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REPUBLIC OF BULGARIA
Ministry of Education and Science

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ILVO

FAST DETECTION OF *YERSINIA PSEUDOTUBERCULOSIS* IN MILK SAMPLES USING LAMP AND DIGITAL DROPLET PCR

EFSA КОНТАКТЕН ЦЕНТЪР
БЪЛГАРИЯ

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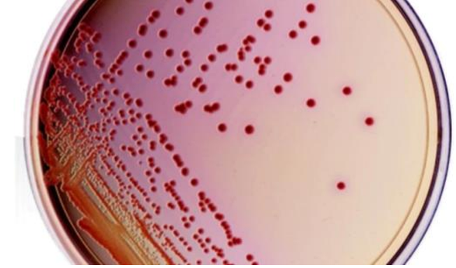
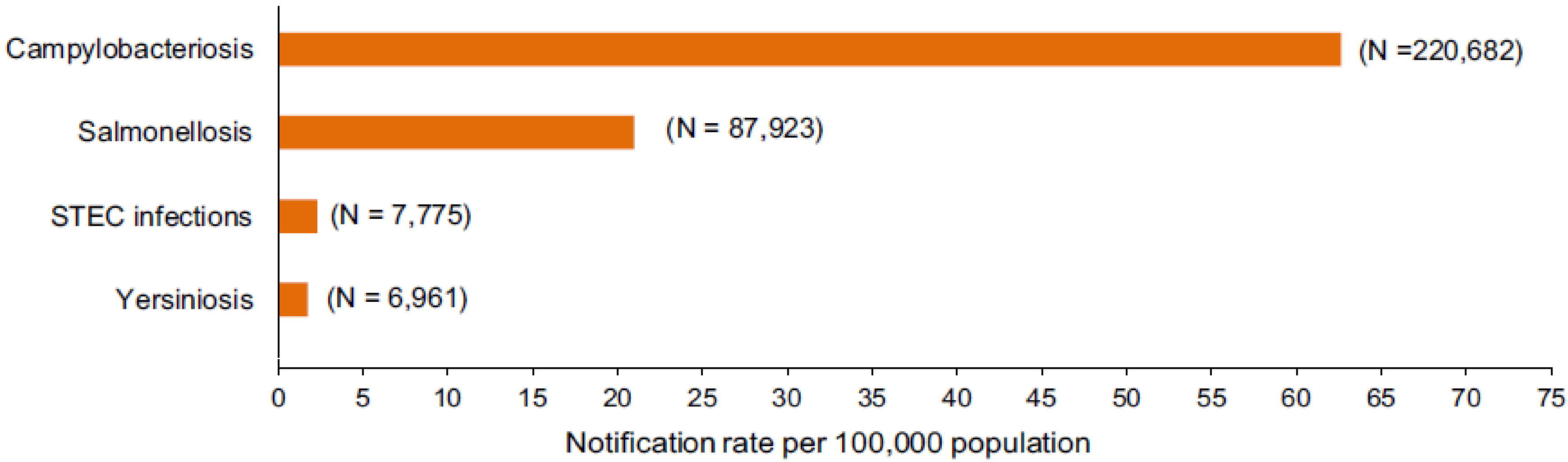
14th Scientific Conference of the Bulgarian Focal Point of EFSA under the motto „Sustainable science for safety food",

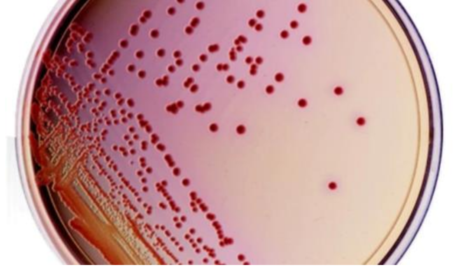
Introduction



- **Yersiniosis** – the **fourth most commonly reported zoonosis** in humans in 2019 with 6961 confirmed cases in the EU and a stable flat trend in the period 2015–2019:
  - EFSA Report on Zoonoses in 2019 and the Annual Epidemiological Report of ECDC on yersiniosis, both published in 2021:



Introduction



- Causative agents of this food-borne disease:
  - ***Yersinia enterocolitica*** and ***Yersinia pseudotuberculosis;***
  - Isolated from meat, vegetables, milk, products thereof, etc.;
- Data on Yersinia to the EC are reported not mandatory in accordance with the Zoonoses Directive 2003/99/EC;
- The **fast identification** of this pathogen in food is crucial for the prevention of outbreaks;
  - The current ISO standards based on the classical microbiological methods are time consuming and laborious.

Aim

- To develop a **fast and robust identification method** based on PCR in samples from raw goat milk:
  - **LAMP** and
  - **digital droplet PCR** (ddPCR).

Experimental design and workflow

1. Processing of a goat milk sample for the optimization of the LAMP and ddPCR protocol

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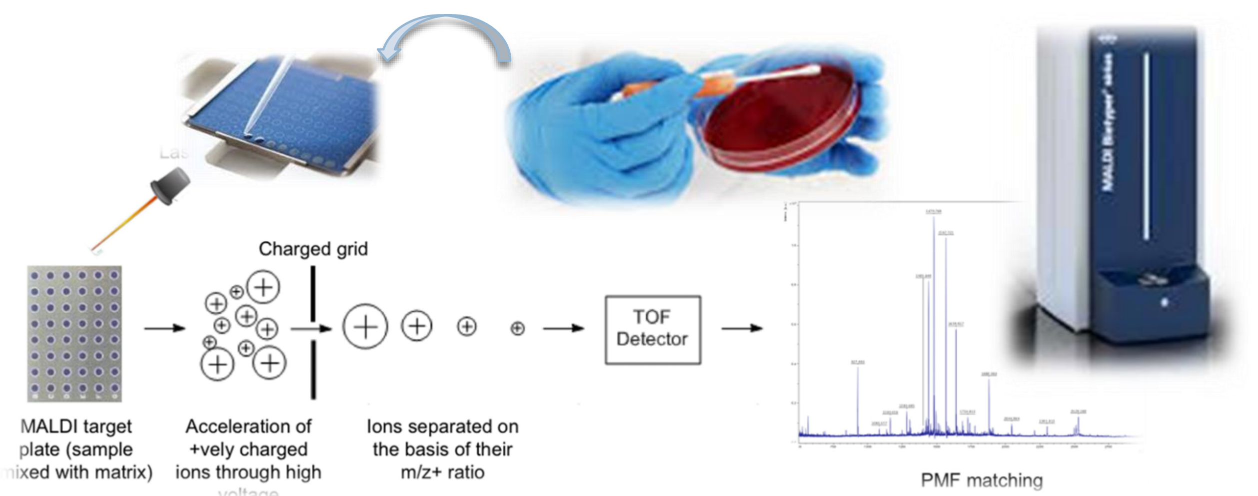
- Raw goat milk was collected from a private farm in the Rhodope Mountains;
- The milk was used for optimization of the LAMP and ddPCR protocols;
- The milk was tested for microbial contamination on selective media after 24 h of enrichment in brain-heart infusion broth;
- Identification of the isolates with MALDI-TOF:



Experimental design and workflow

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2. Selection of primers for LAMP and ddPCR

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3. Selectivity test of the LAMP primers

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- Primers set for *Yersinia pseudotuberculosis* for loop mediated DNA amplification (***Horisaka et al., 2004***):
  - F3......CTCGTCGCGTGATTTCTCC
  - B3 ......GATCTACCCCGACAGTGAGT
  - FIP.......CCAGTTGTGGGAGTGCAGGTAACTATAAAGAGCGCCCAGCC
  - BIP......CACCGGTGAGCGTGTTGCTTTGTGTAATTGATCCCGGCAGT
  - LF.......CATTCGCGCGCAAATCC
  - LB.......GCAACGCAACCCTTATGC
- Probe and primers for ddPCR (Lambertz et al., 2008; Najdenski et al., 2012):
  - Yps 1: 5'-CGT-CTG-TTA-ATG-TGT-ATG-CCG-AAG-3' ;
  - Yps 2: 5'-GAA-CCT-ATC-ACT-CCC-CAG-TCA-TTA-TT-3' ;
  - Yps-Probe: 5'-CGT-GTC-AAG-GAC-GAT-GGG-TAC-AAG-TTG-G-3' (5'-6-FAM), (3'-BHQ-1), Excitation/Emission 498/510 nm
- Selectivity test on:
  - *Yersinia enterocolitica, Campylobacter coli, Campylobacter jejuni, Salmonella dublin, Salmonella enterica, Escherichia coli*

Experimental design and workflow

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4. Artificial contamination of goat milk

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5. Direct DNA isolation

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- *Y. pseudotuberculosis,* strain IP32918:
  - Eight serial ten-fold dilutions were prepared from a 48 h culture (26 °C):
    - -1, -2, -3, -4, -5, -6, -7 and -8
  - Aliquots of 100 µL from dilutions -4, -5 and -6 were seeded onto brain-heart infusion agar;
    - The plates were cultivated 48 h at 26 °C;
  - The colony forming units were counted and the CFU numbers were compared to the results from the ddPCR;
- DNA isolation from the contaminated milk samples with Favorgen kit (Biotech Corp.) – max. volume1ml milk

Experimental design and workflow

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6. ddPCR

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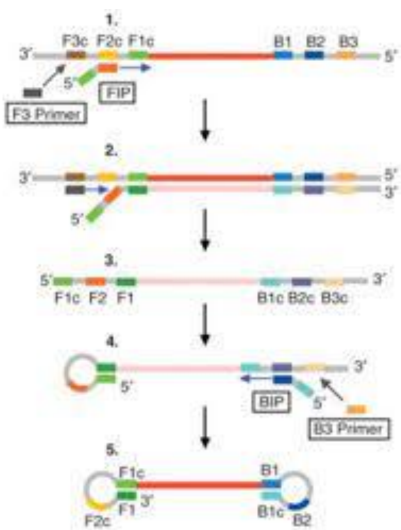
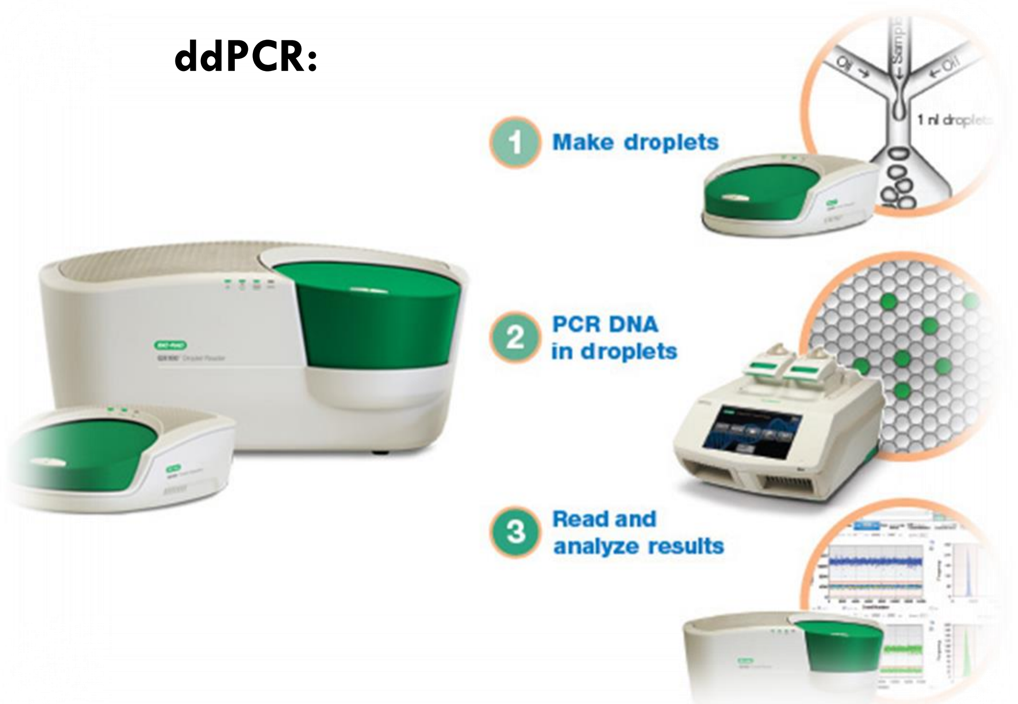
7. LAMP

- Optimization of the LAMP protocol with WarmStart Colorimetric LAMP with Bst polymerase (NEB Inc.);
- Optimization of the ddPCR protocol with Supermix for probes (Bio-Rad) on Bio-Rad QX200 Droplet Digital PCR system:

LAMP:



ddPCR:



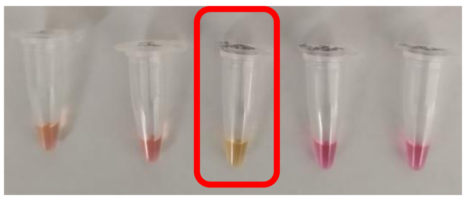
Results: MALDI-TOF identification of bacterial isolates from the goat milk

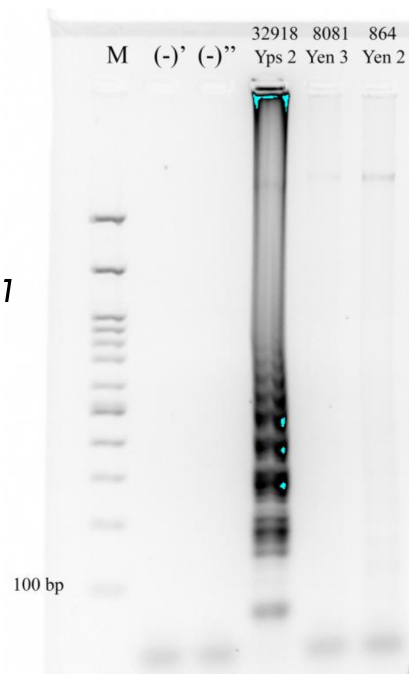
- Four isolates from the goat milk were subjected to MALDI-TOF analysis;
- No Yersinia species were identified in the milk used for optimization of the LAMP protocol, therefore the milk was used for the further experiments.

Sample ID	Organism (best match)	Score value	Organism (second best match)	Score value
M2	*Kocuria salsicia* Often isolated from seafood, catheter-related bacteremia	1.78	No Organism Identification Possible	1.65
M3	*Staphylococcus equorum* Starter culture of different types of cheese	2.01	No Organism Identification Possible	1.43
M4	*Tsukamurella paurometabola* Catheter-related bacteremia, peritonitis, pro-inflammatory activity	2.06	*Tsukamurella tyrosinosolvens*	2.02
M5	*Moraxella osloensis* Opportunistic human pathogen - meningitis, vaginitis, sinusitis, bacteremia, endocarditis, and septic arthritis	2.15	*Moraxella osloensis*	1.80

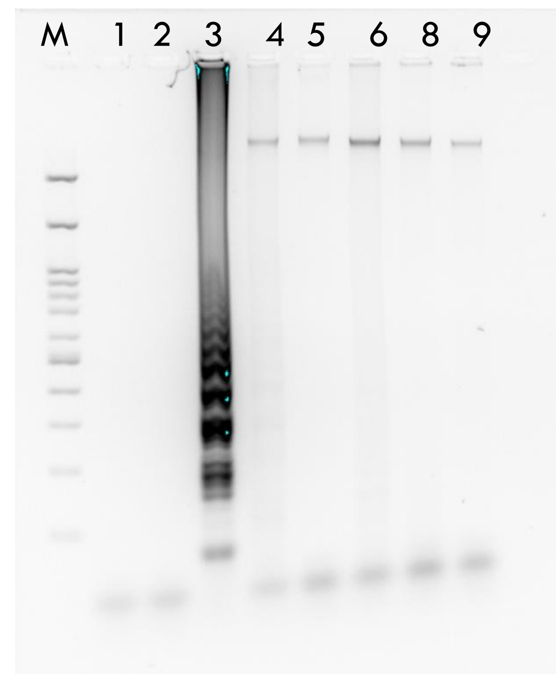
Results: Optimization of LAMP protocol for identification of *Yersinia pseudotuberculosis* – test for specificity

- ✓ Initially, we started with six bacterial species – specificity testing continues with other species;
- ✓ The color identification and the gel electrophoresis showed equal results.





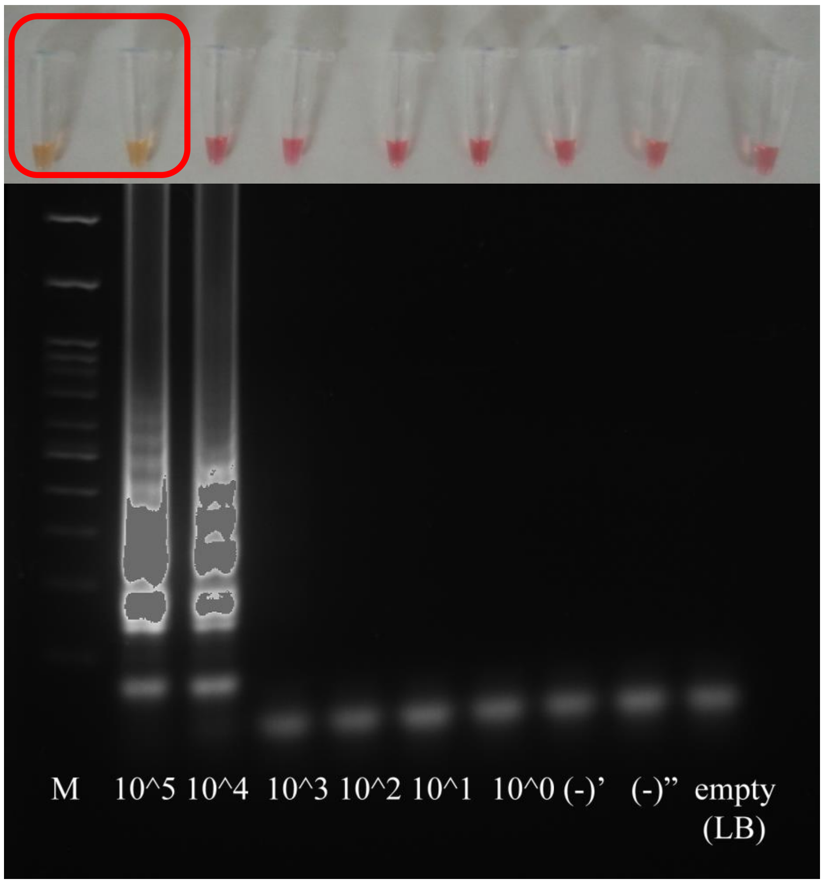
1. Negative control 1
2. Negative control 2
3. *Yersinia pseudotuberculosis*, IP32918
4. *Yersinia enterocolitica, 8081*
5. *Yersinia enterocolitica, 864*



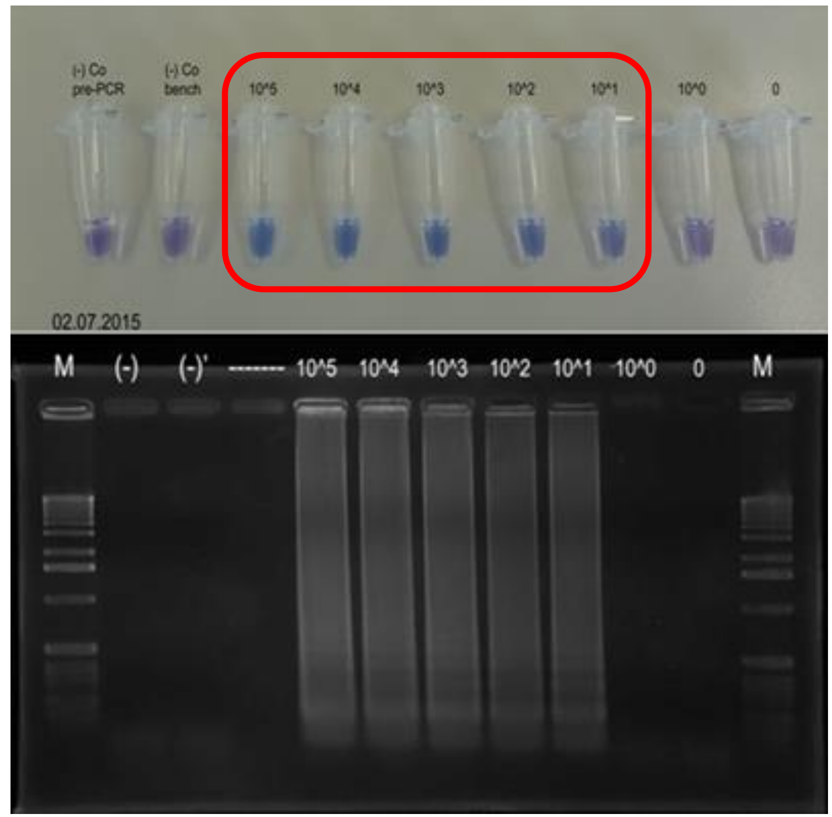
1. Negative control 1
2. Negative control 2
3. *Yersinia pseudotuberculosis*, IP32918
4. *Camplylobacter jejuni*
5. *Campylobacter coli*
6. *Salmonella dublin*
7. *Salmonella enterica*
8. *Escherichia coli*

Results: Sensitivity of the LAMP protocol with different kits

WarmStart Colorimetric LAMP with Bst II polymerase (NEB Inc.), direct application

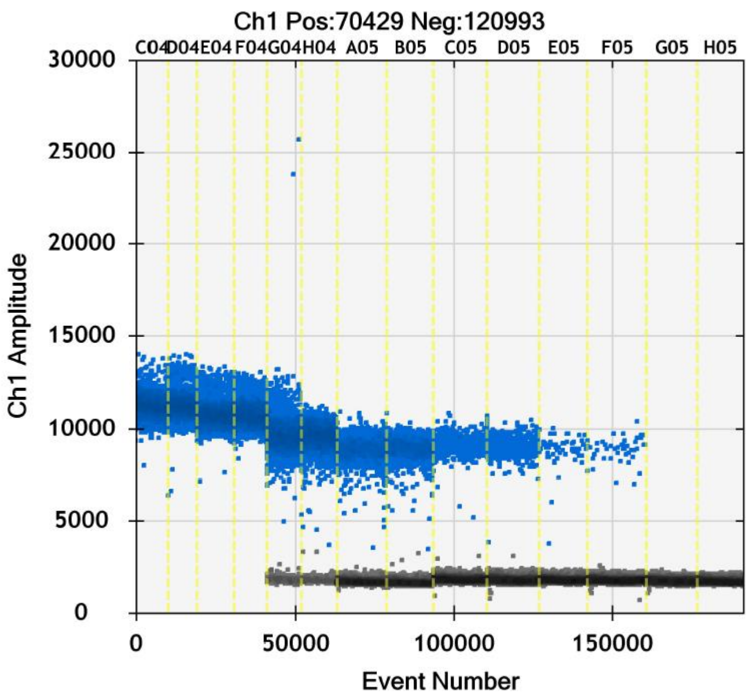


Bst II polymerase (NEB Inc.), optimized protocol with hydroxynaphthol blue



Results: Digital droplet PCR protocol - optimization

Positive control:
Pure DNA of Y. pseudotuberculosis:
10^5 - 10^1

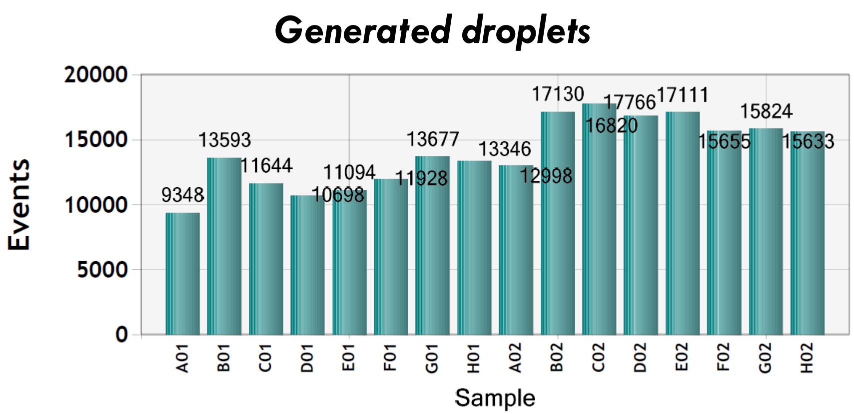


Reference control
Standard dilution
of pure DNA

6.7×10^4
6.7×10^3
6.7×10^2
6.7×10^1
0

Sample ID	**Conc. DNAcopies/µL**		**Conc. DNA copies/reaction**	
D2-10^3	Multi	3310	ddPCR Supermix for Probes	66200
D2-10^3	Multi	3480	ddPCR Supermix for Probes	69600
D2-10^2	Multi	322	ddPCR Supermix for Probes	6440
D2-10^2	Multi	331	ddPCR Supermix for Probes	6620
D2-10^1	Multi	34.8	ddPCR Supermix for Probes	696
D2-10^1	Multi	33.3	ddPCR Supermix for Probes	666
D2-10^0	Multi	4.8	ddPCR Supermix for Probes	96
D2-10^0	Multi	2.9	ddPCR Supermix for Probes	58
Water	Multi	0	ddPCR Supermix for Probes	0
Water	Multi	0	ddPCR Supermix for Probes	0

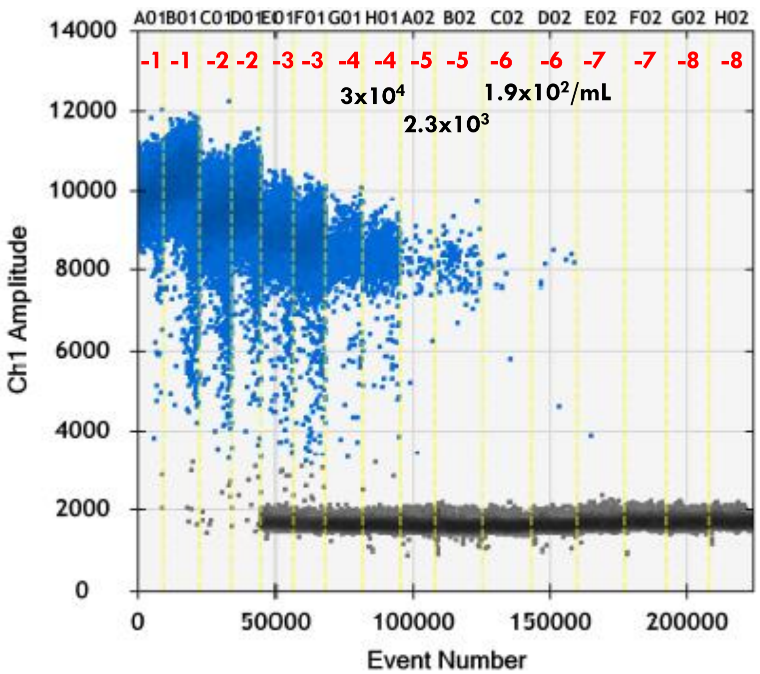
Results: Digital droplet PCR protocol – optimization after artificial contamination with *Y. pseudotuberculosis* and direct DNA isolation



Milk samples,

Dilutions of Y. pseudotuberculosis:

From -1 to -8



Results: Digital droplet PCR protocol - optimization

Artificially contaminated milk samples: DNA copies/mL

T	Target		Status	Conc(copies/µL)	Supermix	Copies/20µLWell	
	M-2	**-2**	Multi	8300	ddPCR Supermix for Probes	166000	**-2**
	M-2	**-2**	Multi	8100	ddPCR Supermix for Probes	162000	**-2**
	M-3	**-3**	Multi	633	ddPCR Supermix for Probes	12660	**-3**
	M-3	**-3**	Multi	752	ddPCR Supermix for Probes	15040	**-3**
	M-4	**-4**	Multi	77	ddPCR Supermix for Probes	1540	**-4**
	M-4	**-4**	Multi	71.1	ddPCR Supermix for Probes	1422	**-4**
	M-4	**-5**	Multi	4.3	ddPCR Supermix for Probes	86	**-5**
	M-5	**-5**	Multi	7	ddPCR Supermix for Probes	140	**-5**
	M-6	**-6**	Multi	0.4	ddPCR Supermix for Probes	8	**-6**
	M-6	**-6**	Multi	0.56	ddPCR Supermix for Probes	11.2	**-6**
	M-7	**-7**	Multi	0.07	ddPCR Supermix for Probes	1.4	**-7**
	M-7	**-7**	Multi	0	ddPCR Supermix for Probes	0	**-7**
	M-8	**-8**	Multi	0	ddPCR Supermix for Probes	0	**-8**
	M-8	**-8**	Multi	0	ddPCR Supermix for Probes	0	**-8**

-4 = 3 x 10^4/mL

-5 = 2.3 x 10^3/mL

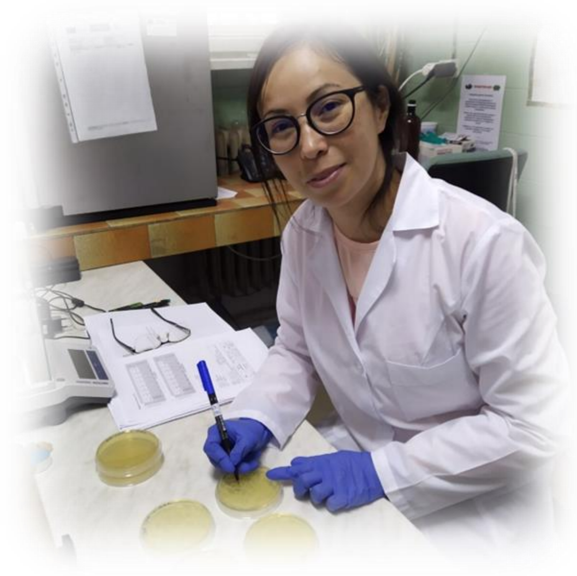
-6 = 1.9 x 10^2/mL

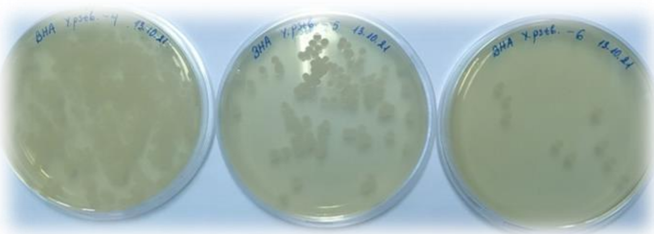
The initial bacterial suspension was ~10^7 CFU/mL

Results: Digital droplet PCR protocol - optimization

- ***Comparison between CFU on agar plates, DNA copies/mL determined with ddPCR and with LAMP***

Sample ID	CFU/mL on agar plates 48 h of cultivation at 26 °C	DNA copies/mL as determined by ddPCR
Dilution -4	**4 = 5.6 x 10^3/mL**	**3 x 10^4/mL**
Dilution -5	**-5 = 5.6 x 10^2/mL**	**2.3 x 10^3/mL**
Dilution -6	**-6 = 5.6 x 10^1/mL**	**1.9 x 10^2/mL**

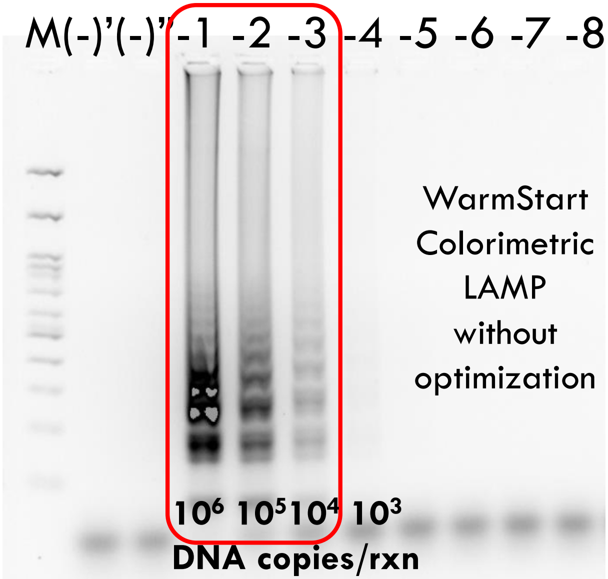




M – DNA marker

(-)’ – Negative control 1

(-)” – negative control 2



Conclusions

- The primers chosen *for* the LAMP assay are specific for *Yersinia pseudotuberculosis*;
- The LAMP reaction sensitivity depends on the chosen kit and the reaction conditions;
- The chosen DNA isolation kit (Favorgen, Biotech Corp.) shows a low detection limit and is suitable for identification of *Y. pseudotuberculosis* in raw milk;
- The detection limit of the ddPCR with the above mentioned DNA isolation kit is ~10^2 DNA copies/mL;
- Further optimizations of the LAMP reaction conditions are needed in order to increase its sensitivity from ~ 10^4 DNA copies/mL to ~10^2 DNA copies/mL.

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Acknowledgements

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