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Comparative detection of Toxoplasma gondii by LAMP, PCR & IFT

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LAMP assays development and applications for the detection of *Cryptosporidium*, *Giardia*, *Toxoplasma* & *Plasmodium*

LAMP Reagents and Heating Devices

Pirmers
FIP; BIP
F3;B3
LF; LB

DNA

25 µl

dCTP
dTTP
LAMP Buffer
dATP
dGTP

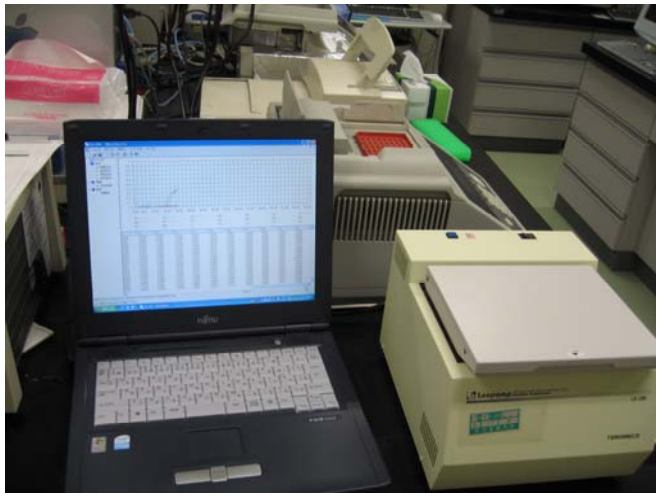
Distilled water

***Bst* DNA polymerase**

Amplification
60⁰C ~ 65⁰C
Maximum 60 min

Loop-mediated isothermal amplification (LAMP)

- **Rapid, simple, highly sensitive.**
- **Uses 4 or 6 primers and Bst DNA polymerase.**
- **Can use a heat block or a water-bath under isothermal conditions.**
- **Visual detection**



In recent years:

Investigations on the detection of *Cryptosporidium, Giardia, Toxoplasma*

Genotyping of *Giardia* and *Cryptosporidium*

Investigated areas

Bulgaria, China, Germany, Greece, Hungary, Japan, Malaysia, Mongolia, Russia, South Africa, Thailand

Identification

Microscopy (IFT, DAPI, DIC, LSM)

PCR-RFLP, Sequence

LAMP

The overall strategy for Development and Application of LAMP identification

1. *Use parasites material from various sources.*
2. *Select the target genes and design the specific primers according to the available sequences in the GenBank.*
3. *Evaluation of specificity & sensitivity of the LAMP assays*

&

4. *Application of LAMP (till now) in:*
   - a) spiked & b) environmental water samples for *Cryptosporidium*, *Giardia*, *Toxoplasma*.
   - c) fecal samples (humans, animals) for the detection of *Giardia* & *Cryptosporidium.*
   - c) blood samples for *Plasmodium* infections

Cryptosporidium generated LAMP products

Sensitivity tests after oocysts' LDM followed by DNA extraction and LAMP

1. 10^6 oocysts
2. 10^5 oocysts
3. 10^4 oocysts
4. 10^3 oocysts
5. 10^2 oocysts
6. 10^1 oocysts
7. 10^0 oocysts
8. 10^{-1} oocysts
9. Negative control

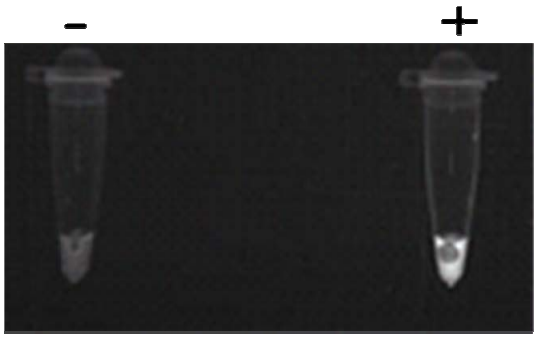
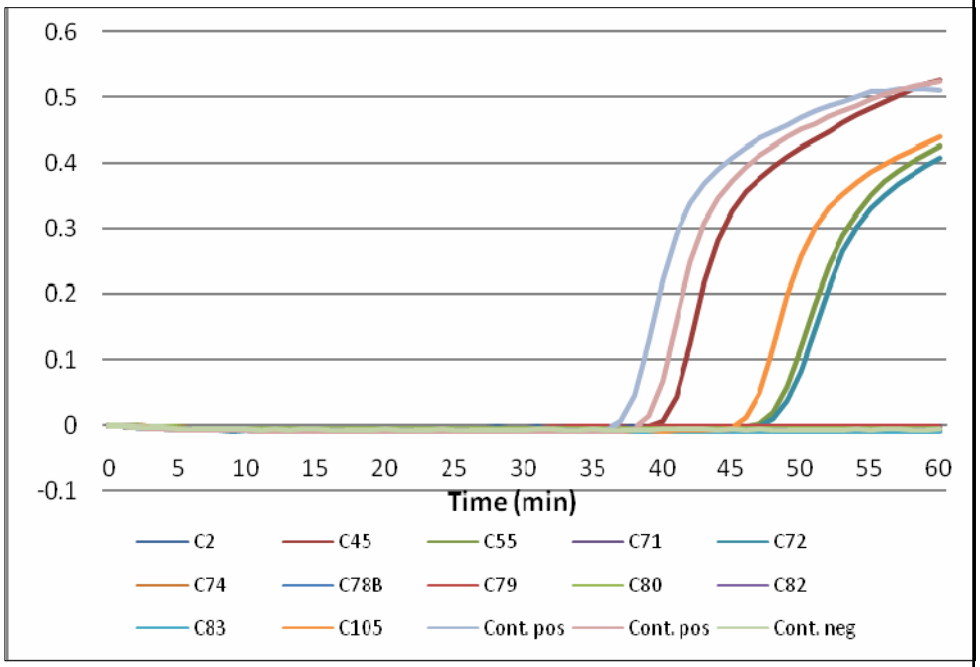
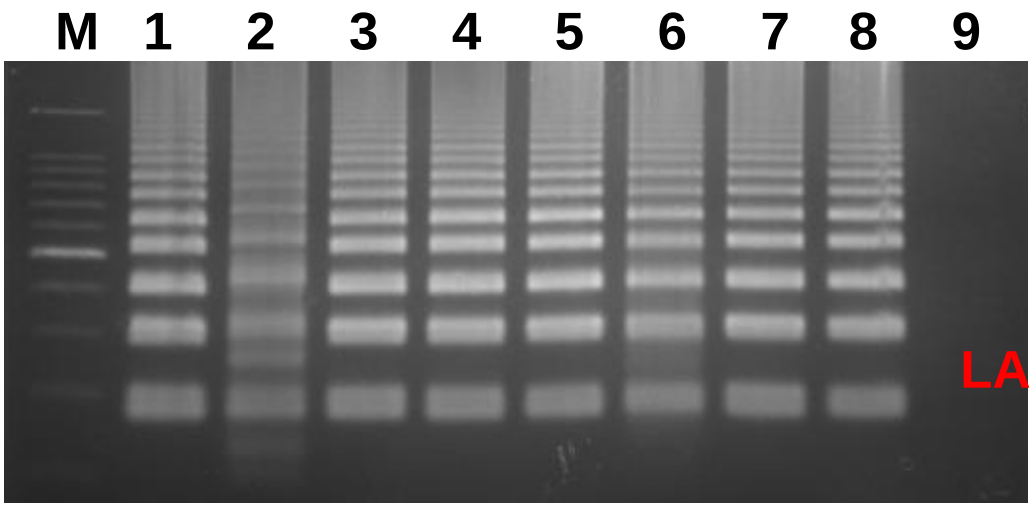
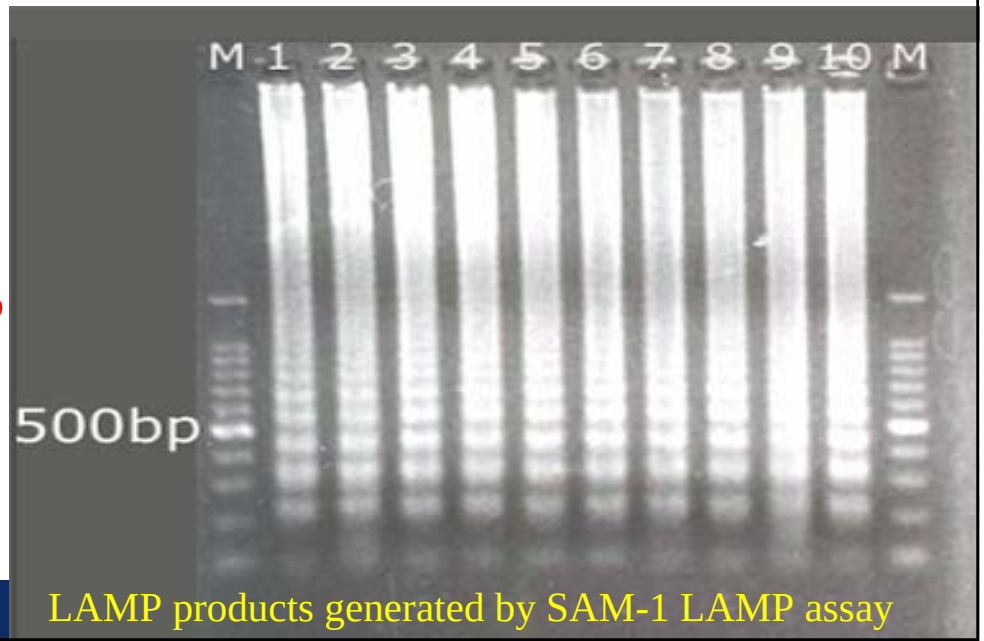


Fig. 4.



Amplification curves of 12 fecal samples using SAM-1 LAMP assay





LAMP products generated by SAM-1 LAMP assay

Part: *Cryptosporidium* findings in environmental samples

LAMP assays based on the S−adenosyl-methionine synthetase (SAM), the 60-kDa glycoprotein (gp-60), the heat shock protein (HSP)-70).

Country	*Source (water)*	*SSU rRNA nested PCR*	*SAM-1*	*GP-60*	*HSP*
Bulgaria	*Well water (Varna)*	*1/1*	*1/1*	*1/1*	*0/1*
	Tap water (Varna)	*1/2*	*2/2*	*2/2*	*2/2*
	Well water (Sofia)	*1/2*	*1/2*	*2/2*	*1/2*
	Sewage effluent (Sofia)	*3/3*	*3/3*	*3/3*	*3/3*
	Raw water (Sofia)	*4/10*	*10/10*	*9/10*	*10/10*
Russia	*Raw water/river*	*1/6*	*5/6*	*2/6*	*3/6*
Hungary	*Effluent sewage*	*2/14*	*1/14*	*0/2*	*0/2*
	Raw water reservoir	*1/6*	*5/6*	*2/6*	*0/3*
	Tap water	*0/1*	*0/1*	*0/2*	*0/2*
Total		*13/45 (29%)*	*23/45 (51%)*	*19/34 (65%)*	*19/34 (65%)*

Verification of LAMP products

It was necessary to verify the LAMP products amplified from the LAMP-positive but PCR-negative samples in order to avoid misinterpretation considering the possibility of LAMP false positives. The specificity of LAMP assays was confirmed by sequencing of the LAMP products generated in positive samples. Sequence products from three Cryptosporidium LAMP assays showed high identity to the target gene sequences confirming the specificity of LAMP.

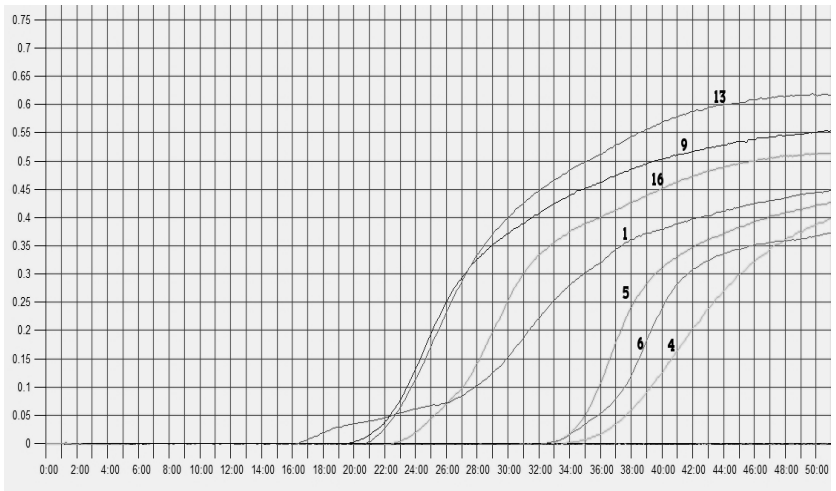
LAMP will be an alternative method to include it in water, food, and/or fecal material analysis after concentrating the (oo)cysts from the samples and combined with a post-Immunomagnetic separation step for further method development.

Giardia duodenalis detection in water & fecal material by PCR / sequence & by LAMP

18 S rRNA - PCR	GDH - PCR	EF1a - LAMP
24 pos/31 exam PCR/sequence	15 pos/31 exam PCR/sequence	20 pos/31 exam
Ass B: 5/19	0/12	-
Ass A: 13/19	8/12	-

GDH *= glutamate dehydrogenase*
EF *= elongated factor 1a*

Detection of *Giardia duodenalis* Assemblage B in samples using real time turbidimeter. The elapsed time versus turbidity are shown in the picture, each curve is different sample including the positive and negative controls. Water samples are curve 1, 4, 5, 6, 9, positive controls are curve 13, 16. The negative samples and negative control are not seen as a curve, since the turbidity in these samples remained zero.



Introduction-*Toxoplasma*

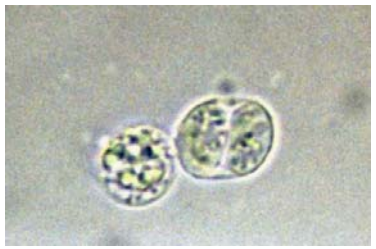
Toxoplasmosis: Worldwide infections in both animals and humans

Felids: the only known hosts excrete environmentally resistant oocysts

Toxoplasma: Water significant transmission?

Association of death in marine mammals with toxoplasmosis

Waterborne outbreaks of toxoplasmosis



No effective method for water detection due to:

a) **Toxoplasma** ***epidemiology;***

b) general limitations in methodology for detection of waterborne protozoan

Aims of the Study

a) Evaluation of a LAMP specific protocol based on two *Toxoplasma* specific genes for the detection of *Toxoplasma* DNA in bench scale experiments using spiked water samples

b) Direct application of the LAMP assay in environmental water samples

c) Comparative findings with nested PCR and IFT

Overview of the study

Primer design based on the B1 and TgOWP *Toxoplasma* genes

↓

Evaluation of specificity and sensitivity of LAMP

↓

Application of IFT, LAMP and Nested - PCR in water samples collected from various sources

↙ ↘

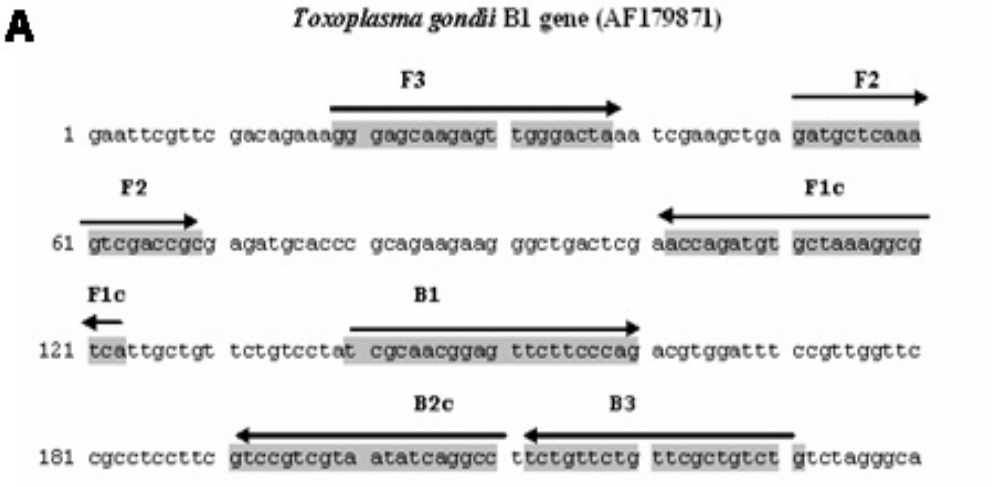
Spiked water pellets | **Natural water sediments**

↘ ↙

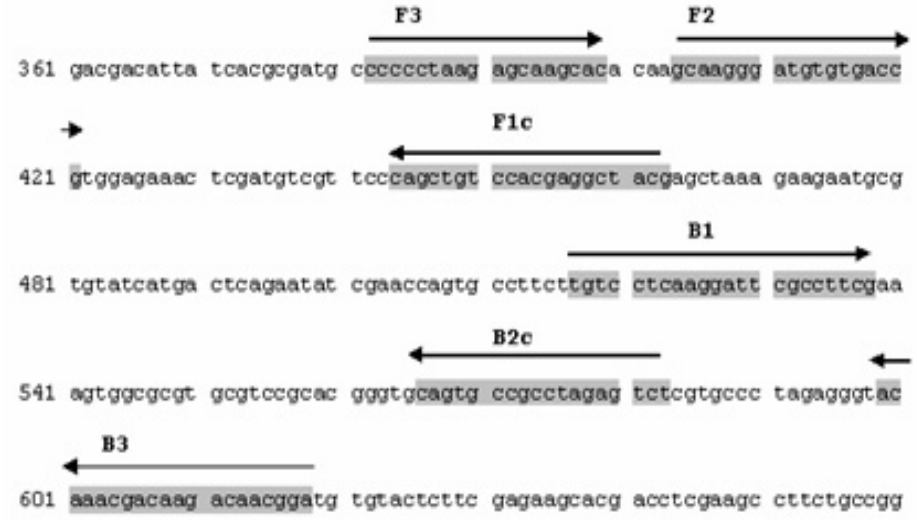
Results

Materials and Methods (cont.)

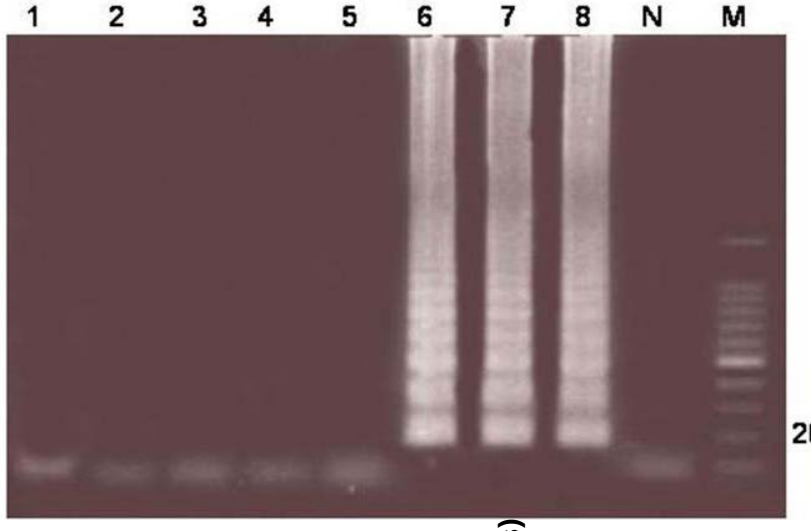
A *Toxoplasma gondii* B1 gene (AF179871)



B *Toxoplasma gondii* putative oocyst wall protein COWP (AY465428)

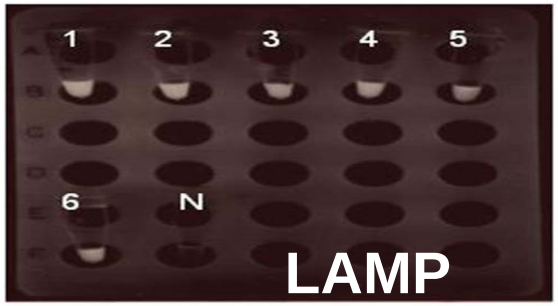


LAMP specificity



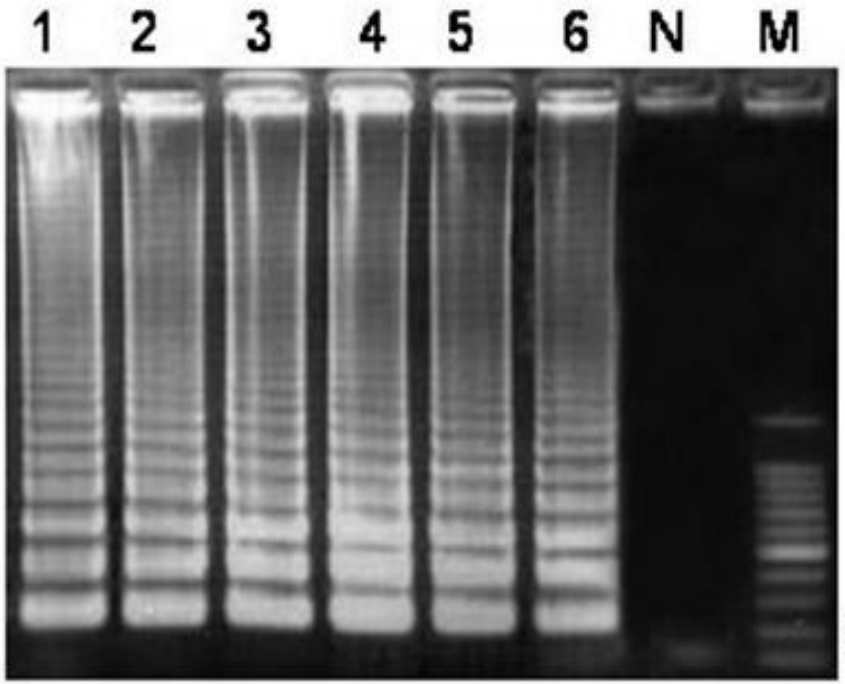
1. *Babesia gibsoni*
2. *Trypanosoma brucei*
3. *Neospora*
4. *Cryptosporidium parvum*
5. *Giardia lamblia*
6. *Toxoplasma* AHC1 (oocysts)
7. *Toxoplasma* PLK (tachyzoites)
8. *Toxoplasma* RH (tachyzoites)
9. Negative control (DDW)
10. Marker

Materials and Methods (cont.)

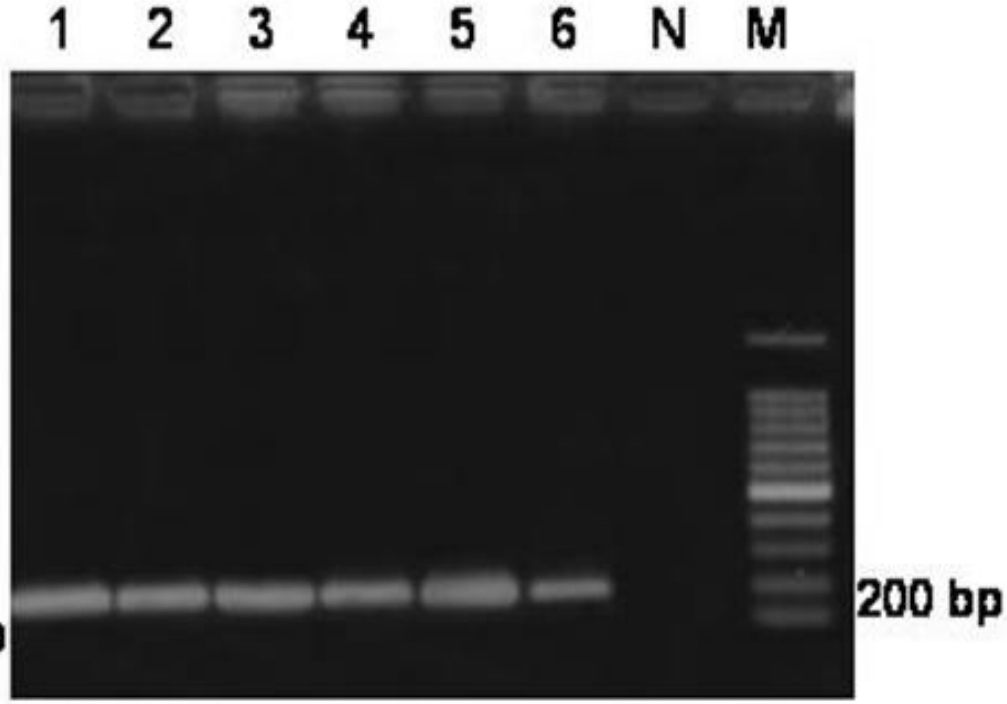


LAMP PCR reaction with Primers F3-B3

LAMP sensitivity



10^4 10^3 10^2 10^1 10^0 10^{-1} Negative Marker



10^4 10^3 10^2 10^1 10^0 10^{-1} Negative Marker

Detection of *Toxoplasma oocysts*

Direct applications of LAMP, Nested - PCR and IFT:

a) in spiked water pellets

b) natural purified pellets

Results

Water quality	LAMP-B1 assay Pos/exam (spiked samples)	Nested PCR Pos/exam (spiked samples)
The origin of samples: Rostov greater area (p 0.0156)		
River	14/14	8/14
Spring	1/1	0/1
Lake	1/1	1/1
Subtotal	16/16	9/16
The origin of samples: Sofia greater area (p 0.0625)		
River	2/2	0/2
Mineral	1/1	0/1
Sewage	3/3	2/3
Well	1/1	1/1
Tap	3/3	2/3
Subtotal	10/10	5/10
Total (%)	26/26 (100%)	14/26 (53.8%)

Results

Water quality	IFT pos/exam (natural samples)	LAMP-B1 assay pos/exam (natural samples)	Nested PCR pos/exam (natural samples)
The origin of samples: Rostov greater area, p 0.0156)			
River	0/14	8/14	2/14
Spring	0/1	1/1	0/1
Lake	0/1	0/1	0/1
Subtotal	0/16	9/16	2/16
The origin of samples: Sofia greater area (p 0.00342)			
River	0/12	7/12	0/12
Mineral	0/3	2/3	0/3
Sewage	0/7	3/7	1/7
Well	0/3	1/3	1/3
Tap	0/9	2/9	3/9
Lake	0/2	1/2	0/2
Subtotal	0/36	16/36	5/36
Total (%)	0/52 (0%)	25/52 (48%)	7/52 (13.5 %)

Remarks

LAMP specificity: Highly specific for the *Toxoplasma* in tests with heterologous genomic DNAs for the detection of *Toxoplasma* B1 and *Tg*OWP genes

LAMP sensitivity: DNA amplification based on the for B1 and *Tg*OWP genes of 1×10^{4} to1×10^{-1} serial diluted tachyzoites

LAMP - PCR: DNA amplification in the serially diluted tachyzoites based the of B1 and *Tg*OWP genes using the F3 & B3 primer pair

Remarks

LAMP:

- **Sensitive in working with „difficult“ DNA templates (g.e. in water samples).**
- **Sequence analysis: exclude the cross reaction with other coccidian.**

Conclusions

Based on the results the LAMP amplification method could currently be placed among the most accurate molecular methods as a specific, sensitive, simple and rapid diagnostic tool for the detection of *Toxoplasma* DNA in water samples.

Investigations on the prevalence of *Plasmodium* species in Thai samples / Northern Thailand

Total blood samples examined = 105	***Microscopy***	***PCR***	***LAMP***	***Any method***
P. falciparum	***37%***	***41%***	***37%***	***44%***
P. vivax	***16%***	***22%***	***21%***	***24%***

Species	Method	Specificity	Sensitivity
Pf	LAMP	100%	91% (100%)[a]
	Microscopy	100%	91%
Pv	LAMP	100%	99%
	Microscopy	98%	65%

[a] Sensitivity calculated by including the results of 5 samples that yielded distinct results when they were retested by nPCR in Japan.

Remarks summary

- LAMP assays developed & established for *Cryptosporidium* spp., *Giardia duodenalis*, *Toxoplasma*, *Plasmodium* spp..
- The LAMP product confirmation is not suitable for routine use. It is laborious and time-consuming and is unnecessary once the specificity of the reaction is established.
- The LAMP assays have major advantages for detection of protozoan at relatively low concentration in any material (water, food, clinical material).
- LAMP: high sensitivity, economic, simple handling.
- LAMP a useful diagnostic tool in the field of parasitology, limited only by the primers design, ingenuity of the technician and application of well-established sampling, sample processing, and control strategy principles.

THANK YOU

TO ALL

CO-WORKERS, SUPPORTERS & COLLABORATORS