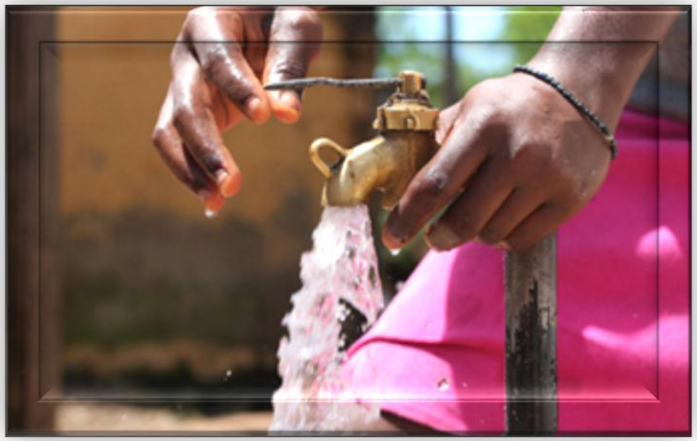
Detection of a microbial source tracking marker by isothermal helicase-dependent amplification and a nucleic acid lateral flow-strip test

Safer water,better health

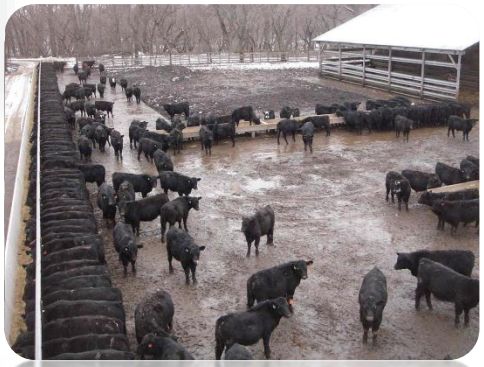
842 000 deaths per year, is attributable to unsafe water supply, sanitation and hygiene and includes 361 000 deaths of children under age five, mostly in low-income countries.



Faecal pollution of water

Origin:

- Human wastewater
- manure from livestock operations
- diffuse sources such as input from wildlife and/or urban runoff



Microbial Source Tracking(MST) is important!

remediate the causes

economic

effective management actions

Assessing associated health risks

Standard Faecal Indicator Bacteria enumeration (SFIB)

Escherichia coli and intestinal *enterococci* were used as general indicators of faecal pollution

this method is based on membrane filtration, subsequent culture on a chromogenic coliform agar medium, and calculation of the number of target organisms in the sample

Molecular microbial source tracking (MST)

Linking faecal pollution to its source using the close association of certain faecal microorganisms with a specific host

⇩

the **order *Bacteroidales*** are currently the most prominent faecal source identifiers in water

⇩

ruminant-associated Bacteroidetes 16S rRNA marker (BacR)

- The results from a geographically expansive comparison study that spanned 16 countries and six continents suggested that the BacR marker was robust worldwide

PCR,quantitative PCR (qPCR)

PCR-based assays have been developed to identify various potential faecal pollution sources

But the widespread use of these powerful tools is often limited

- Have not access to the type of molecular biology equipment
- Molecular MST requires extensive technical training to perform the methods and interpret the results

Helicase-Dependent Amplification (HDA)

- Isothermal DNA amplification → a constant temperature on a standard heating block or in a water bath (~ 65°C)
- without the need for high-end PCR/qPCR instruments
- rapid
- simple-to-use
- low-cost molecular source tracking tools

Material & Method

Faecal sample processing for performance tests

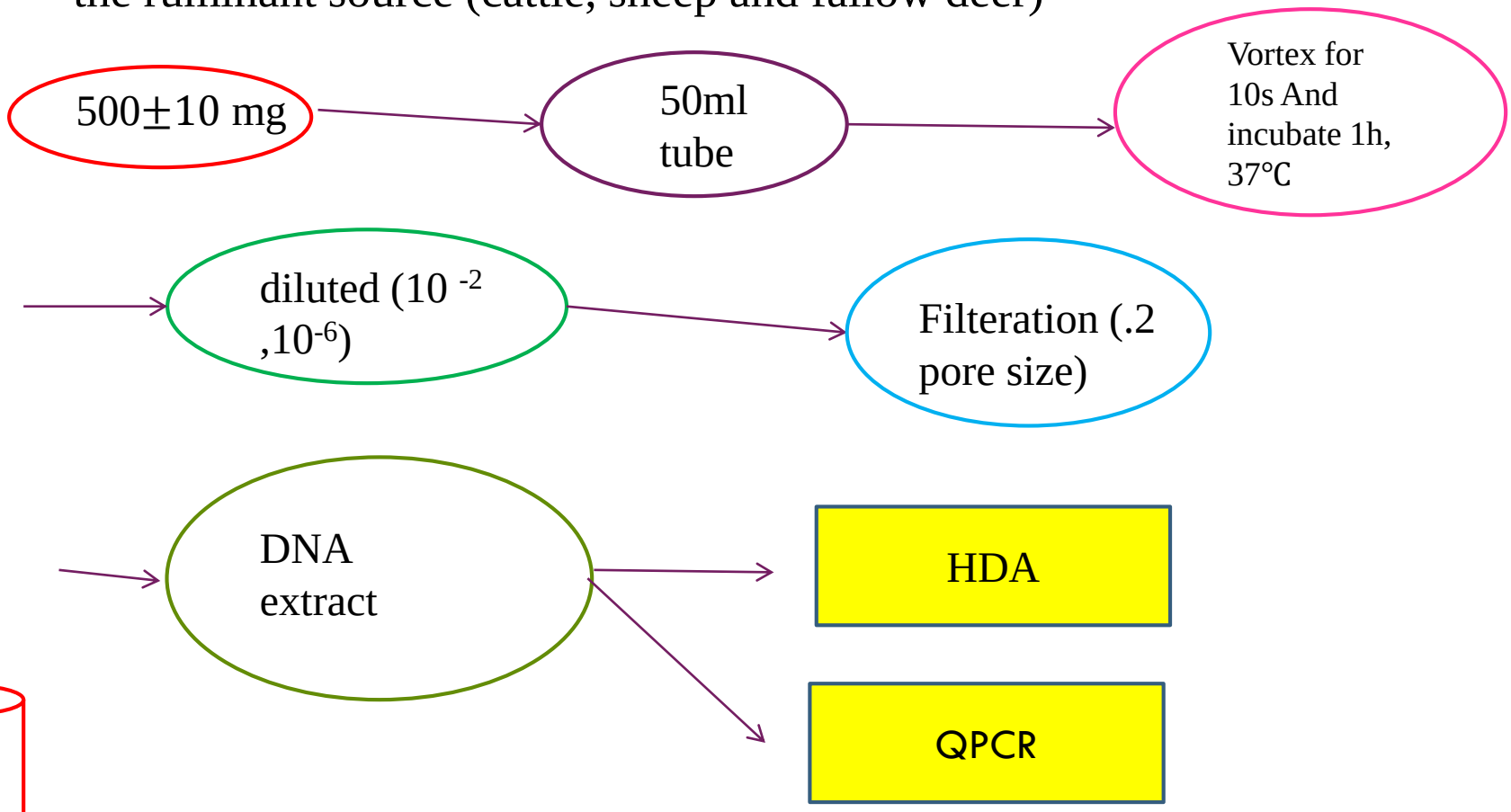
I. single ruminant and non-ruminant faecal samples (stored at −20 °C until further processing)

II. DNA was extracted using the PowerSoilTM DNA isolation kit(stored at −80 °C)

III. DNA concentrations were measured

Sample Limit Of Detection (SLOD)

- pooled faecal samples were created that consisted of 10 single samples of the ruminant source (cattle, sheep and fallow deer)



Primer and probe sequences

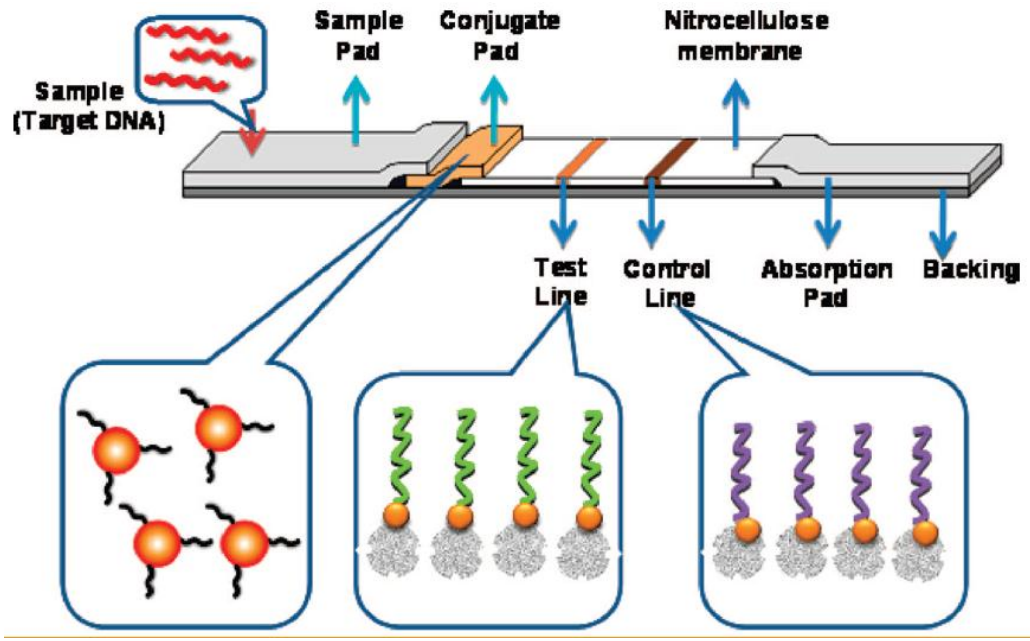
- **All oligonucleotide sequences were synthesized by Sigma-Aldrich**
- **HDA primers were designed using the Primer3 4.1 programme**
- **nucleic acid lateral-flow strip test probes were designed and assessed with OligoAnalyzer 3.1**

Name	Function	Sequence (5′–3′)[a]	Reference	Binding positions[b]
H-BacR_f	HDA forward primer	GTATCCAACCTTCCCGTTAC	This study	91–110
H-BacR_r	HDA reverse primer	ATCCCCATCCGTTACCG	This study	189–205
H-BacR_CP	Strip test capture probe	AAAGGGAGATT$(A)_{20}$-BtnTg	This study	120–127
H-BacR_DP	Strip test detector probe	ThiC6-$(A)_{20}$GCCTTCCG	This study	128–138
H-BacR_CL	Strip test control probe	Btn-$(A)_{20}$GAAGGCTTTTT	This study	—
BacR_f	qPCR forward primer	GCGTATCCAACCTTCCCG	[27]	89–106
BacR_r	qPCR reverse primer	CATCCCCATCCGTTACCG	[27]	189–206
BacR_p	qPCR probe	FAM-CTTCCGAAAGGGAGATT-NFQ-MGB	[27]	122–138

Table 3. Oligonucleotides used in this study. [a]Abbreviations: FAM, 6-carboxyfluorescein; NFQ, non-fluorescent quencher; MGB, minor groove binder; Btn/BtnTg, Biotin; ThiC6, Thiol group. $(A)_{20}$ is a sequence of 20 oligo(dA) used as a spacer. [b]Binding positions refer to GenBank accession number AF233400.

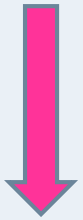
Nucleic acid lateral-flow test strip fabrication

1. the membrane card (laminated card with adhered nitrocellulose membrane)
2. the absorbent pad (cellulose)
3. the conjugate pad (glass fibre)
4. sample application pad (glass fibre)
5. AuNP-DP (functionalized gold nanoparticle with DNA detector probes)
6. streptavidin-biotin-probe conjugates (STV-Bio-capture probe and STV-Bio-control probe)



optimal nucleic acid lateral-flow assay conditions:

a series of experiments was performed to determine the most efficient dispensing volume of AuNP-DP for immobilization on the conjugate pad (10–40 μL cm−1), the dispensing volume of STV-Bio-probe conjugates on the membrane (0.5–1.25 μL cm−1)



AuNP-DP conjugates at a rate of 25 μL cm−1

streptavidin-biotin-probe conjugates at a rate of .75 μL cm−1

BacR HDA-strip test assay :

1. BacR HDA reactions using the IsoAmp II Universal tHDA kit
2. incubated for 90 min at 62 °C

3. HDA products were visualized by transferring 10 µL of the HDA reaction onto the sample application pad

4. test strip in 250 µL running buffer (8x SSC, 0.1% Tween 20, 1% SDS) and incubation for 15 min

5. the results the presence or absence of a coloured test line on the test strip

BacR qPCR assay :

- BacR qPCR reaction using the KAPA PROBE FAST qPCR Kits
- The amplification reactions were run on a 7500 Fast Real-Time PCR system according to the following thermal cycling conditions: an initial step of 5 min at 95 °C,followed by 45 cycles of 15 s at 95 °C and 15 s at 60 °C.
- Score negative:c_q value was undeterminated or $<$ 1 copy

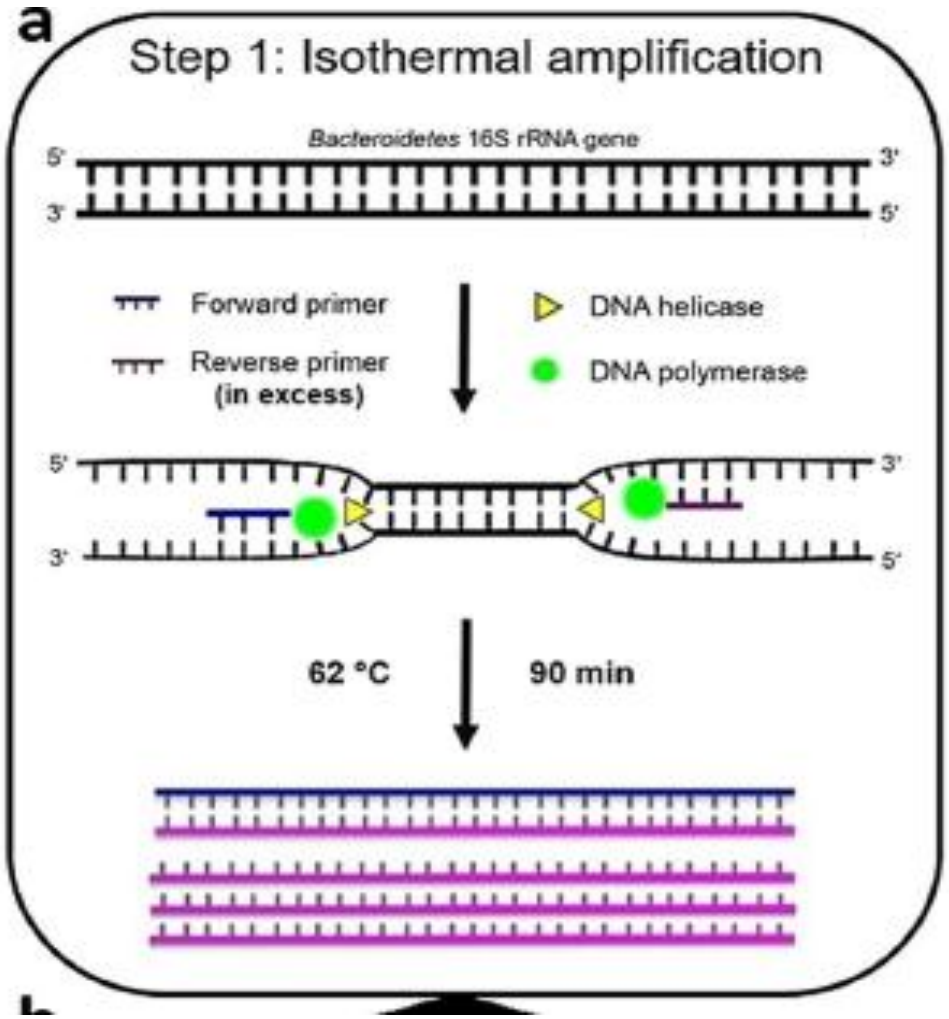
Data analyses:

- ✓ sensitivity (%) = TP/(TP + FN) × 100
- ✓ where ‘TP’ is the number of positive samples and ‘FN’ is the number of false negatives when ruminant samples were used
- ❖ specificity (%) = TN/(TN + FP) × 100
- ❖ where ‘TN’ is the number of negative samples and ‘FP’ is the number of false positives when non-ruminant faecal samples were used

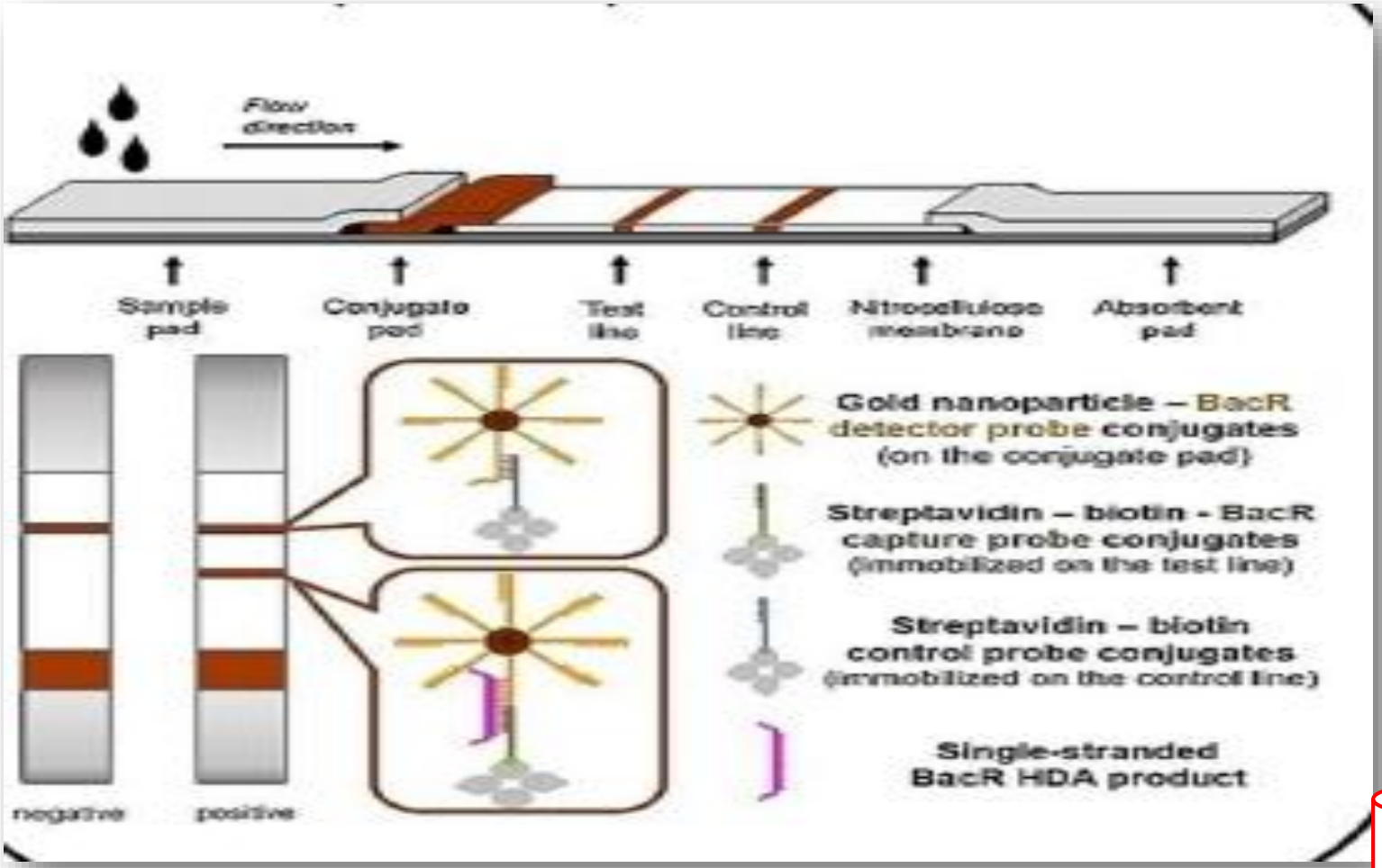
Reasults & Discussion

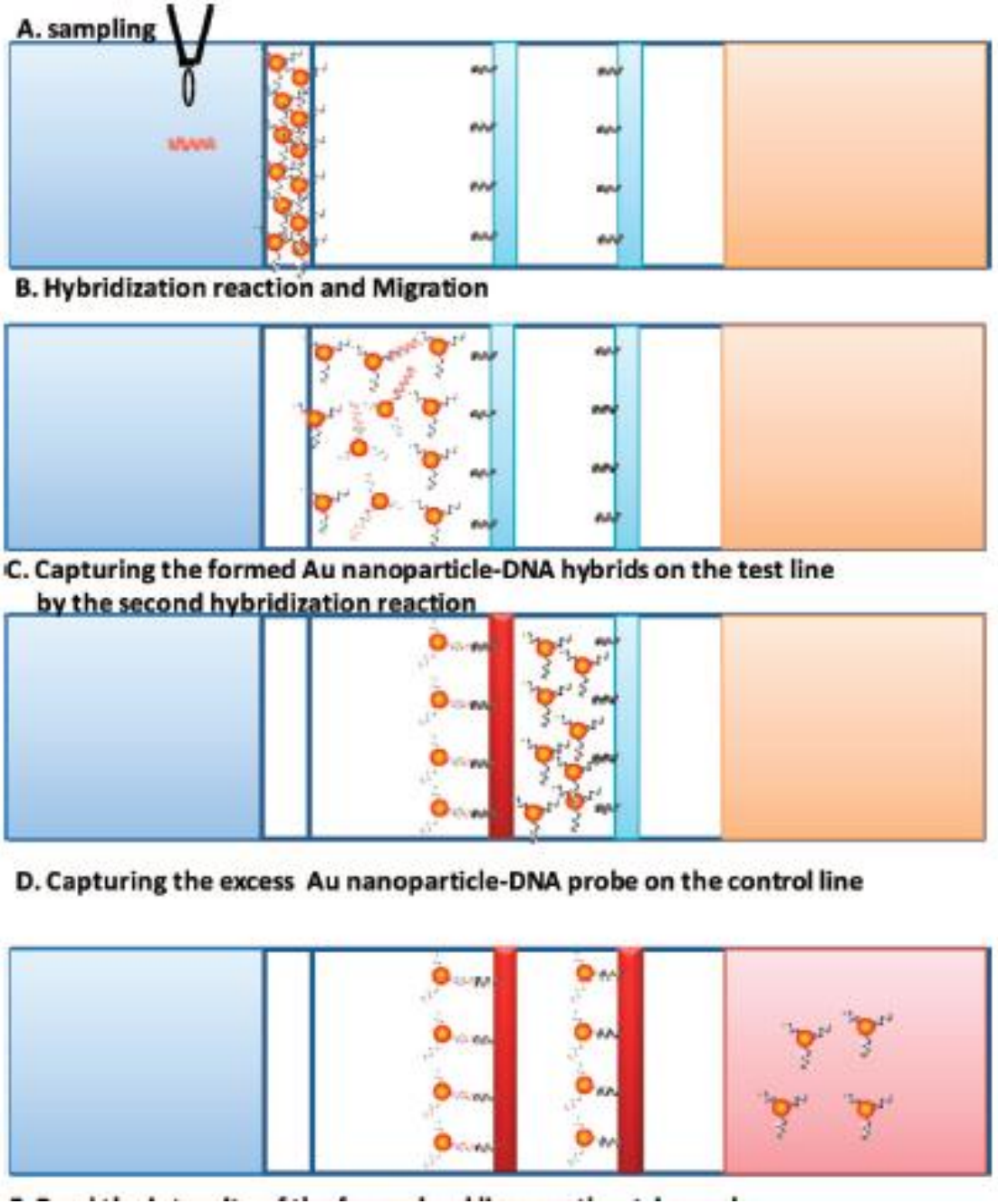
BacR HDA assay design and development

- On a standard heating block
- An asymmetric HDA assay



BacR strip test design and development




A. sampling
B. Hybridization reaction and Migration
C. Capturing the formed Au nanoparticle-DNA hybrids on the test line
by the second hybridization reaction
D. Capturing the excess Au nanoparticle-DNA probe on the control line

Source-sensitivity and source-specificity:

Ruminants				Non-Ruminants			
Source	Scientific name	HDA- strip	qPCR[a]	Source	Scientific name	HDA-strip	qPCR
Cattle 1	*Bos Taurus*	3/3	3/3	Human 1	*Homo sapiens*	0/3	0/3
Cattle 2	*Bos Taurus*	3/3	3/3	Human 2	*Homo sapiens*	0/3	0/3
Cattle 3	*Bos Taurus*	3/3	3/3	Human 3	*Homo sapiens*	0/3	0/3
Cattle 4	*Bos Taurus*	3/3	3/3	Human 4	*Homo sapiens*	0/3	0/3
Red sheep 1	*Ovis aries*	3/3	3/3	Pig	*Sus scrofa domesticus*	0/3	0/3
Red sheep 2	*Ovis aries*	3/3	3/3	Wild Boar	*Sus scrofa*	1/3	0/3
Red sheep 3	*Ovis aries*	3/3	3/3	Horse	*Equus caballus*	0/3	0/3
Red sheep 4	*Ovis aries*	3/3	3/3	Horse	*Equus caballus*	0/3	0/3
Goat 1	*Capra hircus*	3/3	3/3	Chicken	*Gallus gallus*	0/3	0/3
Goat 2	*Capra hircus*	3/3	3/3	Red Fox	*Vupes vulpes*	0/3	0/3
Red deer 1	*Cervus elaphus*	3/3	3/3	Dog	*Canis familiaris*	0/3	0/3
Red deer 2	*Cervus elaphus*	3/3	3/3	Cat	*Felis catus*	0/3	0/3
Red deer 3	*Cervus elaphus*	3/3	3/3	Beaver	*Castor fibre*	0/3	0/3
Red deer 4	*Cervus elaphus*	3/3	3/3	Common carp	*Cyprinus carpio*	0/3	0/3
Roe deer 1	*Capreolus capreolus*	3/3	3/3	Zander	*Sander luciperca*	0/3	0/3
Roe deer 2	*Capreolus capreolus*	3/3	3/3	Common bream	*Abramis brama*	0/3	0/3
Chamois 1	*Rupicapra rupicapra*	3/3	3/3	Brook trout	*Salvelinus fontinalis*	0/3	0/3
Chamois 2	*Rupicapra rupicapra*	3/3	3/3	Greylag goose	*Anser anser*	0/3	0/3
Ibex 1	*Capra ibex*	3/3	3/3	Grey heron	*Ardea cinerea*	0/3	0/3
Ibex 2	*Capra ibex*	3/3	3/3	Duck	*Anas platyrhynchos*	0/3	0/3
Source-sensitivity[b]		100%	100%	Source-specificity[c]		98%	100%

Table 1. Source-sensitivity and source-specificity results of the BacR HDA-strip assay and the BacR qPCR assay. Assays were evaluated on 20 single ruminant (target) and 20 single non-ruminant (non-target) faecal samples, each tested at a concentration of 1 ng extracted DNA per reaction. The results are given as the number of positive reactions from triplicate analyses and as calculated sensitivity and specificity percentages. [a]Marker copy numbers ranged from 10^4 to 10^5 copies per reaction based on qPCR analysis with plasmid standards. [b]Source sensitivity (%): (true positives)/(true positives + false negatives) × 100. [c]Source specificity (%): (true negatives)/(true negatives + false positives) × 100.

Analytical limit of detection (LOD95%)

a

Average plasmid standard copy number per reaction	HDA-strip	qPCR
21	20 / 20	20 / 20
10	20 / 20	20 / 20
4.6	16 / 20	20 / 20
2.1	12 / 20	14 / 20
1.0	4 / 20	14 / 20
0.5	2 / 20	4 / 20
0.2	0 / 20	0 / 20
0.1	0 / 20	0 / 20

b

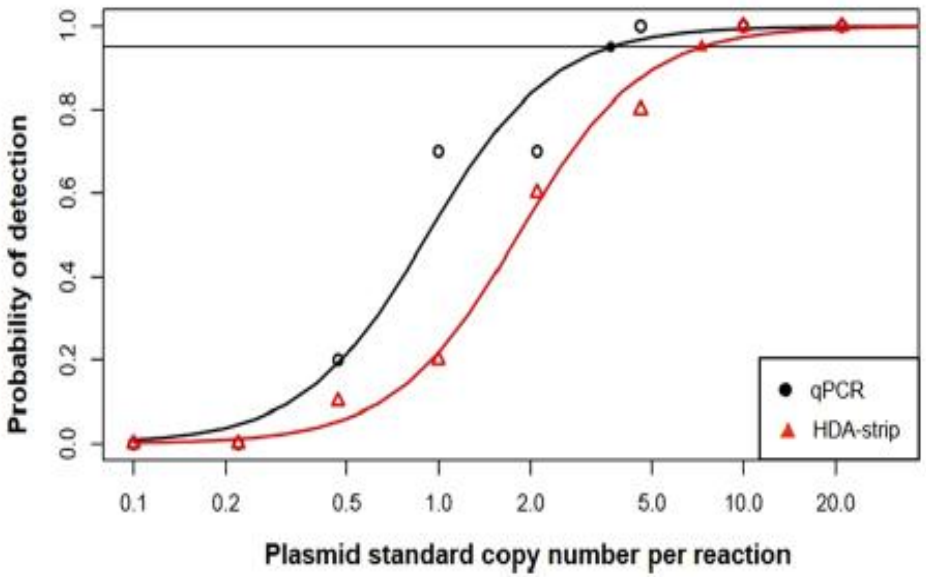


Figure 2. Limits of detection ($LOD_{95\%}$). (**a**) Raw data from the analyses of a dilution series of the BacR plasmid standard that served as the input for statistical calculations. (**b**) Logistic regression model used to determine the $LOD_{95\%}$, which is indicated by filled symbols on the horizontal line. $LOD_{95\%}$ (with 95% confidence interval): HDA = 5.2 ≤ 7.3 ≤ 10.3 copies; qPCR = 2.6 ≤ 3.7 ≤ 5.2 copies.

Sample limit of detection (SLOD):

Source	Faecal dilution	HDA-strip		qPCR		Detected concentration (10^{-8} g faeces per filter)			
						HDA-strip		qPCR	
		Filter 1	Filter 2	Filter 1	Filter 2	$SLOD_{95\%}$	95% CI	$SLOD_{95\%}$	95% CI
Cattle	10^{-2}	10/10	10/10	10/10	10/10	4.9	2.4–10.4	2.8	1.3–5.9
	10^{-3}	10/10	9/10	10/10	10/10				
	10^{-4}	4/10	8/10	5/10	10/10				
	10^{-5}	0/10	2/10	1/10	2/10				
	10^{-6}	0/10	0/10	0/10	0/10				
Sheep	10^{-2}	10/10	10/10	10/10	10/10	2.5	1.2–5.2	2.5	1.2–5.2
	10^{-3}	10/10	10/10	10/10	10/10				
	10^{-4}	4/10	10/10	6/10	10/10				
	10^{-5}	1/10	4/10	1/10	2/10				
	10^{-6}	0/10	0/10	0/10	0/10				
Fallow Deer	10^{-2}	10/10	10/10	10/10	10/10	11.3	4.9–25.9	12.6	5.5–29.1
	10^{-3}	9/10	9/10	9/10	10/10				
	10^{-4}	0/10	8/10	1/10	7/10				
	10^{-5}	1/10	2/10	0/10	1/10				
	10^{-6}	0/10	0/10	0/10	0/10				

HDA-strip assay: 6.2×10^{-8}

qPCR: 6×10^{-8}

Table 2. Sample limit of detection experiments with the HDA-strip and qPCR method. The results are given as number of positive replicates from the analyses of 10 replicates per faecal dilution step. The total number of cates from both filtrations (i.e., n = 20 replicates per faecal dilution) served as input for statistical analysis. ction limits were calculated as the minimum amount of faeces detectable in a filtration volume at a 95% tion probability ($SLOD_{95\%}$) with a 95% confidence interval (CI).

Perspectives and outlook for the developed BacR HDA-strip assay:

- ✓ simplicity and excellent performance, the developed HDA-strip assay provides a promising alternative molecular diagnostic tool
- ✓ an instrument that is readily available in microbiology laboratories (standard heating block)
- ✓ easy to read and interpret by non-experts

However:

both assays (HDA-strip and qPCR) still require water concentration based on membrane filtration and DNA extraction from filters resulting in a similar overall sample testing time of approximately three hours.

Refrence:

SCIENTIFIC REPORTS

OPEN

Detection of a microbial source tracking marker by isothermal helicase-dependent amplification and a nucleic acid lateral-flow strip test

Received: 23 August 2018
Accepted: 23 November 2018
Published online: 23 January 2019

Claudia Kolm[1,2], Roland Martzy[1,2], Manuela Führer[3], Robert L. Mach[4], Rudolf Krska[3,5], Sabine Baumgartner[3], Andreas H. Farnleitner[2,6,7] & Georg H. Reischer[1,7]

Over the last decades, various PCR-based methods have been proposed that can identify sources of faecal pollution in environmental waters. These microbial source tracking (MST) methods are powerful tools to manage water quality and support public health risk assessment. However, their application is limited by the lack of specialized equipment and trained personnel in laboratories performing

Thank you