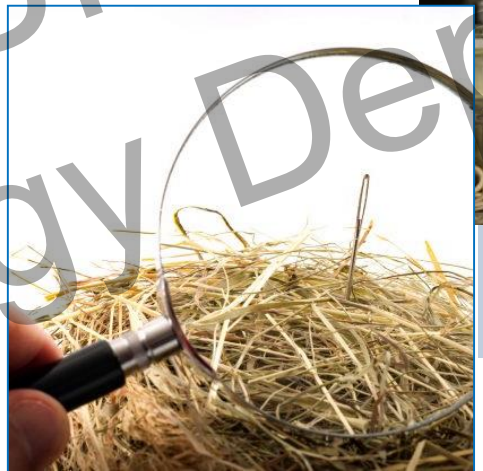
Leiden University
Medical Center

Diagnosing worm infections in a Dutch setting

Looking for a needle in a haystack



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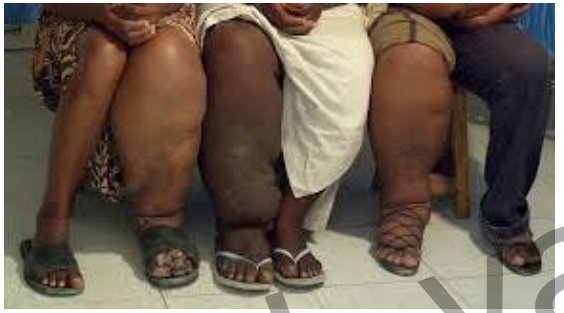
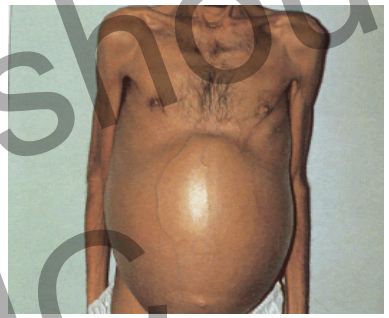
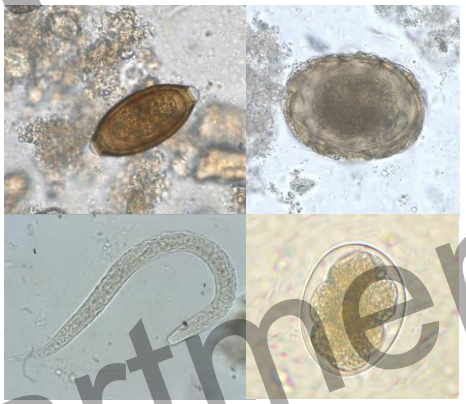
Relevance of helminth diseases - Global estimates
"Living in a wormy world"



Lymphatic filariasis ≈ 30 - 120M

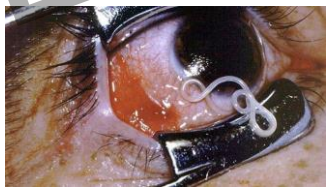


Schistosomiasis ≈ 190-240 M



Soil Transmitted Helminths
≈ 800 - 1,400 M???

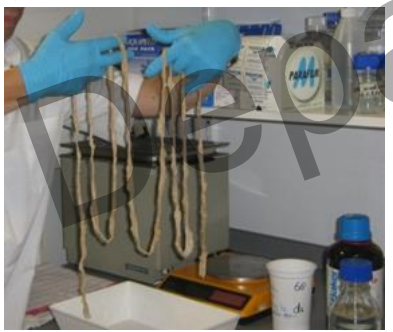
Loiasis and other tissue nematodes, including *Toxocara* ≈ 10 M??



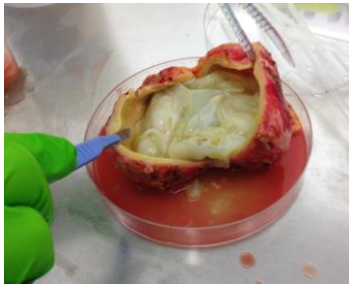
River blindness/ onchocerciasis ≈ 15 M??

Enterobius and other intestinal nematodes ≈ ?? M

Food Born Trematodes
≈ 75 M???



Taeniasis/cysticercosis and other cestodes ≈ 3 - ?? M



Echinococcosis ≈ 1 M??

8 of 17 NTDs are helminths
Top 5 of most prevalent NTDs are helminths

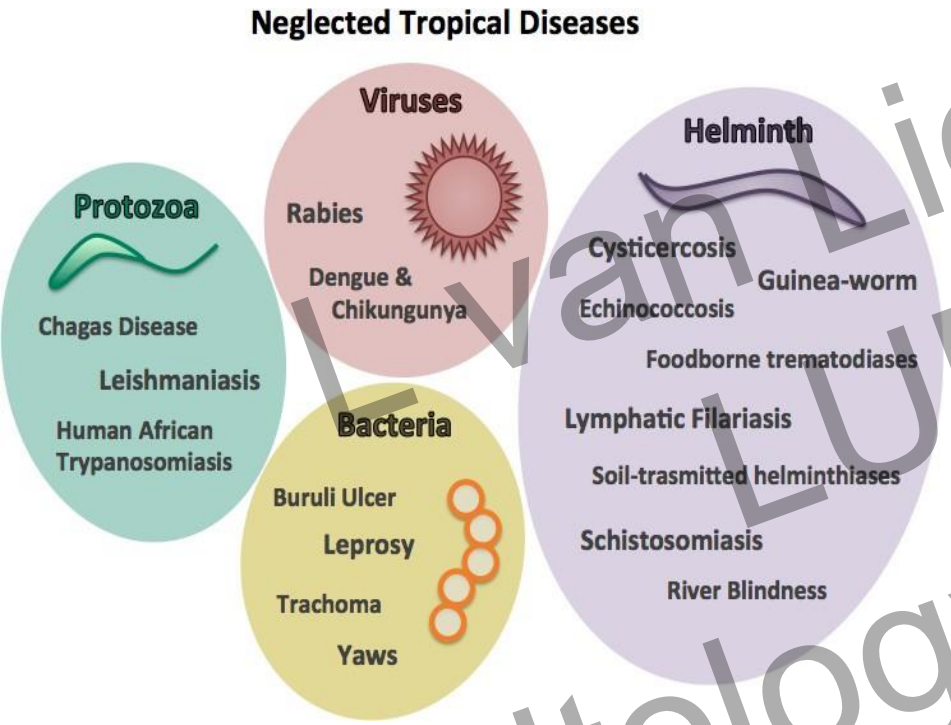


Table 1. The Major Neglected Tropical Diseases Ranked by Prevalence.*

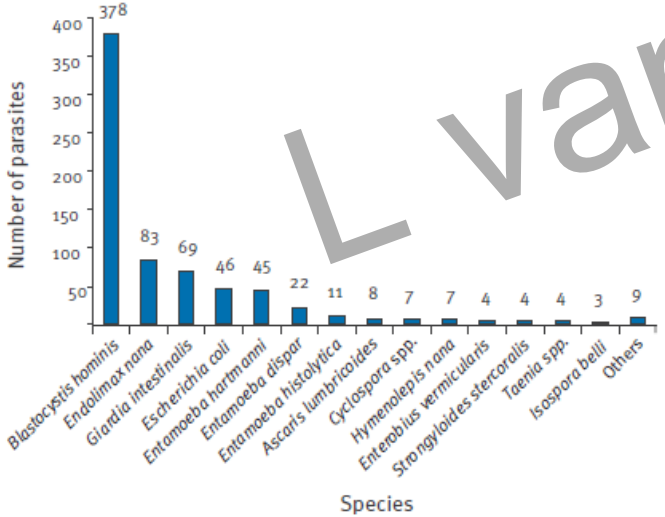
Disease	Global Prevalence (millions)	Population at Risk	Regions of Highest Prevalence	Source
Ascariasis	807	4.2 billion	East Asia and Pacific Islands, sub-Saharan Africa, India, South Asia, China, Latin America and Caribbean	Bethony et al.,[6] de Silva et al.[7]
Trichuriasis	604	3.2 billion	Sub-Saharan Africa, East Asia and Pacific Islands, Latin America and Caribbean, India, South Asia	Bethony et al.,[6] de Silva et al.[7]
Hookworm infection	576	3.2 billion	Sub-Saharan Africa, East Asia and Pacific Islands, India, South Asia, Latin America and Caribbean	Bethony et al.,[6] de Silva et al.[7]
Schistosomiasis	207	779 million	Sub-Saharan Africa, Latin America and Caribbean	Steinmann et al.[8]
Lymphatic filariasis	120	1.3 billion	India, South Asia, East Asia and Pacific Islands, sub-Saharan Africa	Ottesen,[9] WHO[10]
Trachoma	84	590 million	Sub-Saharan Africa, Middle East and North Africa	International Trachoma Initiative,[11] Médecins sans Frontières[12]
Onchocerciasis	37	90 million	Sub-Saharan Africa, Latin America and Caribbean	Basáñez et al.[13]
Leishmaniasis	12	350 million	India, South Asia, sub-Saharan Africa, Latin America and Caribbean	Desjeux[14]
Chagas' disease	8–9	25 million	Latin America and Caribbean	WHO[15]
Leprosy	0.4	ND	India, sub-Saharan Africa, Latin America and Caribbean	International Federation of Anti-Leprosy Associations[16]
Human African trypanosomiasis	0.3	60 million	Sub-Saharan Africa	Févre et al.[17]
Dracunculiasis	0.01	ND	Sub-Saharan Africa	Carter Center[18]
Buruli ulcer	ND	ND	Sub-Saharan Africa	Global Buruli Ulcer Initiative[19]

* ND denotes not determined

Low prevalence of helminths in non-endemic setting

FIGURE

Intestinal parasite counts detected in stool samples from 594 patients, by species, Rome, Italy, 1 May 2006–31 December 2008 (n=700)



'Others' include *Cryptosporidium* spp. (n=2), *Encephalitozoon intestinalis* (n=2), *Dientamoeba fragilis* (n=1), *Trichuris trichiuria* (n=1), *Trichomonas hominis* (n=1), *Chilomastix mesnili* (n=1), and *Iodamoeba bütschlii* (n=1).

Taenia spp. include *Taenia saginata* and *T. solium*.

Masucci et al., 2014

Calderaro *et al. BMC Infectious Diseases* 2014, **14**:264
http://www.biomedcentral.com/1471-2334/14/264

BMC Infectious Diseases

RESEARCH ARTICLE **Open Access**

Intestinal parasitoses in a tertiary-care hospital located in a non-endemic setting during 2006–2010

Adriana Calderaro*, Sara Montecchini, Sabina Rossi, Chiara Gorrini, Flora De Conto, Maria Cristina Medici, Carlo Chezzi and Maria Cristina Arcangeletti

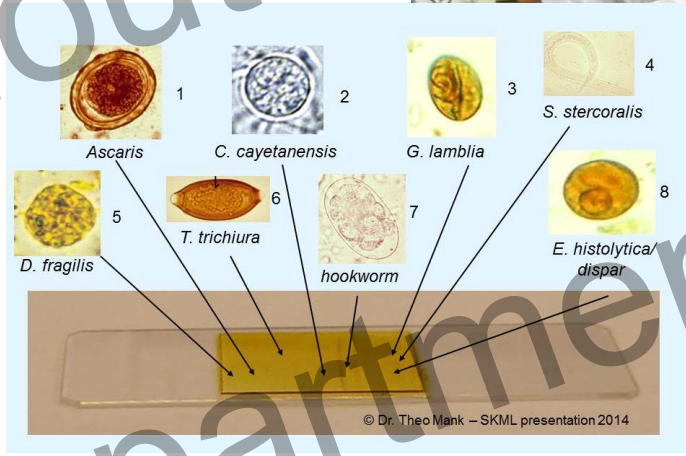
PLOS ONE | https://doi.org/10.1371/journal.pone.0197770 May 30, 2018

RESEARCH ARTICLE

Low incidence of helminth infections (schistosomiasis, strongyloidiasis, filariasis, toxocariasis) among Dutch long-term travelers: A prospective study, 2008-2011

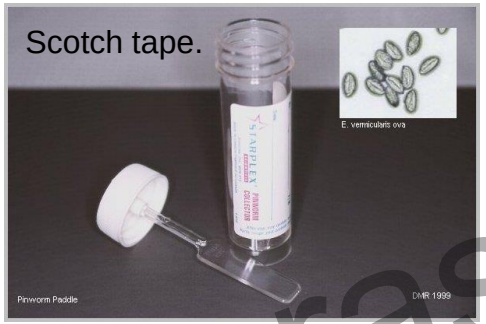
Femke W. Overbosch[1,2]*, Tom van Gool[3,4], Amy Matser[5], Gerard J. B. Sonder[1,2,3]

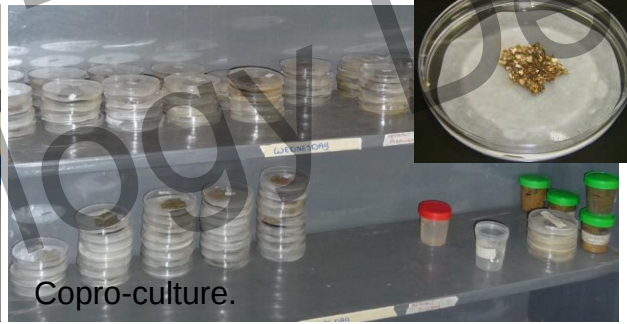
Diagnosis of helminths infection
Key role of microscopy – for most species



Quantification

Lack of sensitivity





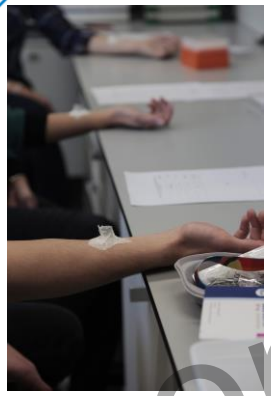
Diagnosing Helminth infections non endemic setting – antibody detection?

Importance of serology:

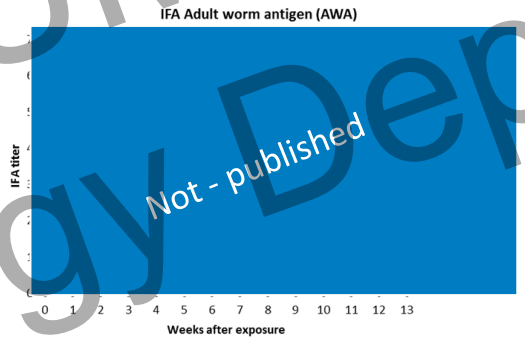
- Schistosomiasis
- Strongyloides
- Filariasis
- Fascioliasis
- Echinococcosis
- Cysticercosis
- Toxocariasis

Controlled Human *Schistosoma* infection (CoHSI) - Antibody detection:

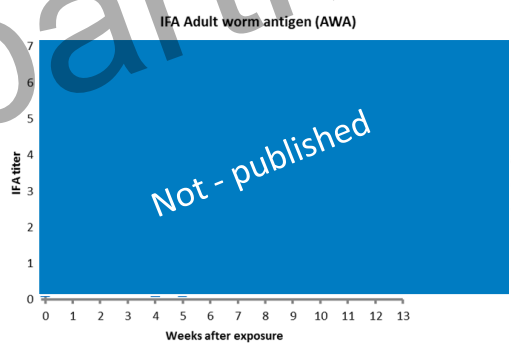
- highly sensitive and specific in first time exposed cases
- sero-conversion 4-6 weeks (IFA)



10 cercariae



30 cercariae



Considerations when introducing NAAT

- Microscopy is laborious and lacks sensitivity
- Prevalence is globally high, but very low in NL => high NPV test for screening
- Serology only for specific species & target populations

Can we replace stool microscopy for helminths by PCR?

Is this beneficial for the lab? – what do we see in the validations?

Is this beneficial for the patient? – which infections can be missed?

What are the challenges? - what more can we learn from EQAS?

Real-time (multiplex) PCR
Examples – helminths stool/urine

Nematodes	Cestodes	Trematodes
Ascaris lumbricoides	*Taenia saginata*	*Schistosoma mansoni*
Trichuris trichiura	*Taenia solium*	*Schistosoma haematobium*
Ancylostoma spp.	*Hymenolepis nana*	*Schistosoma* genus
Necator americanus		*Opisthorchis felineus*
Strongyloides stercoralis		*Clonorchis sinensis*
Oesophagostomum bifurcum		

*) implemented at Dept. Parasitology LUMC
For details see: Verweij & Stensvold; Clin Micr Rev (2014)

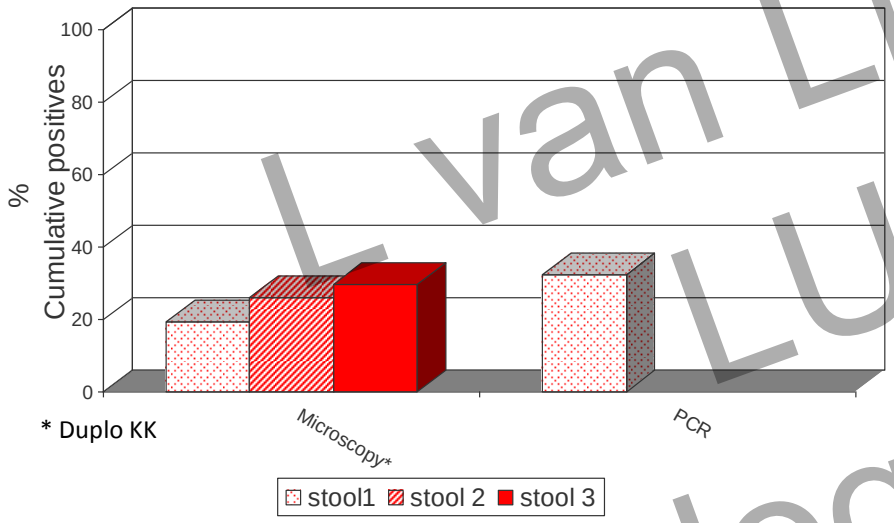
Summary of three LUMC validation studies in a Dutch/Belgium setting

	A		B		C	
	General practitioners		General practitioners and peripheral hospital		Travel clinic	
	N = 722 (ten Hove *et al.* 2007)		N = 397 (Bruijnesteijn van Coppenraet *et al.* 2009)		N = 2591 (ten Hove *et al.* 2009)	
	Microscopy (%)	PCR (%)	Microscopy (%)	PCR (%)	Microscopy (%)	PCR (%)
E. histolytica/E. dispar	0	–	1 (0·3%)	–	99 (3·8%)	–
E. histolytica	–	0	–	1 (0·3%)	–	13 (0·5%)
G. lamblia	41 (5·7%)	67 (9·3%)	29 (7·3%)	44 (11·1%)	95 (3·7%)	149 (5·8%)
Cryptosporidium	–	36 (5·0%)	2 (0·5%)	3 (0·5%)	12 (0·5%)	31 (1·2%)
D. fragilis	8* (3·2%)*	–	69 (17·4%)	122 (30·7%)	–	–
Strongyloides	0	–	0	–	3 (0·1%)	21 (0·8%)
Additional helminths	0	–	1 (0·3%)	–	49 (1·9%)	
Additional protozoa		–	0	–	6 (0·2	
Non-pathogenic protozoa	28 (3·9%)	–	129 (32·5%)	–	74	–

Schistosoma

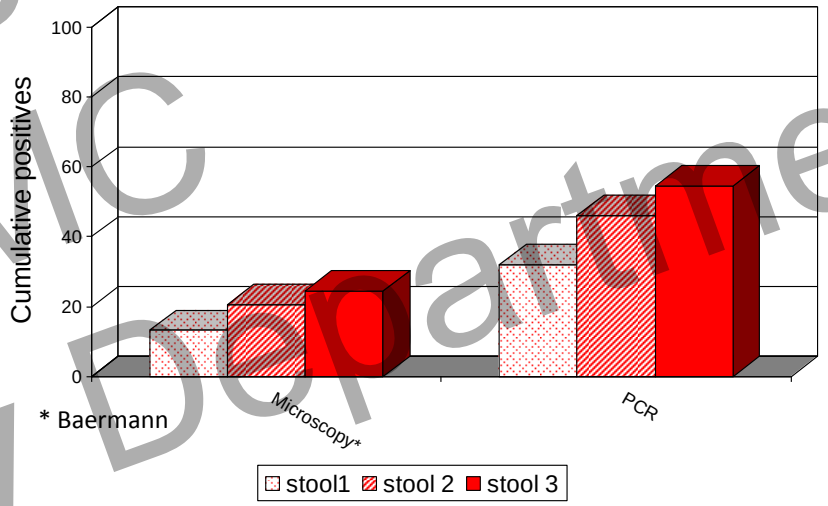
Validation of helminths PCR – endemic setting

Schistosoma; Kenia (Lake Victoria, n=760)



* Duplo KK

Strongloides; Peru (La Merced, n=188)



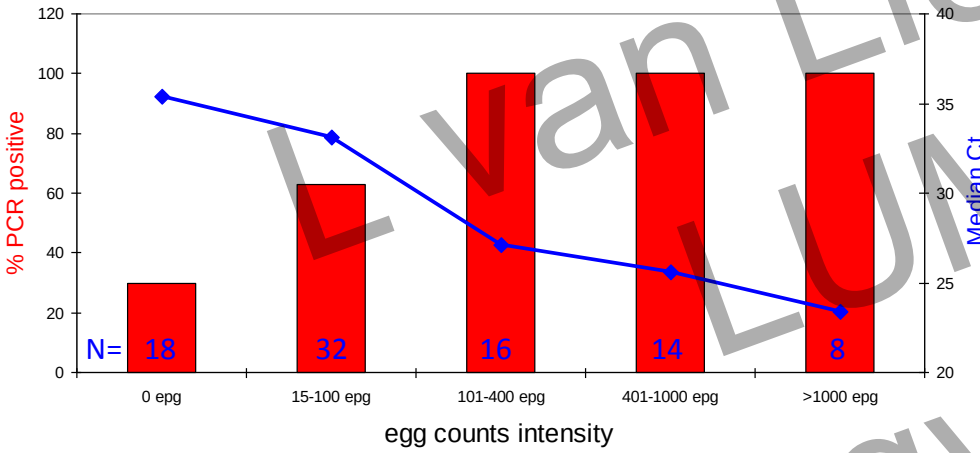
* Baermann

o.a. Verweij & Stensvold (2014); Meurs *et al.*, 2015; 2017

Validation of helminths PCR – endemic setting

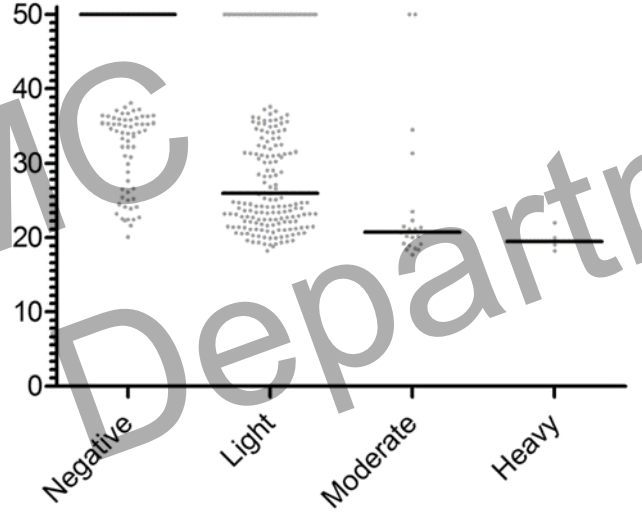
Northern Senegal (N=88, duplo stools microscopy)

S. mansoni PCR



Kenia (N=760, triple stool microscopy)

Schistosoma PCR



1-99 epg 100-399 epg ≥400 epg

Ten Hove *et al* (2008); Meurs et al., (2015)

Validation of helminths PCR – endemic setting

Schistosoma microscopy vs – ITS genus PCR			%pos	%pos
Region	Material	N	Microsc.	PCR
Mozambique	Stool (ethanol)	303	2%	11%
Senegal	Stool (ethanol)	251	70%	72%
Tanzania	urine	422	4%	11%
Ghana	urine	730	8%	21%
South Africa (1)	urine	708	32%	25%
South Africa (2)	urine	394	20%	23%
Kenya(*)	urine	114	83%	100%
Madagascar(*)	urine	79	100%	81%

- (*) selected based on previous screening microscopy
- PCR ≅ to >>>microscopy / quality& quantity microscopy
- intensity of infection; light infections missed by both

o.a. Aryeetey *et al* (2013); Pillay *et al* (2014); Vinkeles et al (2014); Randrianasolo *et al* (2015); Meurs *et al* (2015, 2017) & unpublished data

Influence of sample preparation

Am. J. Trop. Med. Hyg., 90(6), 2014, pp. 1153–1155
doi:10.4269/ajtmh.14-0005

Short Report: Impact of Short-Time Urine Freezing on the Sensitivity of an Established *Schistosoma* Real-Time PCR Assay

Hilaire M. Kenguele,* Ayola A. Adegnika, Anne-Marie Nkoma, Ulysse Ateba-Ngoa, Mirabeau Mbong, Jeannot Zinsou, Bertrand Lell, and Jaco J. Verweij

Centre de Recherches Médicales de Lambaréné (CERMEL), Lambaréné, Hôpital Albert Schweitzer Gabon; Institut für Tropenmedizin, Universität Tübingen, Tübingen, Germany; Department of Parasitology, Leiden University Medical Center, Leiden, The Netherlands; Laboratory for Medical Microbiology and Immunology, St. Elisabeth Hospital, Tilburg, The Netherlands

Abstract. Urogenital schistosomiaisis is a serious public health problem in sub-Saharan Africa. In this study, we have updated an established real-time polymerase chain reaction (PCR) routinely used in our laboratory. *Schistosoma* genus-specific real-time PCR was performed on DNA isolated from 85 urine samples and pellets obtained after centrifugation without and after frozen storage. The results revealed that concentration by centrifugation of the urine samples and freezing of the samples before extracting DNA improves the sensitivity of the PCR.

Helminth PCR – LUMC routine diagnostics

Helminths				
Year of implem.	PCR-target	Replaced microscopy[a,b]	samples/year	indiv. pos/year
2006	*Strongyloides*	yes	≈400	≈2-4
2007	*Schistosoma*[c]	±	≈140	≈4-6

a) as first line of diagnosis
b) serology still in use
c) Stool, urine, semen, biopsy

Helminth PCR – LUMC routine diagnostics

Strongyloides

- Never missed a microscopy positive by PCR
- Serology most sensitive
  - chronic infections
- Serology positive - PCR negative is very common
- PCR more appropriate for follow-up after therapy, days-weeks

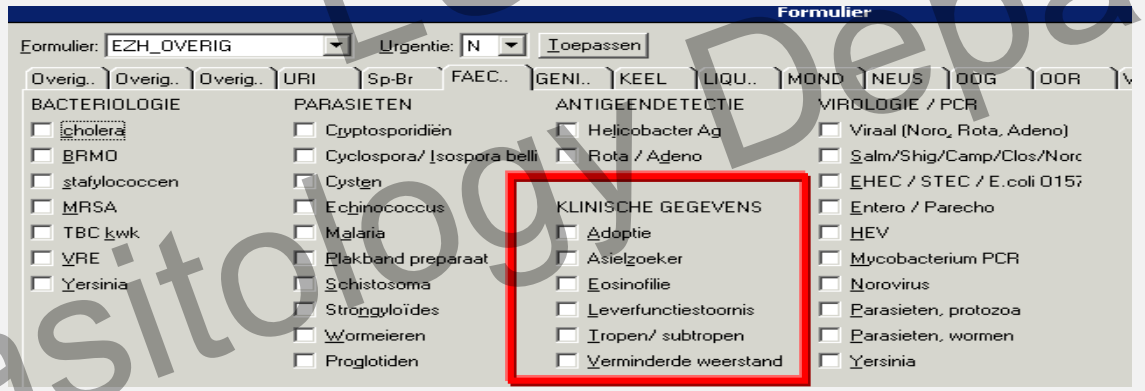
Schistosomiasis

- Hardly missed a microscopy positive by PCR
- Serology very helpful
  - acute infections, travellers
- Serology positive - PCR negative is very common
- PCR very appropriate for tissue, semen, biopsy
- CAA/CCA option

Algorithm parasitological diagnostics

- Standard (Gastroenteritis)
  - PCR protozoa: *E. histolytica, Cryptosporidium, Giardia*
    - Including *Cyclospora* since July 2018
- Standard (Ova & Cysts)
  - PCR protozoa: *E. histolytica, Cryptosporidium, Giardia*
    - Including *Cyclospora* since July 2018
  - PCR helminths: *Ancylostoma, Necator, Ascaris, Trichuris, Strongyloides, Schistosoma*
    - Including *Taenia, Hymenolepis, E. vermicularis* since July 2018

- Adoption, refugee, eosinophilia, hepatic impairment, (sub) tropic, immunosuppression, visual inspection
  - Additional microscopy



Kindly provided by Dr. Jaco Verweij – ETZ Hospital Tilburg

Helminth PCR ETZ hospital 2014-2017 (N=4325)

	Pos/Neg	Median Ct (range)
Ancylostoma spp.	0	
Necator americanus	0	
Ascaris lumbricoides	2	(28.5; 29.0)
Trichuris trichiura	3	(28.2; 27.2; 27.7)
Strongyloides stercoralis	5	24.7 (13.8 - 33.5)
Schistosoma	6	19.7 (17.8 - 24.7)
Negative	4304	
Inhibition	4	

Kindly provided by Dr. Jaco Verweij – ETZ Hospital Tilburg

Additional microscopy ETZ hospital 2014-2017 (N=700)

	Positive
Taenia	3
Enterobius vermicularis	4

Kindly provided by Dr. Jaco Verweij – ETZ Hospital Tilburg

Helminth External Molecular Quality Assessment Scheme (HEMQAS)

Pilot study; following successes of protozoa EQAS

Distribution of real pathogens in stool

- Field samples (& non-endemic controls)
- No lyophilized material nor DNA in artificial matrix

- Ethanol preservative for long stability
- Participants: research laboratories; reference laboratories (control programs); clinical laboratories

Panel:

Hookworm (*N. americanus* & *Ancylostoma spp.*), *Ascaris spp.*, *Trichuris trichiura*, *Stronglyoides stercoralis*, *Schistosoma spp.*

HEMQAS pilot
Study design and sample validation

2017/2018: 12 stool samples

Microscopy, diluted, ethanol

2x4 DNA (purified from isolated adult worms/L3 larvae; high and low concentration)

Sample validation: 6 expert laboratories, 3 continents (4-5 per target)

Stability and Homogeneity

- Targets with high parasite load were consistently detected in 5-plo
- Within accepted range of intra-laboratory variation in Cq
  - True for all 12 prepared samples

Testing of samples: 15 laboratories, 5 continents
(including 6 expert laboratories)

WELLCOME TRUST RESEARCH LABORATORY
Division of Gastrointestinal Sciences

HEMQAS pilot
Principle of scoring system & expert outcome

Sample positive for specific target

- Homogenous & stable, detected by all expert laboratories (by PCR)

Sample negative for specific target

- Not detected by any of the expert laboratories (by PCR)

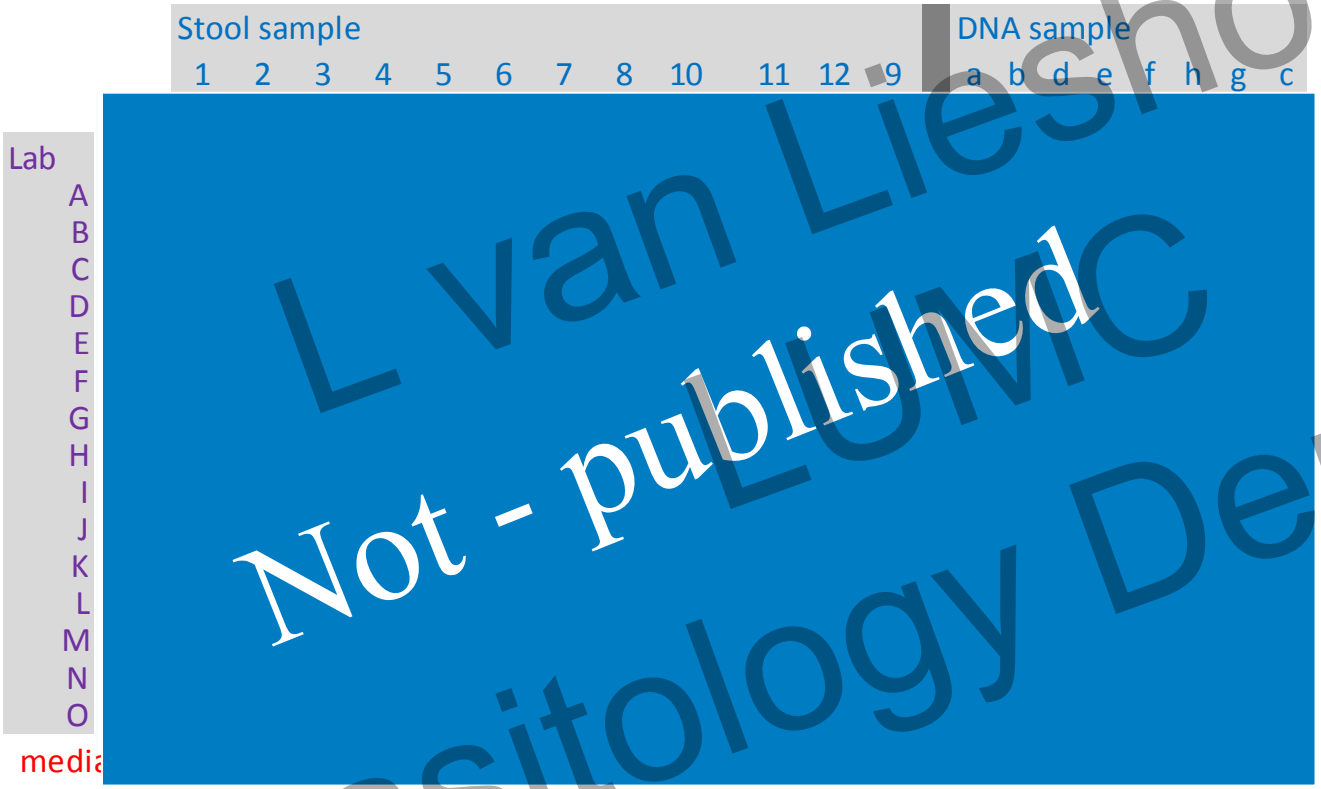
Sample educational for specific target

- Low concentration of a target – not detected by each expert laboratory
- Some of these were (low) microscopy positive!

Outcome of 12 stool samples when tested by 6 experts laboratories:

- 1x completely negative
- 2x with positive target(s) only
- 5x with mixed positive target(s) & educational target(s)
- 4x with educational target(s) only

HEMQAS pilot
participants outcome – *Schistosoma* (*S. mansoni*)



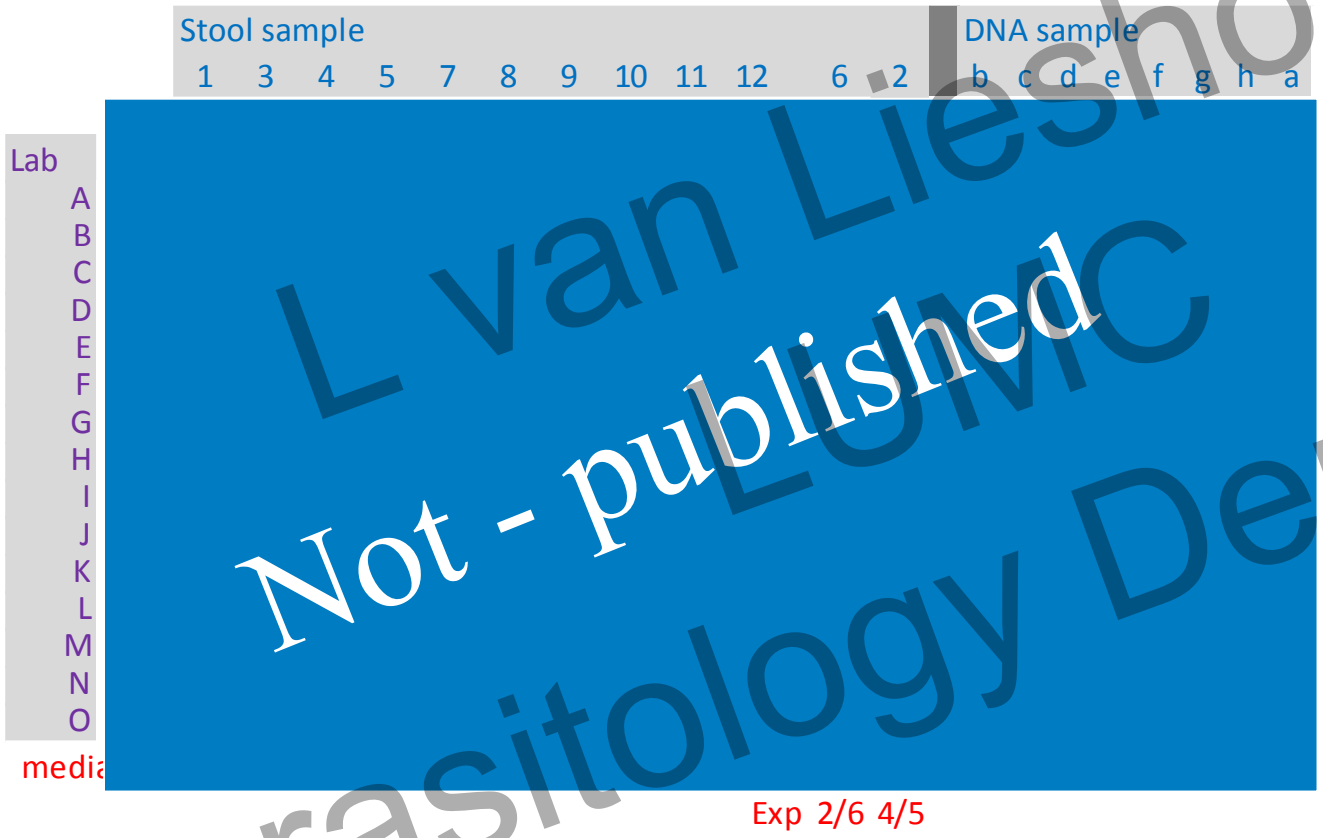
= not performed
I = inhibition

Negative/Positive/DNA samples
= 2 points
= 2 points
N = 0 points
P = 0 points

Educational samples
= not scored
= not scored
N = not scored

x = Δ Ct to median Ct
N = false negative
P = false positive

HEMQAS pilot
participants outcome- *Strongyloides stercoralis*



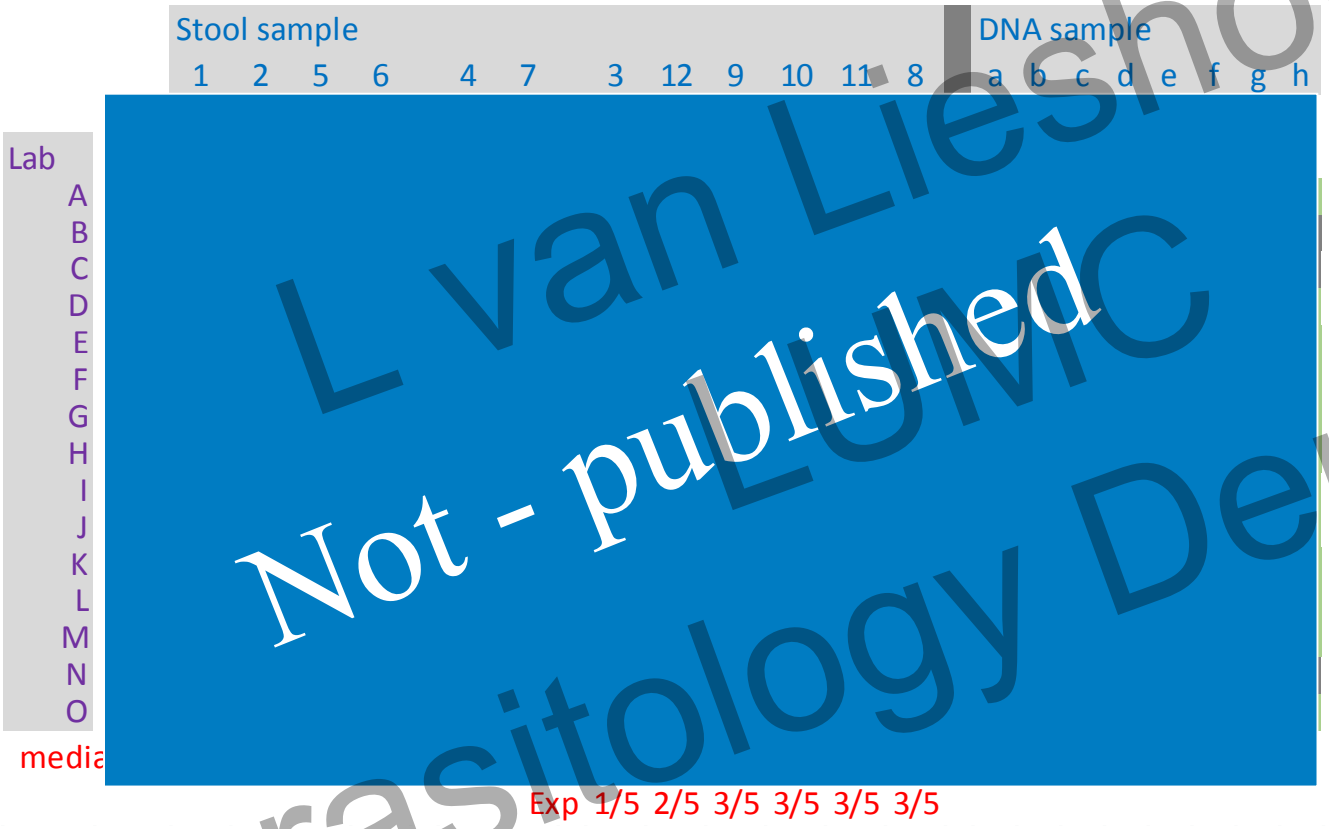
= not performed
I = inhibition

Negative/Positive/DNA samples
= 2 points
= 2 points
N = 0 points
P = 0 points

Educational samples
= not scored
= not scored
N = not scored

x = Δ Ct to median Ct
N = false negative
P = false positive

HEMQAS pilot
participants outcome – *Trichuris trichiura*



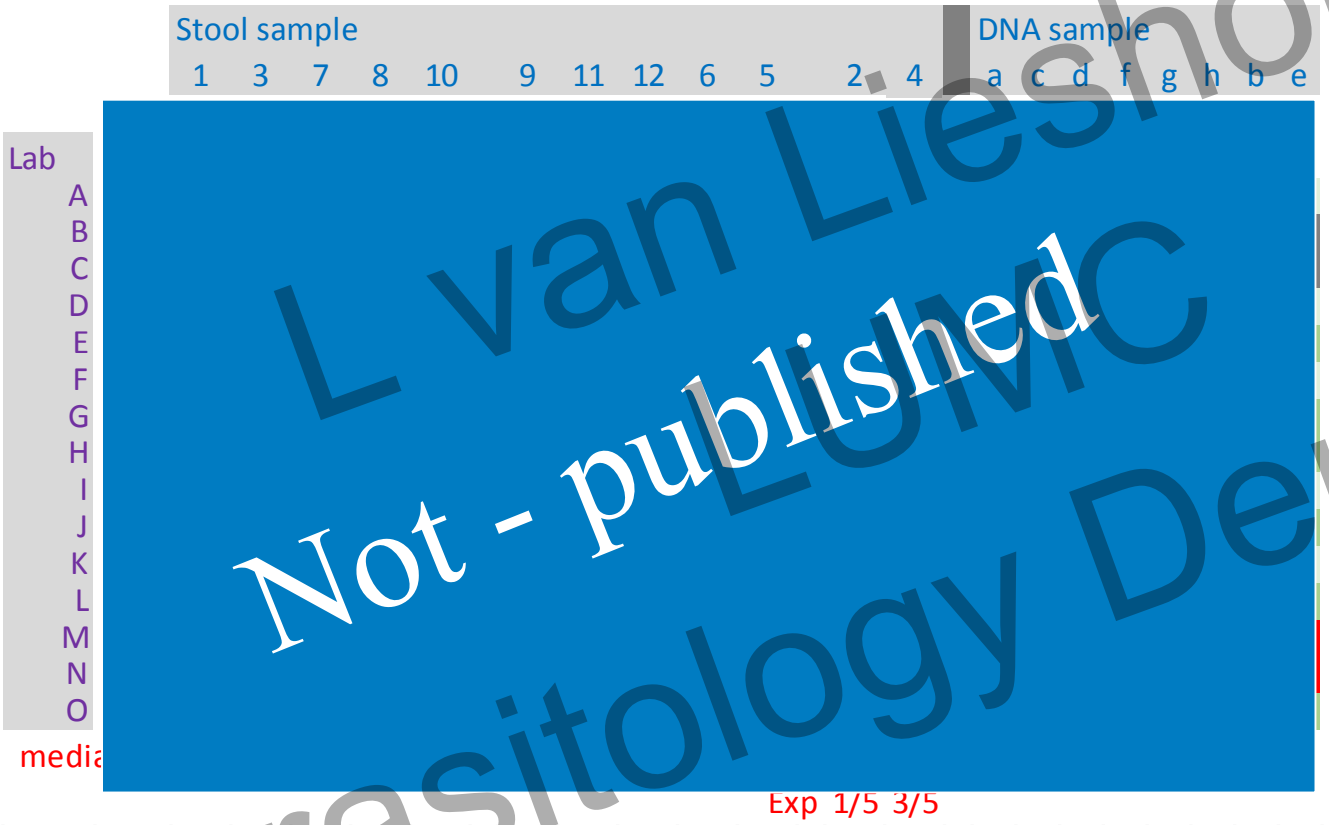
= not performed
I = inhibition

Negative/Positive/DNA samples
= 2 points
= 2 points
N = 0 points
P = 0 points

Educational samples
= not scored
= not scored
N = not scored

x = Δ Ct to median Ct
N = false negative
P = false positive

HEMQAS pilot
participants outcome – *Necator americanus*



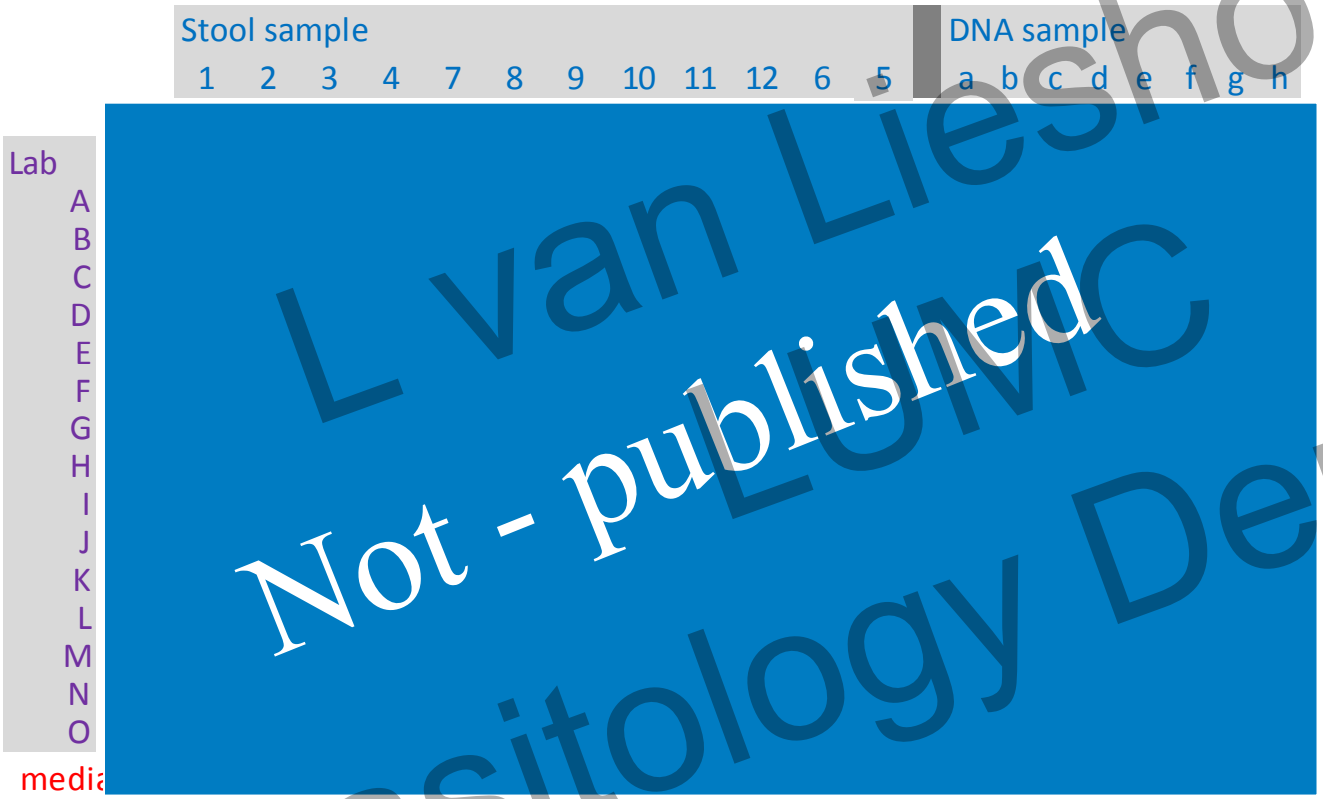
= not performed
I = inhibition

Negative/Positive/DNA samples
= 2 points
= 2 points
N = 0 points
P = 0 points

Educational samples
= not scored
= not scored
N = not scored

x = Δ Ct to median Ct
N = false negative
P = false positive

HEMQAS pilot
participants outcome – *Ancylostoma*



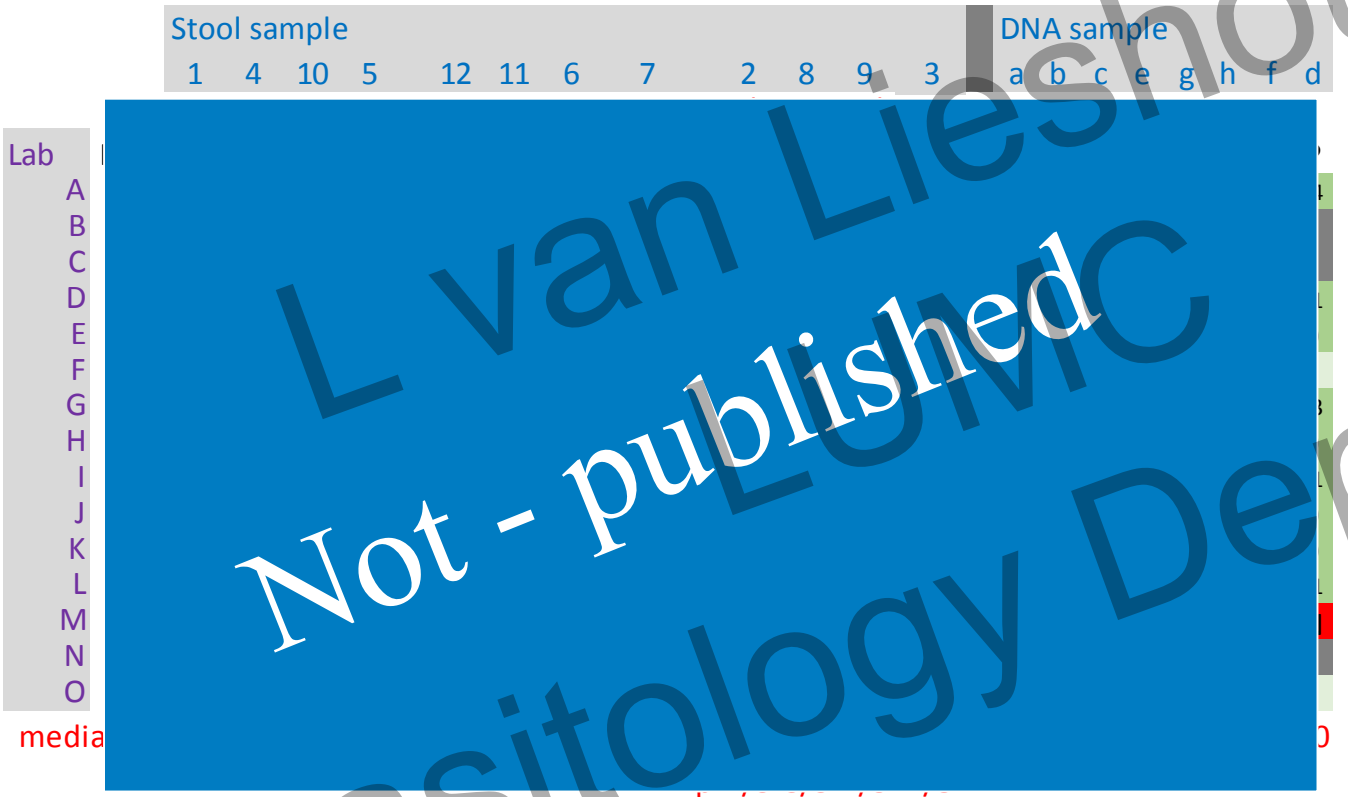
= not performed
I = inhibition

Negative/Positive/DNA samples
= 2 points
= 2 points
N = 0 points
P = 0 points

Educational samples
= not scored
= not scored
N = not scored

x = Δ Ct to median Ct
N = false negative
P = false positive

HEMQAS pilot
participants outcome – *Ascaris*



= not performed
I = inhibition

Negative/Positive/DNA samples
= 2 points
= 2 points
N = 0 points
P = 0 points

Educational samples
= not scored
= not scored
N = not scored

x = Δ Ct to median Ct
N = false negative
P = false positive

HEMQAS pilot participants outcome

- High range in % correctly detected positive targets (33%-100%)
- False negative results (all targets, but mainly *Trichuris* with max 92% pos)
  - intensity of infection (microscopy) related
  - more failures in stool than in DNA samples - importance of correct DNA isolation
- False positive results (in particular *Strongyloides*)
- Clusters of poor-performers and good-performers
  - Cause of variations will be investigated by analysis of differences in methods

HEMQAS pilot
Overall conclusions

- Proof of concept: validated, homogeneous samples
- Feasibility: world-wide EQAS distribution and reporting
- More variation in performance HEMQAS than GI protozoa
- Species-related performance differences, challenging samples
- Quantitative: reported Cq-values differ > 10 cycles (= 2^{10} >1000 fold !)

Need for quality control (and harmonisation) for NAAT methods detecting helminths in stool

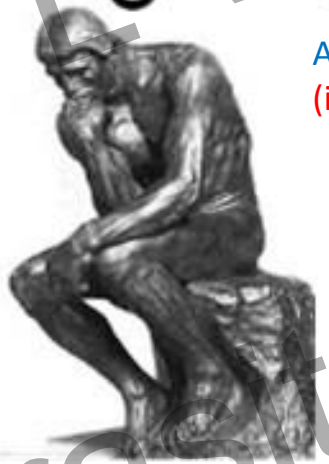
→ Yearly Helminth Molecular EQAS will be started in 2019 by SKML

https://www.skml.nl/en/home/sections/parasitology

Take-home points

- Low prevalence helminths
  - Smart algorithms increases diagnostic efficiency, but species might be missed
  - Increases key-position clinician *(who should be prepared for the unexpected rare cases)*
- NAAT major advantage of high NPP
  - Apply the relevant PCR targets for your population
- Participation in EQAS essential – not all helminths covered
- !!! Shortness of validation material, controls etc.
- Do not forget importance of serology in diagnosing helminths

Helminth diagnostics: be even more critical!



Acknowledgement to: LUMC colleagues, technicians, students, (inter)national collaborators, funding agencies.