BULGARIAN ACADEMY OF SCIENCES
INSTITUTE OF MICROBIOLOGY
"STEPHAN ANGELOFF"

Maya Angelovska

Prevalence and characteristics of enteropathogenic *Y. enterocolitica* strains isolated from pigs

SUMMARY

On a dissertation for the award of the educational and scientific degree "Doctor"

In the field of higher education 4. Natural sciences, mathematics and informatics. Professional direction: 4.3. Biological sciences Specialty Microbiology

Scientific supervisor: Prof. Hristo Najdenski, DVM, DSc,
Corresponding Member of Bulgarian Academy of Sciences

Sofia, 2023

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Abbreviations and symbols used

DNA- Deoxyribonucleic Acid
16S rRNA - 16S ribosomal ribonucleic acid
Kb - Kilobase
Mb - Megabase
kDa – Kilodaltons
bp– base pair
PFGE- Pulsed-field gel electrophoresis
pYV – *Yersinia* virulence plasmid
PCR - Polymerase Chain Reaction
Ail attachment and invasion locus protein
YstA- *Yersinia* heat-stable enterotoxin
YadA *Yersinia* adhesion protein
LPS – Lipopolysaccharide
MyfA- mucoid Yersinia factor
T3SS- type III secretion system
Yop –*Yersinia* outer proteins
EFSA - European Food Safety Authority
ECDC - European Centre for Disease Prevention and Control
PSBB – Peptone Sorbitol Bile Broth
CIN - Cefsulodin–irgasan–novobiocin
KOH- potassium hydroxide
ISO- International Organization for Standardization
LAMP - loop-mediated isothermal amplification
FIP – Forward inner primer
F3 – Forward outer primer
BIP – Backward inner primer
B3 - Backward outer primer
LF – Forward loop primer
LB – Backward loop primer
CFU – colony-forming unit
CR-MOX - Congo red-magnesium oxalate
BLAST- Basic Local Alignment Search Tool
*Spe*I- restriction enzyme of *Sphaerotilus sp.*

I Introduction

Yersinia enterocolitica is a significant zoonotic pathogen, zoonotic pathogen, recognized as the causative agent of Yersiniosis, thereby posing a substantial threat to both animal and human health. Yersiniosis ranks as the third most frequently reported foodborne zoonosis in the European Union. Pigs are considered to be the major reservoir for human pathogenic strains. Bacteria tend to persist within the lymphatic tissue of healthy swine and is frequently disseminated to porcine carcasses during the slaughter process. Human infections typically occur through the consumption of raw or inadequately processed meat, milk, or water that has been contaminated with these pathogens. In accordance with their biochemical characteristics, *Y. enterocolitica* strains are categorized into six distinct groups, denoted as 1A, 1B, 2, 3, 4, and 5. Furthermore, these strains are differentiated into a total of 70 serotypes based on variations within their lipooligosaccharide structure. Among these serotypes, *Y. enterocolitica* bioserotype 4/O:3, commonly referred to as the "pig bio- and -serotype," is the predominant causative agent of human yersiniosis. The pathogenicity of Y. *enterocolitica* is due to chromosomal- and plasmide encoded virulence determinants. Of significant importance for clinical infections are various key determinants, including the

attachment and invasion locus (*ail*), *Yersinia* heat-stable enterotoxin (*ystA*), Yersinia adhesin (*yadA*) genes, invasin (*invA*), *Yersinia* outer membrane protein virulon (*yop*), among others. The genotyping of isolated *Y. enterocolitica* through pulsed-field gel electrophoresis (PFGE) stands as a conventional practice in epidemiological studies. This technique yields comprehensive insights into the genetic relationships among strains and facilitates the tracing of their circulation patterns. In vitro assays have revealed that *Y. enterocolitica* strains, originating from slaughtered pigs, exhibit susceptibility to a broad spectrum of antibiotics, including tetracycline, aminoglycosides, third-generation cephalosporins, and fluoroquinolones and resistance to aminopenicillins and first-generation cephalosporins. Notably, there has been an observable upsurge in the prevalence of multidrug-resistant *Y. enterocolitica* bioserotype 4/O:3 isolates originating from porcine sources in recent times. Conventional methods for *Y. enterocolitica* confirmation entail laborious and time-consuming procedures. The incorporation of molecular-biological methods, known for their sensitivity, cost-efficiency, simplicity, effectiveness, and rapidity, offers a promising avenue to advance the diagnosis of yersiniosis. Loop mediated isothermal reaction- LAMP is a molecular technique for the amplification of DNA under isothermal conditions. This

method is notably distinguished by its exceptional specificity for the target sequence and its ability to detect low quantities of DNA within a short time frame. The advantage of the LAMP method lies in its rapidity and simplicity, rendering it amenable to field conditions without the requisite employment of costly thermal cyclers.

II Aim and Objectives

1. Aim

The aim of this dissertation is to examine the prevalence and distribution of enteropathogenic strains of *Y. enterocolitica* obtained from pigs at slaughter age, employing both traditional and molecular methodologies and to formulate a fast and economically efficient protocol for the qualitative detection of *Y. enterocolitica* in samples directly sourced from the tonsils and feces of slaughterhouse swine.

2. Objectives

2.1. Collection of samples from the tonsils and feces of swine at slaughter age, originating from diverse farms within the geographical expanse of Bulgaria.

2.2. Isolation and identification of *Y. enterocolitica* according to the ISO EN 10273:2003 horizontal method for the detection of *Yersinia enterocolitica* presumed to be pathogenic to human subjects.

2.3. Assessment of the virulence profile of the isolated *Y. enterocolitica* strains.

2.4. Determination of the genetic diversity of the isolated *Y. enterocolitica* strains.

2.5. Evaluation of the antibiotic resistance profiles among the isolated strains of *Y. enterocolitica*.

2.6. Optimization of a Loop-Mediated DNA Amplification (LAMP) protocol utilizing established primer sequences, assessment of the primers selectivity and the protocol's sensitivity.

2.7. A comparative analysis of Loop-Mediated Isothermal Amplification (LAMP) protocol and conventional Polymerase Chain Reaction (PCR) methodology.

III Material and methods

1. Animals and samples

A total of 601 pigs were examined during the five years sampling periods. Palatine tonsils (n = 601) and feces (n = 189) were collected during the slaughtering process in a single slaughterhouse located in Kostinbrod city. The study cohort consisted of swine sourced from seven distinct fattening pig facilities (Farms I – VII), situated within four disparate regions within Bulgaria.

2. Microbiological and biochemical tests for detection of *Yersinia enterocolitica*

The presence of pathogenic *Y. enterocolitica* was detected according to ISO 10273:2003. Presumptive *Y. enterocolitica* were further identified according to the biochemical identification scheme included a test for: determination of arginine, lysine and ornithine decarboxylation, fermentation of carbohydrates, such as sucrose, lactose, trehalose, rhamnose, rafinose, melibiose, hydrolysis of citrate, production of hydrogen sulfide and utilization of manithol, arabitol, inositol.

3. Molecular tests for *Yersinia enterocolitica* identification

- Isolation and extraction of DNA

Isolation of genomic DNA from bacterial colonies, as well as directly from tonsillar and fecal samples, were carried out using commercially available DNA extraction kits. For the LAMP assay optimization DNA was isolated according to Flamm.

- Horizontal gel electrophoresis for the confirmation of the electrophoretic analysis to confirm the integrity of the isolated DNA, an electrophoretic analysis on 0.8 % agarose gel was conducted. Quantitative assays were carried out utilizing both a spectrophotometer and a fluorimeter.

- PCR amplification for detection of *16S rRNA* gene, *ail*, *ystA* and *yadA* detection

The *Yersinia 16S rRNA* gene was detected by genus-specific primers (*YeI* and *YeII*).

- *16S rRNA* gene identification by sequencing

The positive PCR products for the *16S rRNA* gene were sequenced in both directions (Macrogene, Amsterdam, The Netherlands) and the sequenced data were compared with reference sequences of *Y. enterocolitica* in the database of the National Center for Biotechnology Information (NCBI) amplicons using Basic Local Alignment Search Tool (BLAST).

- Gel electrophoresis for PCR and LAMP endpoint detection
- Pulsed-field gel electrophoresis

Macro-digestion of isolated pathogenic *Y. enterocolitica* strains was performed using the restriction enzyme *SpeI*. PFGE was performed according to the PulseNet protocol for *Y. pestis.* PFGE pulsotypes were analyzed using GelCompar software. Clustering of strains was performed by unweighted pair-group method considering using arithmetic averages (UPGMA) with Dice bands correlation tolerance set on 2.5% and optimization of 0.5%.

- Determination of the antimicrobial susceptibility of *Y. enterocolitica* strains by disc diffusion method

Antimicrobial susceptibility tests were carried out by the standard disk diffusion method according to CLSI

recommendations. Pathogenic *Y. enterocolitica* strains were tested for susceptibility to 15 antibiotics, belonging to different pharmacological classes (8 classes antibiotics and 1 unclassified antibiotic). Results were interpreted as susceptible, intermediate, or resistant strains after the diameter of inhibition is calculated.

4. Optimisation of the LAMP assay, selectivity of primers and sensitivity of the reaction

Primers were optimized, and results were detected by both gel electrophoresis and Hydroxyl Naphthol blue dye.

5. Artificial contamination of the porcine feces
6. qPCR

IV Results

1. Detection of *Yersinia enterocolitica*

A total of 601 slaughtered pigs were tested for the presence of *Y. enterocolitica*, and 790 samples were obtained, including 601 tonsil samples (1 tonsil per pig) and 189 fecal samples from 189 pigs only (Figure 1A). The number of pigs examined per visit varied between 7 and 44. A total of 920 colonies onto Cefsulodin-Irgasan-Novobiocin (CIN) agar were obtained from the samples collected. The isolated colonies were not identical with respect to macro morphological parameters (Figure 1)

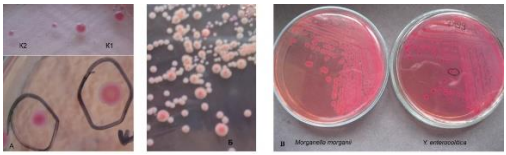

Figure 1 Morphology of the colonies after CIN agar isolation
А: к1 – Typical *Y. enterocolitica* colonies к2- atypical colonies look alike *Y. pseudotuberculosis* Б: Mixed culture 5х. В: Macroscopic difference between *Y. enterocolitica* и *Morganella morganii*

All isolates were assessed for culture purity. Subsequently, each isolate underwent microscopic examination, confirming their Gram-negative status and displaying a rod-shaped cellular structure. Following this, as no significant differences in macromorphology, apart from the aforementioned characteristics, were observed between the two sets of colonies, the isolates were further identified in accordance with the preliminary identification scheme.

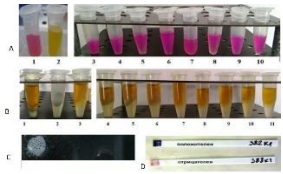

Figure 2 Preliminary identification tests
A- Detection of urease: 1 = positive control *Y. enterocolitica* 8081 (O:8), 2 = negative control *E. coli* ATTC 35218, 3 – 10 Isolates positive for the test; B- phenylalanine deamination test: 1 = positive control *M. morganii*, 2= uniculated, 3= negative control *Y. enterocolitica* 8081 (O:8), 4 - 5 = isolates positive for the test 6-11 = negative isolates; C- Catalase test: left positive reaction, right-negative. D- oxidase test: positive and negative reaction

All of the colonies showed morphological features of the genus *Yersinia*, and 136 of these colonies were determined to be *Y. enterocolitica* presumptive after being analyzed biochemically using characteristics such as degradation of urea, positive catalase test, negative oxidase test, and absence of tryptophan deaminase activity.

Table 1 Isolation and presumptive Y. enterocolitica colonies identification

isolation CIN agar	Urease (+)	Catalase (+)	Oxydase (-)	Deminase of tryptophan (-)	Positive colonies
920	333 (37%)	471 (51%)	181 (19%)	510 (55%)	136 (15%)

(+) positive, (-) negative

16S rRNA gene analyzes confirmed the presence of *Y. enterocolitica* in 43 isolates, with 38 originating from tonsil samples and 5 from fecal samples, all of which were obtained from 40 pigs (Figure 3). The presence of *Y. enterocolitica* was detected in 6.7% (40 out of 601) of the slaughtered pigs.

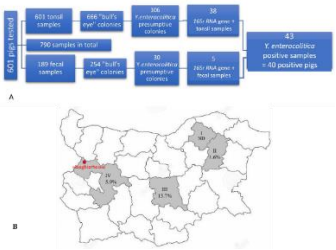

Figure 3 Numbers of pigs and samples tested for *Yersinia enterocolitica*

(A) and the distribution of the positive pigs per region in Bulgaria (B). Regions are presented in roman numerals I: Razgrad region (Farm I), II: Shumen region (Farm II), III: Stara Zagora region (Farm III), and IV: Sofia region (Farms IV, V, VI, and VII). The red dot indicates the location of the slaughterhouse (Kostinbrod city).

The total prevalence of *Y. enterocolitica* in slaughtered pigs was found to be 6.7 % (40 / 601 pigs). As shown in Figure 1B the frequency of *Y. enterocolitica* positive pigs varied according to the region of origin, with the highest prevalence observed in pigs from the Stara Zagora region (13.7 %, 19 / 139 pigs from Farm III), followed by pigs from the Sofia region (5.9 %, 19 / 324 pigs from Farms IV, V, VI and VII), and the lowest prevalence in pigs from the Shumen region (1.6 %, 2 / 126 pigs from Farm II). No positive pigs were found in the Razgrad region (0 / 12 pigs from Farm I). The positive animals were identified from six farms (Farms II – VII), located in Stara Zagora, Sofia, and Shumen regions. Among the farms in the Sofia region, the highest number of positive pigs were found on Farm VI, followed by Farms VII and V, while only one positive

slaughtered pig was identified on Farm IV. It is worth noting that all *Y. enterocolitica* strains were isolated only during the cold season of all sampling periods, from October to March (2016-2021).

Yersinia pseudotuberculosis was not detected in any of the samples.

To confirm the identification, we analyzed the sequenced *16S rRNA* sequences of 43 strains using the BLAST platform. The analysis revealed three top-scoring hits corresponding to a *Y. enterocolitica* strain. When compared to *Y. enterocolitica* strain KNG22703 (CP011286.1), we observed 99% and 100% identity in 39 of our isolates, except for strains -18, -28, -37, and -43. These four strains exhibited 98%, 95%, 92%, and 99% identity, respectively, with the studied sequence.

- Biotyping and serotyping

Bioserotyping was performed on the 43 confirmed isolates of *Y. enterocolitica*, and all isolates were determined to be of the same serotype, namely *Y. enterocolitica* serotype O:3, and biotype 4 based on their biochemical characteristics such as rhamnose and trehalose utilization, tween esterase and pyrazinamidase activity, and esculin and salicin hydrolysis (Figure 4).

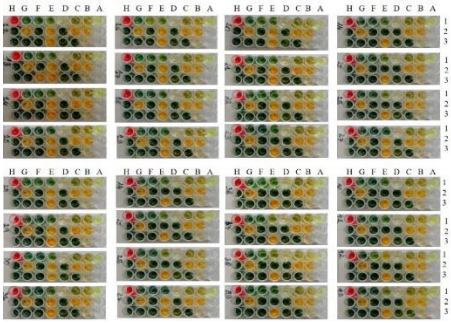

Figure 3 Biochemical test for biotype determination
A1: *β*-galactosidase B1: malonate, C1:citrate, D1: H2S, E1: lysine, F1: ornithine, G1:arginine, H1: urease, A2: *β*-glucorunidase, B2: manitole, C2: trechalose, D2: lactose, E2: celobiose, F2: melibiose, G2: sorbitol, H2: salicine, A3: *β*-xylosidase, B3 esculine, C3: raffinose, D3: inositol, E3: sucharose, F3: arabitol, G3: adonitol, H3 dulcitol.

All examined strains were identified as incapable of fermenting rhamnose, raffinose, melibiose, and lactose. These Y. *enterocolitica* isolates were, however, found to be capable of utilization of sucrose and trehalose, while also exhibiting the production of β-galactosidase, which corresponds to glucose uptake. All strains displayed an absence of lysine decarboxylase, arginine dehydrolase activity, as well as hydrogen sulfide production. None of the tested strains exhibited a positive response to citrate uptake as the sole carbon source. Additionally, the *Y. enterocolitica* strains (numbered 1 to 43) isolated by us were found to be negative for pyrazinamidase activity. The absence of the pyrazinamidase enzyme, along with negative test results for the hydrolysis of

esculin and absorption of salicyl, as well as the absence of the enzyme tryptophanase, which catalyzes the degradation of tryptophan to indole, are all indicators directly associated with the pathogenicity of these strains. These results collectively indicate that there is a 6.7 % carriage rate of potentially pathogenic isolates among the examined pig population. Furthermore, the presence of the virulent plasmid was phenotypically confirmed in a total of 24 strains through the absorption of Congo red dye on CRMOX agar (Figure 5).

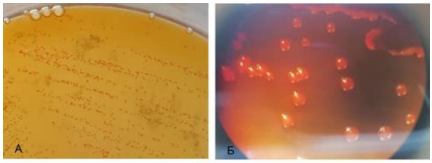

Figure 4 Plasmide positive and Congo red (+) colonies of *Y. enterocolitica*

А by naked eye Б) By microscope 5X.

All isolated strains belonged to biotype 4. After that the serological group was identified by slide agglutination.

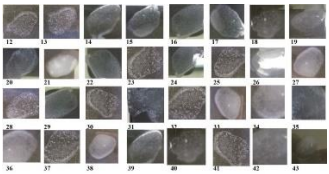

Figure 5 Slide agglutination reaction positive to O:3

Легенда: *Y. enterocolitica* №12 – *Y. enterocolitica* №43,

Isolated strains showed positive reaction to sera O:3.

2. Detection on virulence genes

PCR analysis of the *Y. enterocolitica* isolates (n = 43) revealed that all of them were positive for the chromosomally encoded virulence genes *ail* and *ystA*. The pYV-coded *yadA* gene was detected in 41 isolates by PCR analysis, while 21 isolates were found positive for pYV by phenotypic assay on Congo red-magnesium oxalate (CR – MOX) agar. The prevalence of *ail*- and *ystA*-positive *Y. enterocolitica* isolates among pigs was calculated to be 6.7 % (40 / 601), while the prevalence of the *yadA* gene was 6.3% (38 / 601).

3. Genetic diversity of *Y. enterocolitica* strains determined by PFGE

The genetic relatedness of 43 *Y. enterocolitica* strains isolated from pigs was analyzed by pulsed-field gel electrophoresis using *SpeI* as a restriction enzyme (Figure 7).

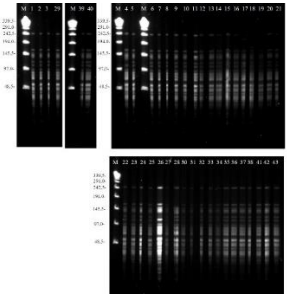

Figure 6 PFGE pulsotypes of the isolated на изолиранатa *Y .enterocolitica* digested by *SpeI*

M= *Pulse Marker* 50 -1000 kb. 1- 41- *Y. enterocolitica*, porcine isolates

From Figure 7, it is evident that following enzymatic degradation, pulse patterns were acquired that appear indistinguishable to the unaided eye. Discernible disparities between these patterns were scarcely discernible. Specifically, strains denoted as 4, 11, 26, 28, and 42, which were selected based on visual observation, were subsequently subjected to *NotI* restriction following *SpeI* restriction (Figure 8).

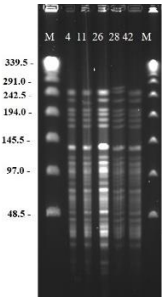

Figure 7 PFGE pulsotypes of *Y. enterocolitica* after *NotI* restriction

M= Pulse Marker 50-1000 Kb *Y. enterocolitica,* подредени по ред 4, 11, 26, 28, 42.

For the purpose of achieving a precise demarcation and comprehensive elucidation of intraspecific diversity, the electrophoresis images underwent computational processing via the GelCompar program (Applied Maths, Kortrijk, Belgium) – Figure 9.

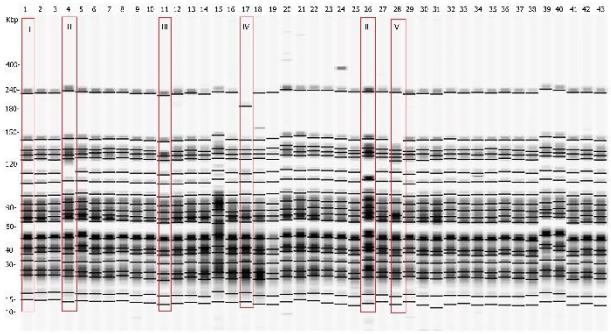

Figure 9 Pulsed-field gel electrophoresis pulsotypes of 43 *Y. enterocolitica* isolated strains after restriction with *SpeI*

Five pulsotypes were observed (surrounded by red rectangles). Pulsotypes II, III, IV, and V differ from the major pulsotype I, which is representative of all analyzed strains except strains 4, 11, 17, 26, and 28. The number of each lane corresponds to the number of the analyzed *Y. enterocolitica* strain (1 – 43). The pulse marker used was 50 – 1000 kb

Five pulsotypes showing minor differences were identified (Figure 9). Pulsotypes were further organized into two main clusters (S01 and S02) and three single pulsotypes (SP1, SP2, and SP3) based on a 97 % similarity (Figure 3). The number of fragments within the pulsotypes varied between 19 and 20 with sizes ranging from 15 kb to 240 kb.(Figure 2). The majority of the strains (88.4 %) belonged to cluster S01, which included strains isolated from tonsils and feces of pigs originating from farms II-VII (Figure 3). Cluster S02 included two strains with the same pulsotype, which originated from farms III and VI in different regions (Figure 3).

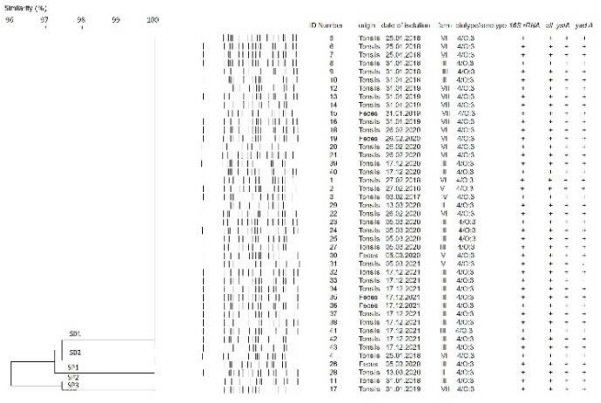

Figure 10 Dendrogram obtained derived by digesting *Y. enterocolitica* genomic DNA with *SpeI*

The DNA of 43 examined strains formed two different clusters, assigned S01 and S02, and three single pulsotypes, named SP1, SP2, and SP3. Clustering was calculated by the Unweighted pair group method with arithmetic mean (UPGMA) with 97 % similarity. Dice correlation was with tolerance of 2.5 % and optimization setting of 0.5. Additional information about dates of isolation, farm distribution, and virulence profile of the strains is given.

The remaining three strains, isolated from tonsils were assigned to single pulsotypes SP1, SP2, and SP3 (Figure 3). SP1 was closely related to S01 and S02 (with 97.4 % similarity) while SP2 and SP3 were closely related to each other (97.4 %). SP1 and SP2 were equidistant from S01 and S02 clusters with 97.4% and 94.7% similarity, respectively. SP3 was the most distant from the other pulsotypes and clustered as follows: 92.3% similarity to SP1, 97.4% similarity to SP2, 95% similarity to S01 cluster, and 92.3% similarity to S02 cluster,

4. Antimicrobial Susceptibility of *Y. enterocolitica* Strains

All 43 strains were tested for susceptibility to 15 antibiotics, and the results are shown in Table 1. All isolates were sensitive to ceftriaxone, amikacin, gentamicin, and ciprofloxacin. None of them was susceptible to ampicillin, novobiocin, cefamandole, and bacitracin. Forty-one strains were also sensitive to tetracycline, nalidixic acid, chloramphenicol, streptomycin, levofloxacin, trimethoprim/sulfamethoxazole, and doxycycline (Table 2).

Table 2 Resistance profiles of Y. enterocolitica, porcine isolates

Antibiotics		**Resistance Profile**		
Class *	Generic name	41/43	1/43	1/43
Penicillins	ampicillin	−	−	−
Cephems	cefamandole	−	−	−
	ceftriaxone	+	+	+
Aminoglycosides	amikacin	+	+	+
	gentamicin	+	+	+
	streptomycin	+	−	+
Phenicols	chloramphenicol	+	−	−
Tetracyclines	tetracycline	+	−	−
	doxycycline	+	+	−
Quinolones	nalidixic acid	+	−	−
	ciprofloxacin	+	+	+
	levofloxacin	+	−	−
Folate pathway antagonist	trimethoprim/ sulfamethoxazole	+	+	−
Aminocoumarins	novobiocin	−	−	−
Other	bacitracin	−	−	−

Legend: * Pharmacological classification defined according to Clinical and Laboratory Standards Institute (CLSI). (+) sensitive (−) resistant.

Two strains were observed to be multidrug resistant, demonstrating resistance to three other antibiotics: tetracycline, nalidixic acid, and chloramphenicol. One of them was also resistant to streptomycin and levofloxacin, and the other one to trimethoprim/sulfamethoxazole and doxycycline, respectively. In general, three resistance profiles were observed (Table 1). The most common profile was ampicillin / cefamandole / novobiocin / bacitracin resistance, detected in 95.3 % of the strains. One strain (2.3 %) demonstrated resistance to ampicillin / cefamandole / novobiocin / bacitracin / tetracycline / nalidixic acid / chloramphenicol / streptomycin / levofloxacin, and one strain (2.3 %) was resistant to ampicillin / cefamandole / novobiocin / bacitracin / tetracycline / nalidixic acid / chloramphenicol / trimethoprim / sulfamethoxazole / doxycycline.

5. Collection of the isolated strains

strain	T/F	Date	Farm	Bioserotype	*16S rRNA*	*ail*	*ystA*	*yadA*
1	T	27.02.2018	VI	4/O:3/VIII	+	+	+	+
2	T	27.02.2016	VI	4/O:3/VIII	+	+	+	+
3	T	03.02.2017	IV	4/O:3/VIII	+	+	+	+
4	T	25.01.2018	VI	4/O:3/VIII	+	+	+	+
5	T	25.01.2018	VI	4/O:3/VIII	+	+	+	+

6	T	25.01.2018	VI	4/O:3/VIII	+	+	+	+
7	T	25.01.2018	VI	4/O:3/VIII	+	+	+	+
8	T	31.01.2018	III	4/O:3/VIII	+	+	+	+
9	T	31.01.2018	III	4/O:3/VIII	+	+	+	+
10	T	31.01.2018	III	4/O:3/VIII	+	+	+	+
11	T	31.01.2018	III	4/O:3/VIII	+	+	+	+
12	T	31.01.2019	VII	4/O:3	+	+	+	+
13	T	31.01.2019	VII	4/O:3	+	+	+	+
14	T	31.01.2019	VII	4/O:3	+	+	+	+
15	F	31.01.2019	VII	4/O:3	+	+	+	+
16	T	31.01.2019	VII	4/O:3	+	+	+	+
17	T	31.01.2019	VII	4/O:3	+	+	+	+
18	T	26.02.2020	VI	4/O:3	+	+	+	+
19	F	26.02.2020	VI	4/O:3	+	+	+	+
20	T	26.02.2020	VI	4/O:3	+	+	+	+
21	T	26.02.2020	VI	4/O:3	+	+	+	+
22	T	26.02.2020	VI	4/O:3	+	+	+	+
23	T	05.03.2020	III	4/O:3	+	+	+	+
24	T	05.03.2020	III	4/O:3	+	+	+	+
25	T	05.03.2020	III	4/O:3	+	+	+	+
26	F	05.03.2020	III	4/O:3	+	+	+	+
27	T	05.03.2020	III	4/O:3	+	+	+	+
28	T	13.03.2020	II	4/O:3	+	+	+	+
29	T	13.03.2020	II	4/O:3	+	+	+	+
30	F	05.03.2020	V	4/O:3	+	+	+	+
31	T	05.03.2020	V	4/O:3	+	+	+	-

32	T	17.12.2020	III	4/O:3	+	+	+	+
33	T	17.12.2020	III	4/O:3	+	+	+	-
34	T	17.12.2020	III	4/O:3	+	+	+	+
35	F	17.12.2020	III	4/O:3	+	+	+	+
36	F	17.12.2020	III	4/O:3	+	+	+	+
37	T	17.12.2020	III	4/O:3	+	+	+	+
38	T	17.12.2020	III	4/O:3	+	+	+	+
39	T	17.12.2020	III	4/O:3	+	+	+	+
40	T	17.12.2020	III	4/O:3	+	+	+	+
41	T	17.12.2020	III	4/O:3	+	+	+	+
42	T	17.12.2020	III	4/O:3	+	+	+	+
43	T	17.12.2020	III	4/O:3	+	+	+	+

T = tonsils, F = feces

6. Optimisation of the LAMP protocol

- **Selectivity test**

The genomic DNA of the 32 strains was extracted, measured and used for the evaluation of the LAMP reactions. The optimal concentration of Mg2SO4 was needed for the reaction was determined to be 8 mM, and all experiments were conducted under same conditions at a temperature of 65°C for a duration of 30 minutes.

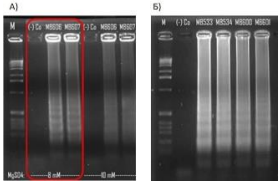

Figure 11 Selectivity of the LAMP primers

А – Mg_2SO_4 different concentrations on two pathogenic strains *Y. enterocolitica*: MB606 = *Y. enterocolitica* 4/O:3 (IP864); MB607 = *Y. enterocolitica* 4/O:3 (IP8944). Б – LAMP specificity of the primers to other pathogenic *Y. enterocolitica* strains: MB533= *Y. enterocolitica O:3*; MB534= *Y. enterocolitica O:9*; MB600= *Y. enterocolitica* O:8; MB601= *Y. enterocolitica* O:5,27 (IP22981).

The amplification assay, targeting the *phoP* gene, effectively discerned the DNA of all six pathogenic*Y. enterocolitica* strains. Subsequently, our LAMP protocol unequivocally identified all six pathogenic *Y. enterocolitica* strains as positive results. To ascertain the specificity of the employed primers, a rigorous evaluation was conducted, involving primer application other no *Y. enterocolitica* strains. All 26 of them showed no reaction product was obtained from any of the strains subjected to the test. Consequently, it was ascertained that the primers exhibited selectivity for the *phoP* gene specific to pathogenic *Y. enterocolitica*, thereby failing to recognize regions of genetic similarity within other strains encompassing the genus *Yersinia* or among the various bacterial strains examined outside the genus. By incorporating hydroxynaphthol blue (HNB) dye into the reaction mixture, we assessed its ability to differentiate between positive and negative samples. The color transformation within the reaction mixture occurred upon completion of the reaction, enabling immediate result interpretation. This visual assessment of the color change not only expedites the process but also eliminates

the need for intricate electrophoretic analysis techniques (Figure 12) For the internal control in all tests from the optimization scheme we used three negative controls: one with the water added before master mix is added, second one with the water after master mix and the third one in the DNA application room.

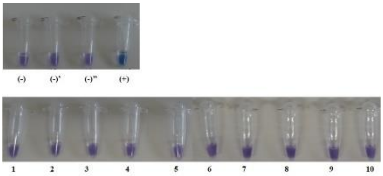

Figure 12 Visualization of the reaction by color change
M = DNA marker, (-), (-)', (-)'' = different negative controls (master Mix + water); (+) = positive control *Y. enterolitica* IP864). 1= *Y. mollaretii*, 2= *Y. bercovierii*,; 3= *Y. frederiksenii*,; 4 = *Y. kristensenii*, 5= *Y. intermedia*,; 6 = *Y. rhodei*, O.8; 7 = *Y. aldovae*; 8 = *Y. pseudotuberculosis*; 9= *Y. pseudotuberculosis*; 10 = *Y. pseudotuberculosis*.

As depicted in the accompanying figure, none of the tested strains within the *Yersinia* genus exhibited a color shift when compared to the positive sample. Negative samples exhibited a purple hue, while positive samples displayed a blue coloration. Our evaluation revealed that all tested strains were negative for the presence of the pathogenic *Y. enterocolitica*'s *phoP* gene when visualized using the dye. Importantly, these dye-based results perfectly aligned with those obtained through electrophoretic analysis of the reaction mixture.

- **Sensitivity test**

Figure 13 showed that electrophoretic analysis revealed a positive product at DNA dilutions of 10^1, 10^2, 10^3, 10^4, and 10^5 per 50 µL reaction mixture. The smallest dilution, which signifies the limit for confirming a positive result in the LAMP reaction, was a mere 10 copies of DNA in a 50 µL reaction mixture. Figure 13 – A clearly illustrates the dye's capability to differentiate between positive and negative samples. Both methods for detecting LAMP products exhibited equal sensitivity in detecting the *phoP* gene of pathogenic *Y. enterocolitica.*

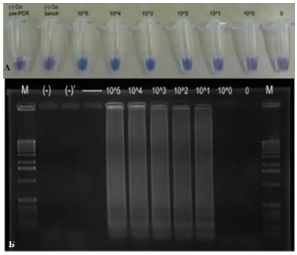

Figure 13 Sensitivity of the LAMP detection

A Visualisation by HNB dye. B Gel electrophoresis result detection M= DNA marker, (-) and (-)' = negative controls, *Y. enterocolitica* IP864 in different concentrations (10^5, 10^4, 10^3, 10^2, 10 и 0 DNA copies).

- **qPCR for LAMP product detection**

The results indicate that amplification was observed only when using DNA solutions with a concentration ranging from 10^5 to 10 copies for the control strain *Y. enterocolitica* IP684

DNA (Figure 7 - A). Conversely, amplification was not observed in strains other than *Y. enterocolitica* (Figure 14 - B).

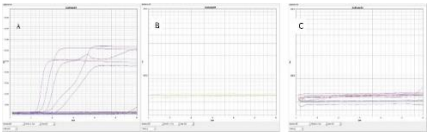

Figure 14 LAMP sensitivity measured by qPCR
A Serial dillutions of *Y. enterocolitica* IP684 from 10^5 до 10 DNA copies / reaction; B Negative control C *Yersinia spp. ;*

The LAMP primers exhibited a fluorescent signal, detectable after approximately 12 cycles of the reaction when diluted with 10^5 DNA copies, with the maximum signal occurring at 15 cycles (Figure 14 - A). When *Y. enterocolitica* DNA was diluted to 10 copies, a fluorescent signal was detected at 22 cycles, reaching its maximum at approximately 40 cycles of the reaction.

- **Artificial contamination of the samples**

The results of LAMP reactions using DNA templates isolated from contaminated feces are depicted in Figure 15. The resulting LAMP products were assessed both through gel electrophoresis and dye staining. As illustrated in the figure, a distinct product was evident in the electrophoresis analysis, and a blue color was observed at four different dilutions: 10^-4, 10^-3, 10^-2, and 10^-1.

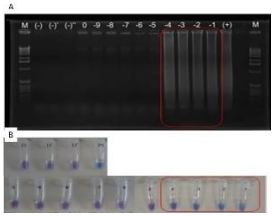

Figure 15 Lamp assay for artificial contaminated porcine feces

A LAMP wisualisation by gel electrophoresis B LAMP visualization by HNB. M= ДНК маркер, (-), (-)' (-)''= negative control (+) = positive control *Y. enterocolitica* 4/O:3 MB60; -9 до 1: DNA in serial delutions

These positive results established the lower detection limit of the *phoP* gene in the DNA of the utilized strain, which was determined to be at a dilution of 10^-4. The DNA isolated from 9 fecal samples, each infected with bacterial suspensions numbered from -1 to -9, served as the matrix for confirming the presence of *Y. enterocolitica* using qPCR. The optimized LAMP protocol is capable of detecting *Y. enterocolitica* even in the presence of DNA at a concentration of 41 copies in 5 µl and a bacterial dilution of 9.6 x 10^4 CFU/ml. These findings demonstrate agreement in the limit of *Y. enterocolitica* detection between the LAMP and qPCR methods.

- **Evaluation on the LAMP protocol on naturally contaminated samples**

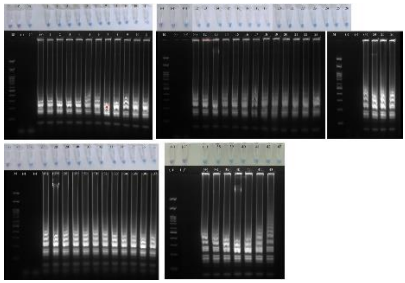

Figure 16 *phoP* gene detection from DNA isolated from *Y. enterocolitica* strains

1-43 *Y. enterocolitica* strains (-), (-)'' = negative control; (+) = positive control *Y. enterocolitica* 1B / O:8 IP8081. M = DNA marker

All 43 strains were detected as positive as the positive reaction for *phoP* gene in their DNA. Both methods revealed the equal results (Figure 16).

Genomic DNA isolated directly from the tonsils (n=30) and feces (n=30) of thirty previously characterized positive pigs was utilized to confirm the presence of pathogenic *Y. enterocolitica* using an optimized LAMP protocol. The isolated DNA served as a template for the detection of the ail gene through conventional PCR.

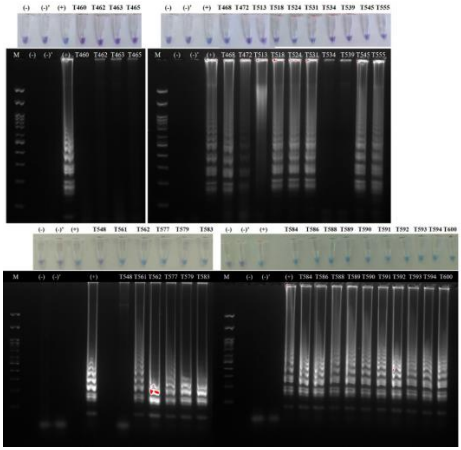

Figure 1 Detection of *phoP* gene in DNA isolated directly from porcine tonsil tissues

(-), (-)'' = negative control; (+) = positive control *Y. enterocolitica* 1B / O:8 IP8081 10^3. M = DNA marker T= tonsils.

Among the thirty tonsil samples examined, positive LAMP products for the *phoP* gene were detected in twenty-five samples (25/30) (Figure 17). The results of the tests for detecting the *phoP* gene in DNA isolated from the feces of positive pigs are depicted in the figure. A positive outcome was observed in twenty-seven out of the thirty samples (28/30) of DNA isolated directly from the feces that were tested (Figure 18).

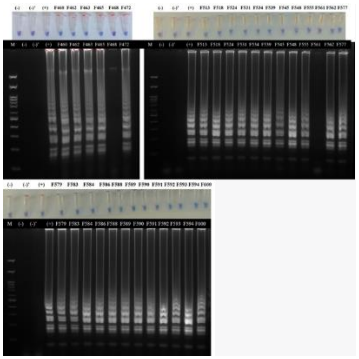

Figure 18 Detection of *phoP* gene in DNA isolated directly from porcine feces

(-), (-)'' = negative control; (+) = positive control *Y. enterocolitica* 1B / O:8 IP8081 10^3. M = DNA marker F = feces

In both tonsil and fecal samples, the detection of the phoP gene using the optimized LAMP protocol exhibited a high frequency. The percentage of pathogenic Y. enterocolitica in DNA isolated from pig tonsils was 83.3%, based on the analysis of 30 samples. Our optimized LAMP protocol successfully detected pathogenic *Y. enterocolitica* in 93% of the 30 total fecal DNA samples tested. These findings demonstrate the high sensitivity of the LAMP protocol.

Using conventional PCR, we successfully detected the ail gene in eight out of thirty DNA samples isolated directly from tonsils and four out of thirty DNA samples isolated directly from feces. We also demonstrated the presence of pathogenic *Y. enterocolitica* through PCR in only twelve samples.

In comparison to the conventional PCR method, our optimized LAMP protocol for direct detection of pathogenic Y. enterocolitica in pig tonsil and fecal tissues, without the need for a microbiological protocol involving colony enrichment and isolation, exhibited a higher sensitivity level.

V. Discussion

To the best of our knowledge, this is the first five-year study presenting data on the prevalence of Y. *enterocolitica* in pigs slaughtered in Bulgaria. The tested samples were collected in a single slaughterhouse, and originated from seven farms, located in four regions of Bulgaria. These data could be used for detailed epidemiological analysis of distribution patterns of pathogenic *Y. enterocolitica* in animals and humans in Bulgaria. The estimated 6.7 % prevalence of pathogenic *Y. enterocolitica* in healthy slaughtered pigs in this study is relatively lower compared to other European countries. The high prevalence in some countries could be explained by the different isolation meth-ods, different age of the pigs, technical parameters, etc. It is well known that molecular methods are more sensitive and precise in comparison to culture methods Conven-tional microbiological methods of isolation with enrichment step followed by a PCR method for confirmation reduce the likelihood of false positive results due to dead cells. Here, we combined microbiological, biochemical, and PCR methods

with enrich-ment step in Peptone Sorbitol Bile Broth (PSB broth) to ensure the most successful possible isolation and detection of pathogenic *Y. enterocolitica* strains. To enhance recovery of pathogenic *Y. enterocolitica*, the enrichment period was reduced from five to two days, and isolation was performed on selective agar after alkali treatment of PSB broth Alt-hough we believe that the choice of farm type can also affect the detection of pathogenic *Y. enterocolitica*, specific types of farms that could be responsible for the occurrence of *Y. enterocolitica* among pigs were not explored herein and remain to be further researched. Porcine tonsils are well recognized as a source of pathogenic *Y. enterocolitica*, in view of their lymphoid tissue tropism. In line with previous studies we detected pathogenic *Y. enterocolitica* mainly in tonsils and in pig feces. Different studies have demonstrated that shedding of yersiniae in the feces increases in piglets younger than 30 days and decreases when pigs reach slaughter age. *Y. enterocolitica* is a psychrophilic bacterium and can withstand cold temperatures over long periods of time. Indeed, in the current study *Y. enterocolitica* strains were isolated during the cold period of the year similarly to other studies reporting isolation of *Y. enterocolitica* from tonsil samples mostly during cold months. Sequencing data analysis revealed different similarity of our strains to the 16S rRNA nucleotide sequence of Y.

enterocolitica strain KNG22703, complete genome (GenBank ac-cession number: CP011286.1), and the 16S rRNA gene, partial sequence originated from uncultured bacteria clone 05-951_IBD.37307 (GenBank accession number: GQ965064.1). A very high similarity (98–99%) was established for most isolated strains in this study. Some of the isolated strains, such as Y. enterocolitica strains 18, 28, and 43, demonstrated similar-ities to KNG22703 of 97%, 93%, and 95%, respectively. Although small, the deviation of similarity among our isolates and the reference strain KNG22703 may be due to the different origin of the isolates, as KNG22703 has been adapted to humans. Like other authors, we have shown that the most commonly isolated biotype among pigs is *Y. enterocolitica* biotype 4 with the O:3 serotype confirmed in all 43 isolates. Moreover, in line with similar studies, we support the hypothesis of serotype O:3 predominance within biotype 4 of pig isolates. *Y. enterocolitica* 4/O:3 seems to be the most frequently detected and isolated bioserotype from healthy pigs in European counties, with the exception of the United Kingdom. Our study identified *Y. enterocolitica* 4/O:3 as the only bioserotype isolated among healthy pigs in a slaughterhouse in Bulgaria. The prevalence of bio/serotype 4/O:3 poses a risk to human health, as this path-ogen can easily enter the food chain through meat processing, spread to consumers, and eventually cause yersiniosis in

humans. Thus, our findings underline the importance of pigs in the epidemiology of yersiniosis. The Ail protein and yersiniabactin are important factors of virulence in *Y. enterocolitica*, both chromosomally encoded. Genes responsible for their expression—ail and ystA, re-spectively—are one of the most commonly used chromosomal targets for determination of pathogenicity. In our study, all genetically proven *Y. enterocolitica* strains harbored the genes of virulence ail and ystA, indicating a high pathogenic potential of the pig *Y. enterocolitica* isolates. Of note, the presence of pYV is based on the detection of different genes, and the complete virulence of *Y. enterocolitica* depends on plasmid availability as well. The pYV is unstable and its detection is faced with some difficulties. We detected the plasmid-borne gene yadA by both PCR and culture methods, and, as expected, the PCR method revealed a higher detection rate. It seems that Y. enterocolitica 4/O:3 is a less genetically diverse bioserotype. Indeed, we found a high degree of similarity between the macro-restriction pulsotypes of the *Y. enterocolitica* strains, suggesting a pronounced genetic homogeneity among the population of this species with only few isolates distinguishable from the predominant genotype. Our study found a 100% similarity within two clusters. This finding is in accordance with other studies which indicate a high degree of similarity and a minor genetic variation among 4/O:3

bioserotype strains, restriction enzyme applied (SpeI, NotI, or XbaI). In previous studies, we found that the genome of *Y. enterocolitica* is the most stable com-pared to the other two pathogenic species, *Y. pestis* and *Y. pseudotuberculosis*. Obviously, the geographic location is an important factor for the spread of a given pulsotype and enables the emergence of new branches. The high genetic similarity observed in our study suggests well pronounced homogeneity and conservation in the genome structure of the strains. The majority of strains belong to close pulsotypes, pointing to the wide distribution of one genotype of *Y. enterocolitica* 4/O:3. The results showed the predominance of this genotype for all observed farms, and its persistence over time, indicating low genetic variation. The few other PFGE pulsotypes detected could be contamination with these genotypes on a farm level. Clustering with 100% similarity of *Y. enterocolitica* 4/O:3 isolated from palatine tonsils confirmed the relevance of palatine tonsils for direct Y. enterocolitica contamination in carcasses. The overuse of antibiotics in veterinary medicine as growth promoters in farm ani-mals, including pigs, amplifies the significance of antimicrobial resistant *Y. enterocolitica*. Three profiles of resistance were detected among the isolated *Y. enterocolitica*. Our results showed that all tested strains were resistant to ampicillin, novobiocin, cefamandole, and bacitracin. In 2 out of

the 43 isolated *Y. enterocolitica* strains, we detected resistance also to tetracycline, nalidixic acid, and chloramphenicol, and only in 1 (out of 43) to streptomycin, trimethoprim/sulfamethoxazole, levofloxacin, and doxycycline. Multi-resistant *Y. enterocolitica* strains can be a serious threat to human health after transmission in the food chain and food contamination.

The loop-mediated isothermal amplification (LAMP) reaction is a method that offers significant potential, making it suitable for diagnostic applications in resource-limited laboratory settings. This technique was initially introduced by Notomi *et al.* 2000. Its notable advantages include ease of execution and the capacity for highly specific DNA amplification within a relatively short timeframe, typically ranging from 15 to 60 minutes (Notomi et al., 2000). Consequently, we have optimized a LAMP protocol specifically for the direct detection of the *phoP* gene associated with *Y. enterocolitica* pathogens in porcine samples. While the literature contains several studies aimed at detecting Y. enterocolitica in food products by targeting various genes (Gao et al., 2009; Xu et al., 2014), only one study has focused on the detection of the phoP gene (Li et al., 2010). The results of primer testing, as demonstrated in the study by Li et al. (2010), exhibit a high degree of specificity towards a particular region

within the *phoP* gene, which is exclusively present in pathogenic *Y. enterocolitica* strains. This unique specificity renders these primers well-suited for the detection of pathogenic *Y. enterocolitica* strains. The optimization of the running time for the LAMP reaction revealed a direct correlation with the incidence of false positive outcomes. Shortening the duration of the reaction resulted in a notable decrease in the occurrence of false positives. In contrast to traditional PCR, the LAMP method exhibited a remarkable twenty-fold increase in the yield of the final amplified product (Nagamine et al., 2001; Parida et al., 2008). An examination of real-time reaction data readings indicated the specificity of the LAMP primers for *Y. enterocolitica*. The lower detection limit of DNA in LAMP assays contributes to the method's heightened sensitivity. Saharan et al. (2014) conducted an analysis of the limit of detection across various LAMP protocols employed for the detection of diverse bacterial strains. Their findings reported a sensitivity threshold of merely six copies per reaction when utilizing a pure DNA template. Our research findings demonstrate the capability of DNA detection at a remarkably low concentration of only 96 copies per µl (192 CFU), which closely aligns with the detection rate achieved by the quantitative polymerase chain reaction (qPCR) method. This heightened sensitivity inherent to our approach frequently

presents challenges in the form of sample contamination, leading to subsequent false-positive results and diminished repeatability. Regarding the utilization of internal controls, within the framework of our protocol optimization experiments, we introduced three negative controls, each incorporating the addition of water in distinct physical locations. The negative control involving the introduction of water into the container simultaneously with the instillation of DNA was particularly relied upon. Notably, our experimental results indicated the absence of contamination when employing this specific control. The interpretation of results through agarose gel electrophoresis proves to be straightforward, as a positive signal within the sample corresponds to the presence of a columnar product, readily comparable to a positive control. The incorporation of non-intercalating dyes, such as hydroxynaphthol blue, serves the dual purpose of preventing contamination arising from post-amplification tube opening while also possessing non-fluorescent characteristics. This allows for the interpretation of results through a visual assessment, without the need for specialized instrumentation, by relying on the human eye (Wong et al., 2018). In our experimental aproaches, it was demonstrated that the methodology employed for reading the reaction did not exert an influence on the minimum concentration of DNA copies that were subject to analysis. The

utilization of colorimetric assessment in the LAMP reaction expedited the time required to obtain results. Subsequently, the outcomes derived from the optimized protocol were employed to assess its viability for clinical isolates, specifically 43 strains of *Y. enterocolitica* that had been previously isolated and confirmed in accordance with the ISO 10273:2003 standard. Notably, these strains exhibited enhanced DNA yield, attributed to their isolation from colonies of enteropathogenic Y. enterocolitica. Additionally, we applied the proof-of-concept protocol to DNA samples isolated directly from tonsil and fecal specimens. In this context, it is worth noting that the DNA yield was notably lower, primarily owing to the reduced likelihood of the presence of pathogenic bacteria in extractions obtained from 1 gram of tissue. Our research findings underscore the advantages associated with the LAMP method in the detection of Y. enterocolitica in comparison to conventional PCR when applied to pigs that have tested positive for the pathogen. This suggests the potential for implementing the LAMP protocol as a routine diagnostic method for the expedient identification of *Y. enterocolitica* in pigs, well in advance of confirmation by conventional diagnostic techniques. Notably, the LAMP method's capability to detect low concentrations of Y. enterocolitica DNA renders it suitable for early-stage infection screening, especially in asymptomatic cases such as those in

slaughter pigs. Detection of the enteropathogenic Y. *enterocolitica* in food samples adheres to the ISO 10273:2003 standard, which is characterized by time-consuming procedures and relatively lower sensitivity. Consequently, there exists a compelling need to introduce novel, swifter methodologies characterized by enhanced sensitivity, which can directly confirm the presence of pathogens in food or pig samples. In light of its manifold advantages, LAMP emerges as a justifiable diagnostic method, particularly in resource-constrained laboratory settings and field conditions.

VI Conclusions

1. Pathogenic *Y. enterocolitica* was detected in 6.7 % of the examined slaughtered pigs from Bulgaria.
2. The geographical region of origin of slaughter pigs does not exert an influence on the presence of *Y. enterocolitica.*
3. *Yersinia enterocolitica* was detected only during the cold period of sampling (October to March).
4. Bioserotype 4/O:3 was the only identified bioserotype among slaughtered pigs.
5. *Yersinia enterocolitica* harbor a great pathogenic potential according to the detected virulence genes *ail, ystA* and *yadA.*

6. Three profiles of resistance against the most commonly employed antibiotics, alongside the detection of multiresistant strains of *Y. enterocolitica*, were identified.

7. A predominant *Y. enterocolitica* genotype, persistently observed throughout a five-year study period, was ascertained.

8. LAMP protocol for detection of pathogenic *Y. enterocolitica* directly from tonsils and feces of slaughtered pigs was developed.

9. The sensitivity of the optimized LAMP protocol for the detection of pathogenic *Y. enterocolitica* is equal to that of qPCR for the detection of the pathogens.

VII Contributions

Original contributions

1. The present study marks the intraspecies genetic similarity among strains of *Y. enterocolitica* isolated from pigs across diverse farms in Bulgaria over a comprehensive five-year research endeavor.

2. Pioneering research in Bulgaria has unveiled the isolation of multi-resistant strains of *Yersinia enterocolitica* from slaughter pigs, characterized by resistance profiles spanning more than four distinct antibiotic groups.

3. A collection comprising *Y. enterocolitica* bioserotype 4/O:3 strains, sourced from slaughtered pigs, has been successfully established.

Recommendations for the practice:

1. Reporting of results using a dye allows the optimized LAMP protocol to be implemented in field settings as well as in laboratories with limited resources.

VIII Publications related to the dissertation

1. Prevalence, Genetic Homogeneity, and Antibiotic Resistance of Pathogenic *Yersinia enterocolitica* Strains Isolated from Slaughtered Pigs in Bulgaria. **Maya Angelovska**; Maya Margaritova Zaharieva; Lyudmila L. Dimitrova; Tanya Dimova; Irina Gotova; Zoltan Urshev; Yana Ilieva; Mila Dobromirova Kaleva; Tanya Chan Kim; Sevda Naydenska; Zhechko Dimitrov; Hristo Najdenski. Antibiotics 2023, Volume 12, Issue 4, 716 **Q1**

2. *Yersinia enterocolitica* - Isolation, Pathogenicity, and Prevalence in Farms for Slaughtered Pigs. **Maya Angelovska**, Maya M. Zaharieva, Hristo Najdenski. Acta microbiologica bulgarica 2023, Volume 39, Issue 2, 118-129. **Q4 –-**

Scientific conferences:

1. 8th Balkan Congress of Microbiology Microbiologia Balkanica 2013. Prevalence of Human Enteropathogenic *Yersinia spp*. in Pigs at Slaughter. Maya Gatzovska, Tanya Dimova, Maya M. Zaharieva, Trayana Draganova, Iva Tsvetkova, Hristo Najdenski. October 2-5. *2013,* Veliko Tarnovo, Bulgaria. Poster presentation..

2. Thirteenth Congress of Bulgarian Microbiologists with International participation 2014. Prevalence of Enteropathogenic *Yersinia* in tonsils of Slaughter pigs. Maya Gatzovska, Tanya Dimova, Maya M. Zaharieva, Trayana Draganova, Iva Tsvetkova, Hristo Najdenski. 07-10 October 2014 Tryavna, Bulgaria. Poster presentation..

3. Second National Food Conference with International Participation. Study of Pigs at slaughter for pathogenic *Yersinia* strains. Maya Gatzovska Tanya Dimova Maya M Zaharieva Trayana Draganova Iva Tsvetkova Hristo Najdenski. March 20th-21st 2015, Sofia Poster presentation..

4. Workshop on food-borne Pathogens and Food safety. Detection of *Yersinia enterocolitica* in pig faeces by using Loop mediated DNA Amplification. Maya M. Zaharieva, Maya Gatzovska, Els van Collie, Mark Heyndricks, Hristo M. Najdenski. 26.05.2016 - 27.05.2016. Poster presentation.

5. International Scientific Conference Microbiology for better health and Industry. 2017 Seventy Years The Stephan Angeloff Institute Of Microbiology-BAS. Detection of *Yersinia enterocolitica* in pig faeces and tonsils by LAMP. Maya M. Zaharieva, Maya Gatzovska, Victoria Teneva, Els van Collie, Mark Heyndricks, Hristo M. Najdenski. March 14-15.2017. Poster presentation.

6. Food 3 International Conference the challenges for quality and safety along the food chain. 2017. Detection of *Yersinia enterocolitica* pig faeces and tonsils by LAMP. Maya M. Zaharieva, Maya Angelovska, Victoria Teneva, Els van Collie, Mark Heyndricks, Hristo M. Najdenski. March 23-25, 2017 NBU, SOFIA, BULGARIA. Poster presentation..

7. 10th Scientific Conference of the Bulgarian Focal Point of EFSA "10 years of food science in service of consumers" 2017. New approaches for fast detection of *Yersinia enterocolitica* and surveillance in slaughter pigs, Maya Zaharieva, Maya Angelovska, Lyudmila Dimitrova, Iva Tsvetkova, Victoria Teneva, Els van Collie, Mark Heyndricks, Hristo Najdenski. 31 October – 2 November 2017, Sofia. Oral presentation.

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11. Научна конференция 120 години Национален Диагностичен Научно-Изследователски Ветеринарно Медицински институт и 140 години от рождението на неговия патрон проф. д-р Георги Павлов. Доказване на *Yersinia enterocolitica* в тонзили и фецеси от свине за клане с класически микробиологични и PCR базирани методи. Мая Захариева, Мая Ангеловска, Людмила Димитрова, Мила Калева, Елз Ван Коли, Марк Хендрикс, Христо Найденски. 21.10 2021, София, България. Poster presentation.

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