMP probe: FITC-5'-TGAGGGGGTGGCG-3'
bioZBIP: Biotin-5'-TGGTTGGGAAGCGACGAGAGTCGGAGAGGGAGAAGATGTTTCC-3'
```

Methods

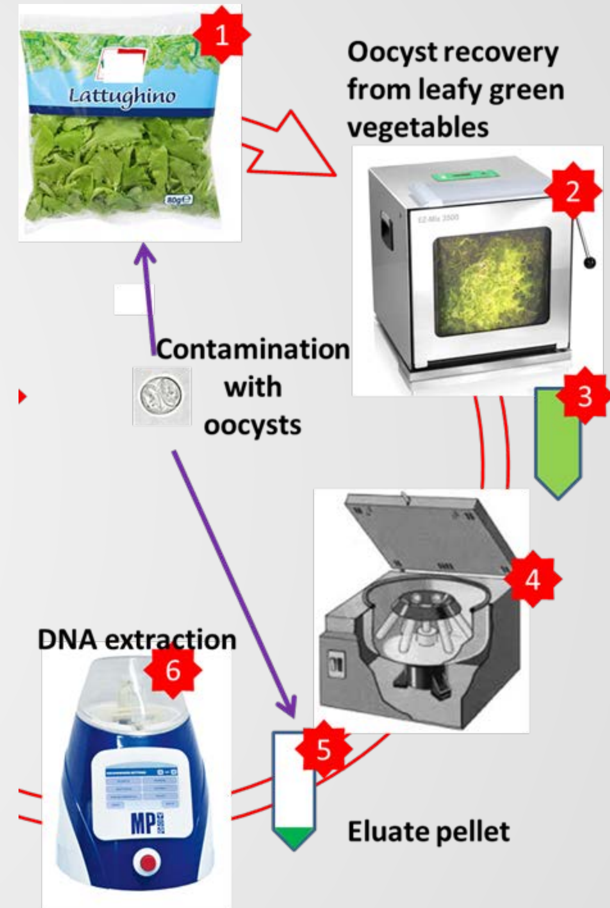
❖ A procedure for oocyst recovery was adapted from ISO 18744:2016 (*Detection and enumeration of Cryptosporidium and Giardia in fresh leafy green vegetables and berry fruits*)

❖ DNA isolation from vegetables with FastPrep 24 5G homogenizer and FastDNA™ SPIN Kit for Soil

❖ LAMP and Bio-LAMP-LFD sensitivity were determined using:

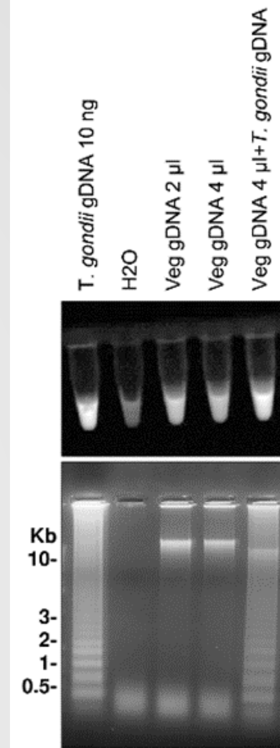
➢ serial dilution of *T. gondii* RH DNA

➢ DNA isolated from sediment of baby lettuce contaminated with T. gondii oocysts at different stages of sediment preparation: 5, 10, 50 oocysts/ml sediment or 10, 25, 50, 100 oocysts/50g of baby lettuce.



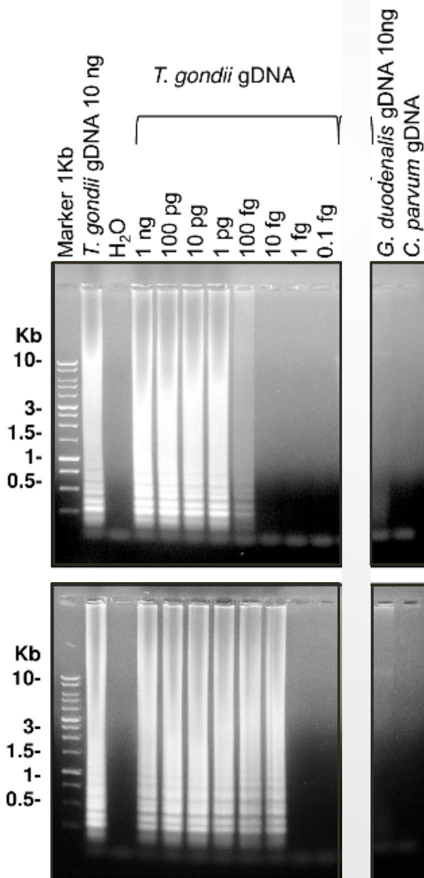
Optimization of LAMP

Addition after the reaction of the SYBR Green dye allow visualization of positive reaction directly in the vials. However, **reaction negative vials containing DNA from control plant sediment fluoresce** but a Ladder-like bands were evident in agarose gel electrophoresis only if the amplification product was present. **High amount of vegetable DNA background!**



1h

2h



Specificity

No positive LAMP reaction was observed on gel electrophoresis using human, *C. parvum*, *G. duodenalis* or baby lettuce DNA!

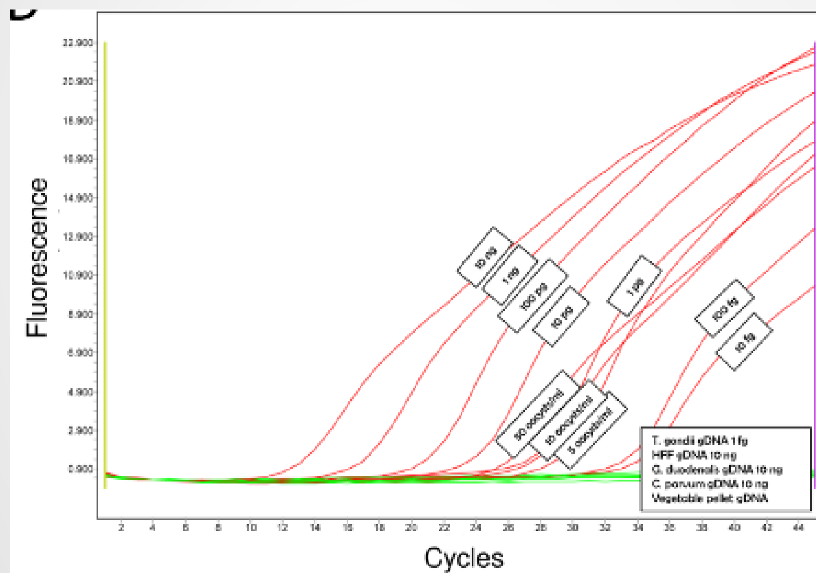
Sensitivity

Optimum condition for LAMP were **2h at 63°C**, improving the detection limit! (B and C)

- From 100 fg to 10 fg of gDNA

LAMP vs qPCR

LAMP was as sensitive as qPCR, targeting the same locus, with a 6-FAM-labelled Taqman probe in LightCycler480 thermal-cycler (Opsteegh et al., 2010).

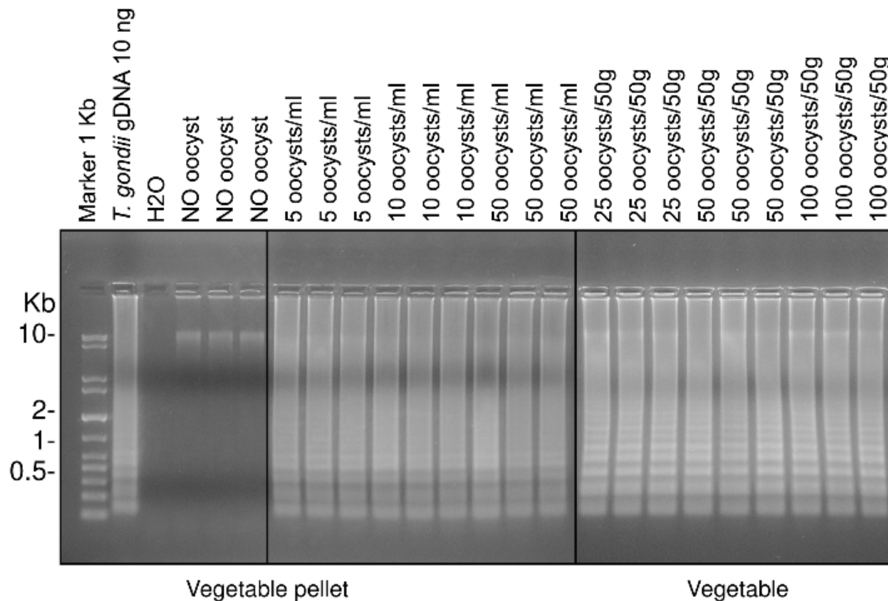


Sample	*N. of T. gondii* haploid genomes	Mean Cp (± SD)
HFF	NA	NA
Giardia duodenalis	NA	NA
Cryptosporidium parvum	NA	NA
T. gondii (RH strain) 10 ng	140000	12.72±0.38
T. gondii (RH strain) 1 ng	14000	16.50±0.25
T. gondii (RH strain) 0.1 ng	1400	20.33±0.37
T. gondii (RH strain) 10 pg	140	23.70±0.38
T. gondii (RH strain) 1 pg	14	27.31±1.05
T. gondii (RH strain) 0.1 pg	1.4	31.07±1.33
T. gondii (RH strain) 10 fg	0.14	34.74±1.51
T. gondii (RH strain) 1 fg	0.014	NA
Vegetable pellet suspension+50 oocysts	16	26.19±1.12
Vegetable pellet suspension+10 oocysts	3.2	27.34±0.43
Vegetable pellet suspension+5 oocysts	1.6	27.65±1.47
Vegetable pellet suspension	NA	NA

Detection limit of oocysts contamination in vegetable by LAMP

LOD evaluation with baby lettuce artificially spiked with 25, 50 and 100 T. gondii oocysts per 50 grams of leaves (six samples for each condition).

Contaminated leaves were subjected to recovery procedure with glycine wash buffer and stomaching, following by DNA isolation.



Sample type	LAMP positive[a]/Total
Leaves (50g)	
25 oocysts	5/6 (83%)
50 oocysts	6/6 (100%)
100 oocysts	6/6 (100%)
Pellet suspensions (1 mL)	
5 oocysts	4/6 (66%)
10 oocysts	5/6 (83%)
50 oocysts	6/6 (100%)

LAMP LOD 25 oocysts/50 gr (or 0.5 oocyst/gr) of contaminated leaves and 5 oocysts/mL of vegetable pellet suspension

In lettuce spiked with *T. gondii* oocysts, LOD was 10 to ≥100 oocysts per microliter by PCR targeting the B1 gene or the 529-bp repeat element (Marchioro et al., 2016)

A 33 oocysts/g LOD was reported using artificially contaminated basil applying immunomagnetic separation followed by qPCR targeting 529-bp repeat element (Hohweyer et al., 2016)

A LOD of 100 oocysts by B1 qPCR targeting was reported for an undefined amount of artificially contaminated radish (Lass et al., 2012).

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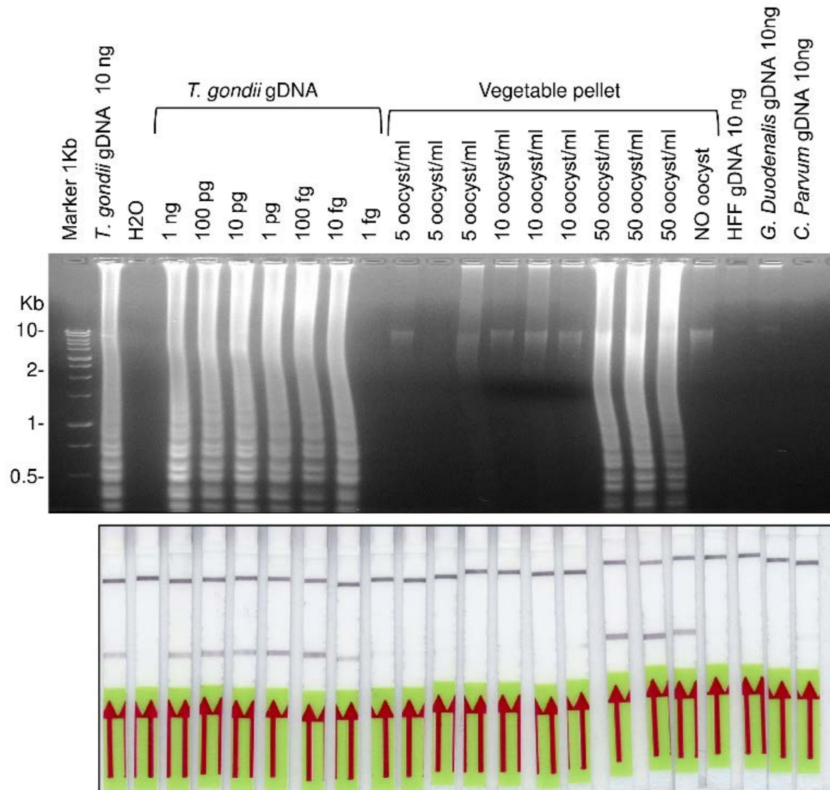
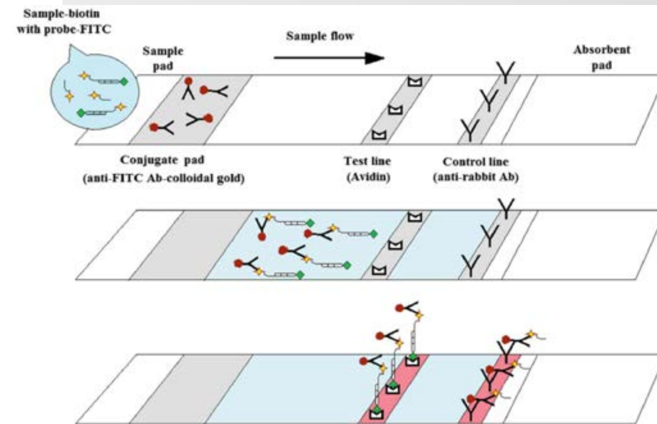
Faster visualization of LAMP by lateral flow deepstick





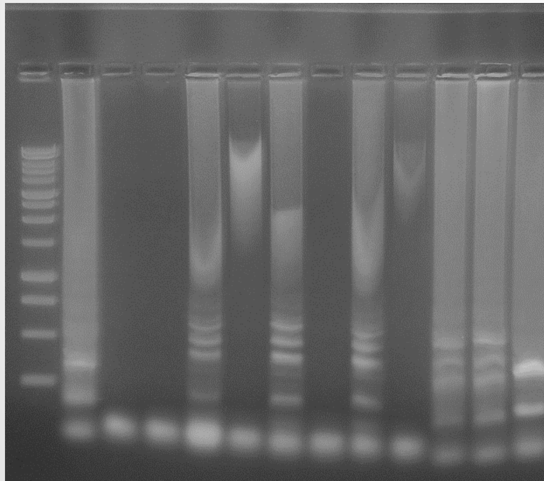
- BIO-LAMP and LAMP showed a comparable sensitivity on T. gondii gDNA
- BIO-LAMP was 10 times less efficient than LAMP (LOD 50 vs 5 oocysts) in detecting parasite gDNA in vegetable pellet suspension samples spiked with T. gondii oocysts

Kaya Stollberg (BfR)
2019 STSM 1 week

MED • VET • NET
ASSOCIATION

Detection of *T. gondii* in wild boar meat

1 Kb Marker
Toxo 10 ng
Neg
Neg
WB 307H1
WB 269H1
WB 146H1
WB 72H1
WB 48H1
WB 268H1
WB 269H1 spiked
WB 146H1 spiked
WB 72H1 spiked



Main preliminary results

- Extending LAMP assay to 2 hours improved sensitivity
- DNA preparation do not contain inhibitors

- Samples will be further tested by qPCR for comparison with LAMP.

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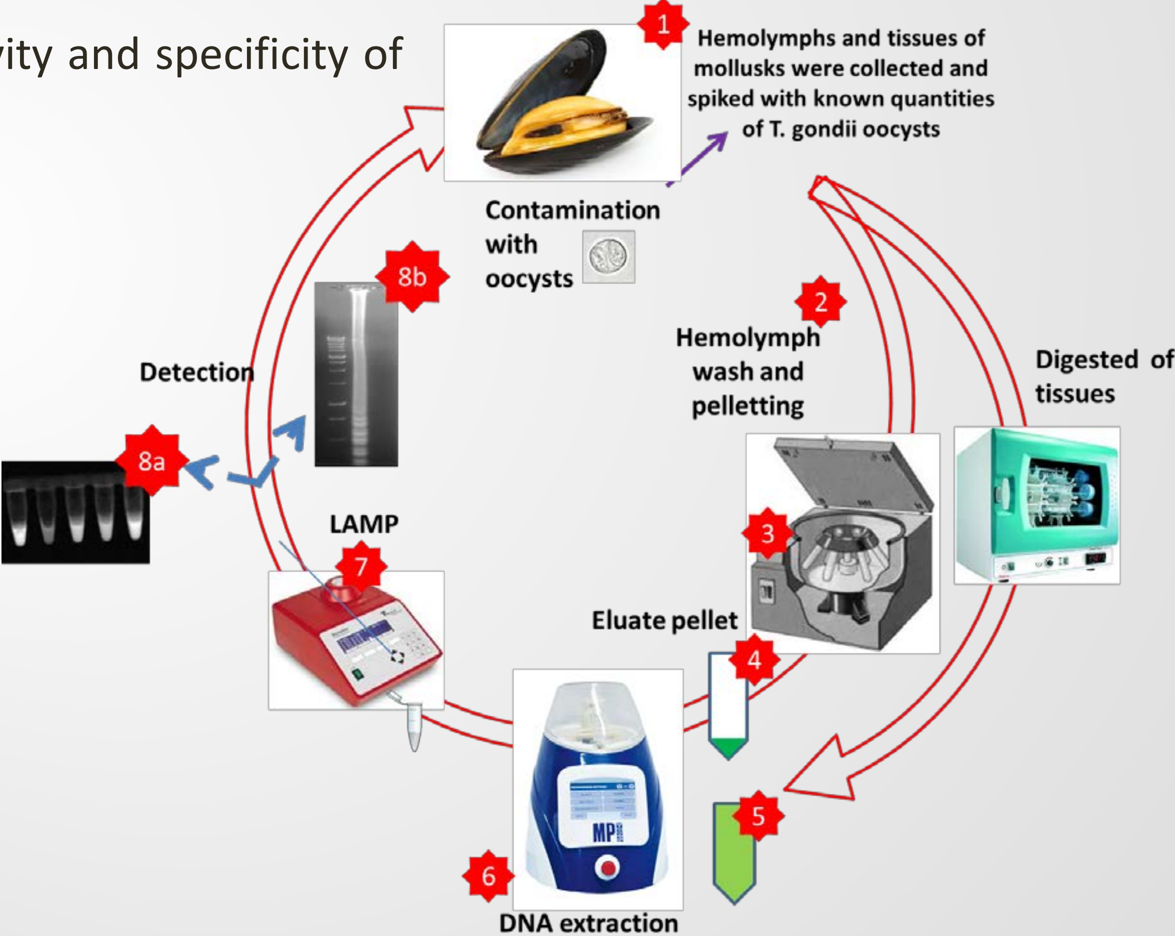
European Union Reference Laboratory for Parasites

Loic Durant (ACTALIA)
2018 STSM 2 weeks

Aim

- Preliminary development of LAMP based procedure to detect *T. gondii* oocysts in mussels
- Evaluation of sensitivity and specificity of the LAMP assay



Materials

- Toxoplasma gondii oocysts of the VEG strain
- gDNA from tachyzoites of the RH stain
- Fresh blue mussels (hemolymph and tissue) as matrix
- LAMP assay targeting the 529 bp repetitive region (up to 300 copy per genome) was conducted using 6 primers (Zhang et al. 2009)

Methods

- DNA isolation from mussels tissue and emolymph with FastPrep 24 5G homogenizer and FastDNA™ SPIN Kit for Soil

- LAMP sensitivity was determined using:

- DNA isolated from hemolymph and tissue contaminated or not with different amount of *T. gondii* oocysts 5 and 50 for 700 µl hemolymph, 10 and 100 oocysts/2g tissue.

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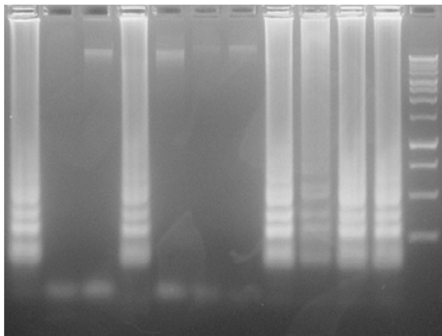
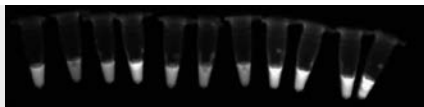
European Union Reference Laboratory for Parasites

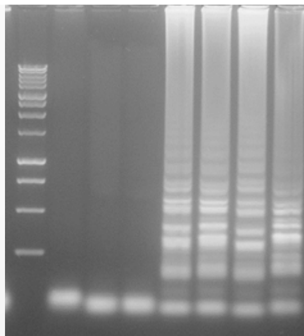
Detection limit of oocysts contamination

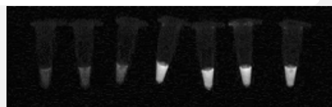
1 : Pos control (20ng)
2 : Neg control
3 : Neg vegetable
4 : Pos vegetable
5 : Hemolymph 3 without spiking
6 : Hemolymph 8 without spiking
7 : Hemolymph 15 without spiking
8 : Hemolymph 3 with 5 oocysts
9 : Hemolymph 8 with 5 oocysts
10 : Hemolymph 15 with 50 oocysts
11 : Hemolymph 8 with 50 oocysts

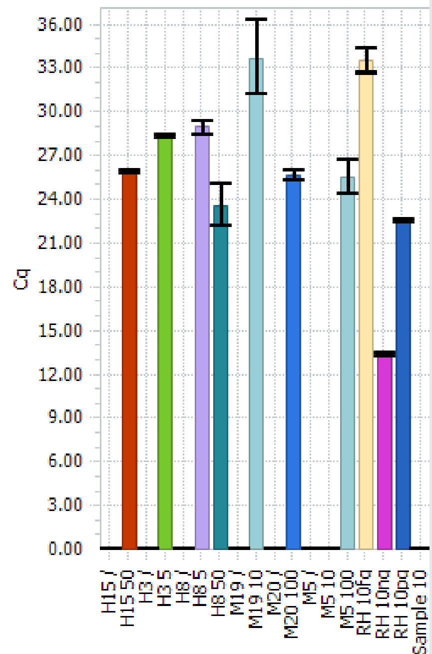




Mussel 5 without spikes
Mussel 19 without spikes
Mussel 20 without spikes
Mussel 19 with 10 oocysts
Mussel 5 with 10 oocysts
Mussel 5 with 100 oocysts
Mussel 20 with 100 oocysts







qPCR

LAMP LOD 10 oocysts/2 gr (or 5 oocyst/gr) of contaminated tissue and 5 oocysts/mL of washed hemolymph suspension

LAMP vs qPCR

	LAMP-PCR (2h)	LAMP-PCR (2h)	LAMP-PCR (1h)	LAMP-PCR (2h)	qPCR				qPCR				
	09/02/17	10/02/17	14/02/17	16/02/17	13/02/17				14/02/217				
Sample ID	Tube 1	Tube 1	Tube 1	Tube 1	Well 1	Well 2	Mean	SD	Well 1	Well 2	Well 3	Mean	SD
H3 /	-		-		N/A	N/A			N/A	N/A	N/A		
H3 5	+		+		27,81	27,37	27,59	0,22	28,36	28,08	28,35	28,26	0,13
H8 /	-		-		N/A	N/A			N/A	N/A	N/A		
H8 5	+		+		29,53	28,92	29,23	0,31	28,59	29,35	28,91	28,95	0,31
H8 50	+		+		25,81	26,13	25,97	0,16	22,95	22,43	25,27	23,55	1,23
H15 /	-		-		N/A	N/A			N/A	N/A	N/A		
H15 50	+		+		N/A	26,27	26,27	0,00	25,84	25,95	25,94	25,91	0,05
M5 /		-	-		N/A	N/A			N/A	N/A	N/A		
M5 10		+	+		N/A	N/A			N/A	N/A	N/A		
M5 100		+	+		30,21	NA	30,21	0,00	26,87	25,16	24,38	25,47	1,04
M19 /		-	-		N/A	N/A			N/A	N/A	N/A		
M19 10		+	+		N/A	N/A			30,94	34,14	35,96	33,68	2,08
M20 /		-	-		NA	39,19	39,19	0,00	N/A	N/A	N/A		
M20 100		+	+		25,19	22,01	23,60	1,59	25,34	26,05	25,45	25,61	0,31

Preliminary results suggest that by applying the same extraction procedure and by targeting the same locus, LAMP is more sensitive and than qPCR especially to detect low amount of spiked oocyst in mussel tissue

Conclusions

- LAMP assay targeting the 529 bp repetitive region can be applied to detect *T. gondii* gDNA in meat, leafy green vegetables and mussels

- A well defined LOD was calculated in different conditions

- Despite qPCR is faster than LAMP (1h versus 3h), the comparable sensitivity of the two assays and the cheapest equipment required for LAMP makes the latest a valuable molecular test to be performed also in resource limited setting.

- Further optimization are necessary to apply BIO-LAMP to complex matrices as leafy green vegetable in order to provide a molecular detection tool more suitable to facilitate timely and effective food-borne disease outbreak investigations and response.

ISTITVTO SVPERIORE DI SANITA

Acknowledgments

Alessia Possenti
Furio Spano

European Union Reference Laboratory for Parasites

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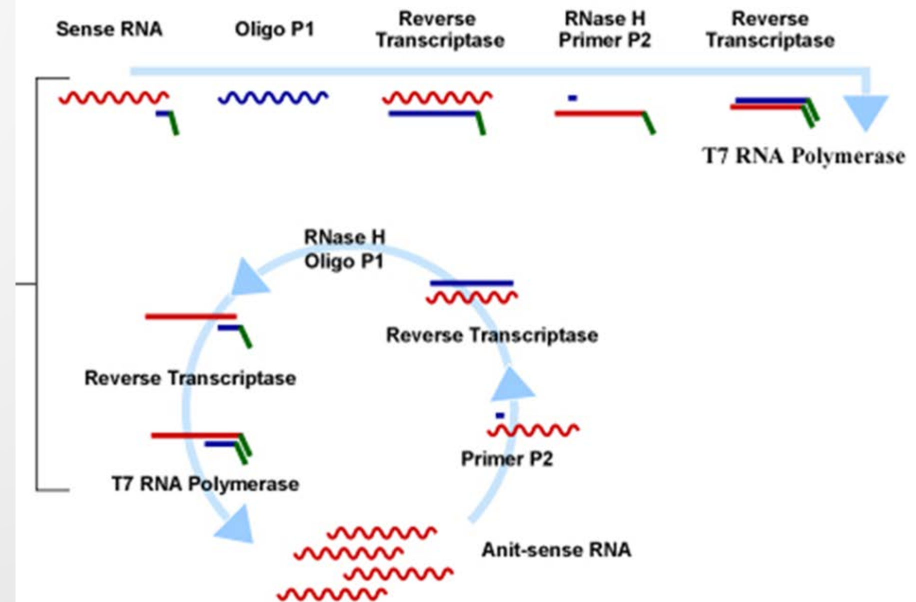
Kaya Stolberg
Nadja Bier

Federal Institute for Risk Assessment

Nucleic Acid Sequence-Based Amplification (NASBA)

- Specifically **designed to detect and amplify RNA** in the presence of DNA background without the use of a thermal cycler.
- 2 specific primers flanking the sequence to be amplified.
- 3 enzymes required: avian myeloblastosis virus reverse transcriptase (**AMV RT**), **RNase H**, and **T7 RNA polymerase**
- **Exponentially accumulate RNA/cDNA can be detected**

1. The primer P1 carries the T7 RNApol binding sequence at its 5′ end, it initiates the RT reaction
2. The RNAse H then degrade the RNA strand in the RNA/cDNA hybrid molecules
3. Primer P2 bind cDNA and initiates the synthesis of the complementary strand.
4. T7 RNA Polymerase, docks the double strand DNA on P1 5′ end and transcribing RNA copies.
5. The process is repeated indeterminately starting from the newly transcribed RNA



- **Advantages**
- Lab equipment is less expensive
- not require a thermal cycler (a water bath or an isothermal block at 41°C is sufficient)
- High sensitivity (>10^9 copies of target in 90 min) compared to qPCR
- Suitable for lab-on-chip or portable apparatus
- Reduced sample prep compared with PCR due to less inhibition
- Can provide information of pathogen viability since the RNA is less stable than DNA.

- **Disadvantages**
- RNA integrity is the main cause of concern
- a single initial melting step is sometimes required.
- Temperature sensitive, use of thermolabile enzymes
- Amplified RNA target sequence 120–250 nucleotide

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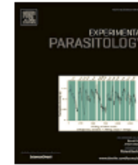


Full length article

Real-time nucleic acid sequence-based amplification (NASBA) assay targeting *MIC1* for detection of *Cryptosporidium parvum* and *Cryptosporidium hominis* oocysts

Birgitte K. Hønsvall [a, b, *], Lucy J. Robertson [c]

Both *C. parvum* and *C. hominis* are often associated with cryptosporidiosis in humans (humans are the main host for C. hominis, C. parvum is zoonotic and able to infect a variety of species.)

A real-time NASBA, targeting the MIC1 transcript in *C. parvum* (CpMIC1) and *C. hominis* (ChMIC1).

LOD of *C. parvum* and *C. hominis* oocysts (down to 5 oocysts in 10 micl, and down to 1 oocyst using diluted RNA samples),

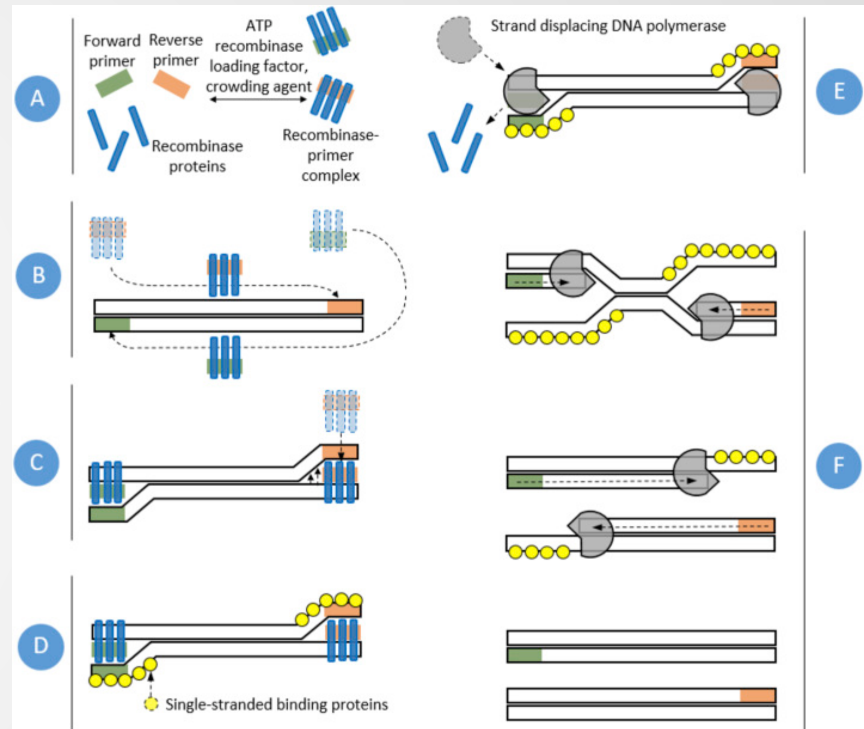
Distinguish between them. One of the primer sets targeted an exon only occurring in CpMIC1, distinguishing *C. parvum* from other *Cryptosporidium* spp.

NASBA assay detected MIC1 mRNA also in inactivated oocysts. RNA does not seem to be a suitable marker for assessing oocyst viability.

Recombinase polymerase amplification (RPA)

- RPA was first reported in 2006
- produced and under the intellectual license of TwistDx Limited (Cambridge, UK)

- **RPA utilizes:**
  - a bacterial recombinase, and a single-stranded DNA binding protein (SSB) to match primers to their target on the template DNA
  - a Staphilococcus aureus Sau polymerase
  - 2 primers of 30–35 mer (even shorter)
  - ATP and a crowding agent (a high molecular polyethyleneglycol), forming a recombinase-primer complex.



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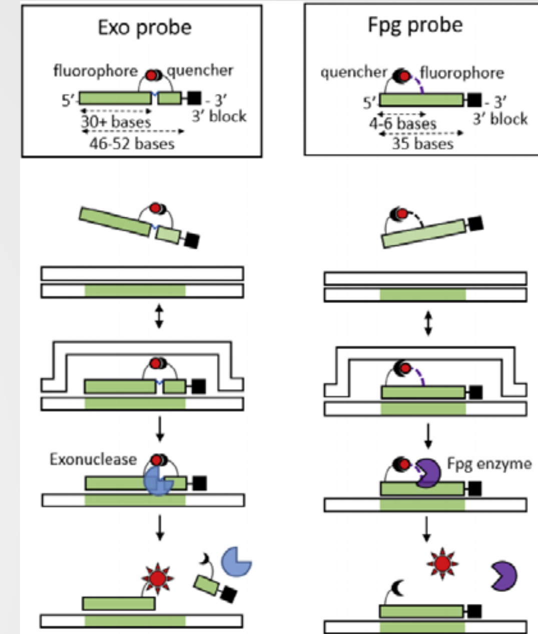
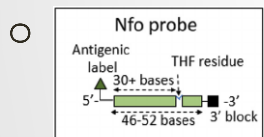
- The recombinase enzyme forms a nucleoprotein complex with oligonucleotide primers and scans for homologous sequences in a DNA template.
- Recognition of a specific homologous sequence leads to the initiation of strand invasion and the opposing oligonucleotides are then extended by isothermal strand displacement amplification via Sau polymerase.

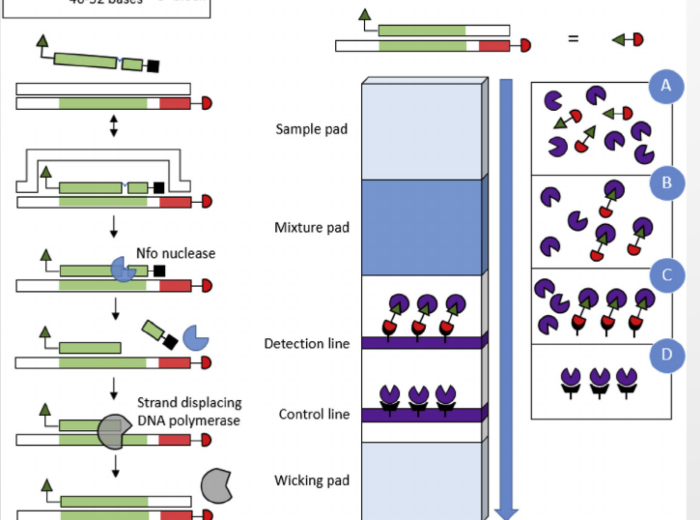
RPA can be monitored by end point detection (following amplification) or in real time (during amplification) and probes may be used depending on the detection strategy

- **The exo probe** (generally around 50 bases) contains an internal fluorophore and quencher quencher is cleaved by exonuclease III only upon binding to the template DNA. The probe contains a blocked 3′ (not extendable)

- **The fpg probe** (generally 30–35 bases) contains a 5′ quencher that is a few bases away from an abasic deoxyribose to which a fluorophore is linked. A nuclease (Fpg) specifically recognizes and cuts the linked fluorophore when the probe is annealed



- 



The nfo probe here the quencher is cleaved by endonuclease IV (also called Nfo). This unblocks the probe 3′ end of the and it can be extended by a polymerase to eventually join a segment amplified by a primer with 5′ biotinylation. The biotynilated amplicon allows for easy application on a lateral flow

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❑ **Advantages**

- the fastest isothermal amplification methods to date with minimal sample preparation, amplification of as low as 1–10 DNA target copies in less than 20 min
- Reaction is performed at 37–42°C with no requirement for a tight control of the temperature within this range
- Robustness in the presence of traditional PCR inhibitors (haemoglobin, ethanol and heparin)
- A reverse transcription step can be included to target RNA
- Multiplexing possible but highly dependent on target sequences, amplicon size and primer design
- The reagents necessary for RPA are sold in pellets, stable up to 6 months when stored at room temperature (22-28°C)

❑ **Disadvantages**

- Tolerance to mismatches can lead to cross-reactivity.
- Can be inhibited by high concentrations of genomic DNA,
- Kits are only sold by one company, (impact on pricing) and the user also has limited flexibility in the kit formulation.
- Requires purification/protein digestion following amplification (smearing or impaired flow in agarose gel electrophoresis and lateral flow)

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Recombinase Polymerase Amplification-Based Assay to Diagnose *Giardia* in Stool Samples

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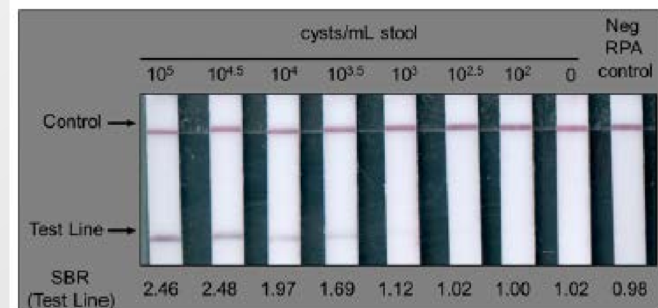
- RPA assay for Giardia utilizes DNA primers that target a unique 183 bp sequence on the beta giardin gene. Highly specific no cross amplification
- Detect as few as 10 Giardia cysts/ml spiked into PBS, similar to the analytical sensitivity of qPCR.
- The RPA assay detected both the A and B assemblages of Giardia
- Detected as few as 10^3 cysts per milliliter stool (50 cysts per reaction).
- RPA assay yielded 73% sensitivity and 95% specificity when tested using DNA extracted from clinical stool samples in comparison with gold standard (PCR microscopy)
- Lateral flow RPA has demonstrateda limit of detection of as few as 3000 Giardia cysts per gram of stool, slightly better than PCR-based assays



Conclusions

- There are a number of flexible, highly sensitive isothermal techniques that have been be adapted to detect foodborne parasite at least in some matrices
- Most of the tests however are still confined to the labs where originally developped and still need to pass the test of wide and in intense application to really evaluate their suitability in the field expecially in the point-of-need (industry, sloughterhouse, farm)
- There are, however, limitations in the applicability of such techniques that will be difficult to overcome, as these are inherently linked to the nature of the samples under study.