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VALIDATION OF METHOD FOR *YERSINIA ENTEROCOLITICA* ISOLATION FROM RAW MATERIALS OF ANIMAL ORIGIN

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SUMMARY

Upward trend in the number of human yersiniosis cases, caused by bacterium *Yersinia enterocolitica*, is globally observed nowadays. This microorganism is widely spread in the environment, able to persist for prolonged periods in animal products and propagate under low temperatures. Basic infection sources are meat and meat products. In order to isolate *Yersinia enterocolitica* from food and feed samples horizontal method for the detection pursuant to GOST ISO 10273-2013 was used. It was noted, that *Yersinia enterocolitica* isolation is associated with certain difficulties, because the sample contains only small quantities of the agent and only the use of special techniques allows removing the concurrent microflora. It was proposed to use cold enrichment (4 ± 1) °C of the test material before conventional technique is started. The technique was validated pursuant to GOST ISO 16140-2011. As a result, it was established that validated method for *Yersinia enterocolitica* bacteria detection in food products, performed at the Microbiology Laboratory, is specific. The method sensitivity is 10 CFU/cm^3. Intralaboratory reproducibility and repeatability were confirmed by relevant tests. Additional culture step at (4 ± 1) °C allows complete inhibition of non-psychrophilic microorganisms' growth.

Key words: yersiniosis, *Yersinia enterocolitica*, psychrophilic microorganisms, cold enrichment.

INTRODUCTION

Recently human enteric yersiniosis has become increasingly important problem. According to the literature, yersiniosis takes the second place after salmonellosis in the Russian Federation. About 4–5 thousand cases of the said diseases are reported annually. In the Republic of Crimea and Sevastopol city yersenioses are detected almost every year [1, 13–15]. The disease is caused by *Yersinia enterocolitica* bacterium.

The agents were firstly isolated from diseased humans and animals in the Europe in the first half of 20th century and initially classified to *Pasteurella* genus as *Pasteurella* X. In 1946 J. Van Loghem proposed to classify the above mentioned bacteria in a new genus – *Yersinia*. In 1954 H. Mollaret and E. Thal proposed to classify *Yersinia* genus to *Enterobacteriaceae* family. Later, *Yersinia enterocolitica* (*Pasteurella* X), yersiniosis causative agent, was added to *Yersinia* genus [1, 24].

Yersinia genus comprises 11 species, including three species that are pathogenic for humans: *Y. pestis*, *Y. pseudotuberculosis*, *Y. enterocolitica* [1, 24].

Y. enterocolitica is a gram-negative rod-shaped bacterium with rounded tips or coccobacillus 0.8–1.2 μm in length and 0.5–0.8 μm in width, readily stained with all aniline dyes and motile at 18–20 °C, becomes non-motile at cultivation temperature of 28–37 °C, has a lot of peritrichously arranged flagella. It is a facultative aerobe, does not grow under the total anabiosis conditions and does not form any spores. Some *Y. enterocolitica* strains have fimbria [1, 3, 16, 18, 24].

The bacterium grows at temperature of 4–40 °C, is capable of growth at 5% NaCl and pH 5.2–9.0. Yersinia are propagated in meat peptone agar, Hottinger's, Martin, Endo agars. CIN-agar, SSDC-agar, yersinia-agar are used for its selective isolation [1, 3, 16, 18, 24].

Y. enterocolitica produces hydrogen sulphide and ammonia, shows positive reactions with methyl red in a sample, reduces nitrates to nitrites, has no fibrinolytic, plazma-coagulating and proteolytic properties and causes rabbit red blood cell hemolysis [1, 3, 16, 24].

Yersinia are widely distributed in the environment. *Y. enterocolitica* was isolated from almost all mammal, bird, fish, amphibian, mollusk and insect species. In wildlife, Yersinia are mainly isolated from rodents [1, 6, 9]. The bacterium is also isolated from plant products (vegetables, root vegetables, leaf vegetables, fruit) [2, 23].

According to A. V. Moskalev, et al., yersiniosis is not apparently endemic in the Republic of Crimea. However, enteric yersiniosis agent is found to circulate in 10–20% of small mammals in the majority of administrative raions [17].

In domestic animals, *Y. enterocolitica* is mostly isolated from dogs, pigs and cattle [1, 6, 9].

According to data provided by A. K. Bkhunia, pigs are the main reservoir for pathogenic strains responsible for infectious disease in humans (as the bacteria are commensal in pigs). Pathogenic *Y. enterocolitica* are isolated from 35–70% of pigs [3]. Therefore, meat and meat products are the source of infection for humans. It is accounted for the *Yersinia* ability to persist in animal products for a long time and propagate at low temperatures as well survive under various environment conditions [7].

Enteric yersiniosis has become a great concern due to its widespread, rapid adaptation to the environment and difficult diagnosis. Investigation of the disease prevalence in animals is hampered by the fact that the disease is not to be mandatory reported in the Russian Federation as a separate nosological unit [6, 12, 21].

Customs Union Regulation No. 021/2011 on food product safety lays down requirements for *Yersinia* spp.

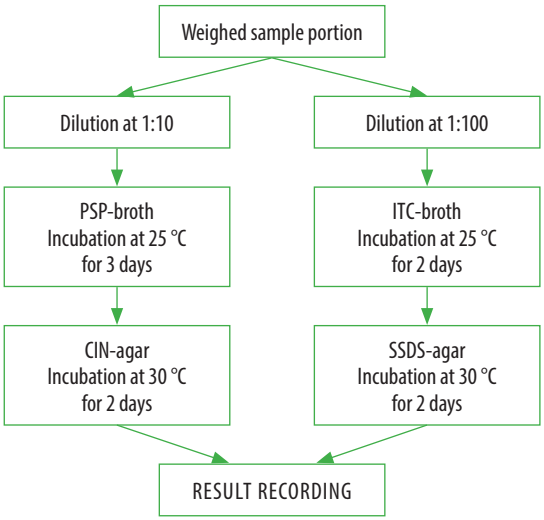

Fig. 1. Test procedure according to GOST ГОСТ ISO 10273-2013

Table 1
***Y. enterocolitica* No. 9610 ATCC and *S. typhimurium* No. 14028 ATCC growth patterns onto agar medium**

Strain	CIN-agar	SSDS-agar
Yersinia enterocolitica No. 9610 ATCC	Small colonies with smooth edges and red centre	Small transparent light-creamy colonies
Salmonella typhimurium No. 14028 ATCC	Small creamy colonies with smooth edges and darker centre	Small colorless colonies with dark centre

amounts in dried vegetables, potatoes and products thereof, raw vegetable products and cut, blanched vegetables and fruit including frozen ones but only in case of unfavorable epidemic situation associated with the said products in the area of production [22]. However, there is a lot of evidence of *Y. enterocolitica* presence in meat, milk and products thereof in literature [3, 4, 8–10, 18, 19].

Y. enterocolitica isolation is difficult. The agent concentration in a sample is often very low and the agent can be detected only using special methods allowing its differentiation from other concurrent microorganisms. Therefore, researchers have to use various selective enrichment techniques [6].

Currently food and animal feed samples are tested according GOST ISO 10273-2013 "Microbiology of food and animal feeding stuffs. Horizontal method for the detection of presumptive pathogenic *Yersinia enterocolitica*" is as follows: a sample is enriched in liquid selective medium, subsequently reinoculted onto agar plates and specific colonies are collected and subjected to identification [4].

According Methodical Guidelines 4.2.3019-12 "Organization and procedure for laboratory tests for yersiniosis at local, regional and federal levels", cold enrichment shall be used in the tests. For this purpose, tested materials are inoculated to one of enrichment media and incubated in a refrigerator at temperature of (6 ± 2) °C for 2–15 days. Within the said period they are reseeded onto dense nutrient media on day 2–3, 5–7 and 10–15 after first inoculation. The inoculated media are incubated at temperature of (26 ± 2) °C for 24 hours and observed up to the first positive results but not longer than for 15 days [11].

Considering the abovementioned, our study was aimed at evaluation of applicability of cold enrichment at (6 ± 2), as an additional stage, for lowering concurrent microorganism concentration during tests of raw animal materials performed according to GOST ISO 16140-2011.

In accordance with modern requirements to tests, any modification in the standard method requires validation. Relative accuracy, specificity and sensitivity of the method are to be validated [5].

The goal of the study was to validate the method for *Y. enterocolitica* horizontal detection according to GOST ISO ГОСТ ISO 10273-2013 as suitable for testing.

MATERIALS AND METHODS

Tests were performed at the Microbiological Laboratory of the FGBI "Federal Centre for Animal Health" subordinated to the Rosselkhoznadzor.

Animal raw material and product specimens previously tested for *Salmonella* и *Y. enterocolitica* bacteria with standard method with negative results were used as materials for artificial infection.

Y. enterocolitica strain (No. 9610, ATCC) and *S. typhimurium* strain (No. 14028, ATCC) were used.

The following was used for tests for *Y. enterocolitica* bacteria according to GOST ISO 10273-2013: ITC-broth and PSB-broth (HiMedia, India), Yersinia selective agar (CIN-agar, Merk, Germany) as well as SSDS-agar for Yersinia (HiMedia, India) were used as dense media for re-inoculation (Fig. 1) [20].

Table 1 shows patterns of control strain growth in nutrient media used for the test.

Modified standard method including cold enrichment according to MG 4.2.3019-12 "Organization and procedure for laboratory tests for yersiniosis performed at local, regional and federal levels" [22] was used for tests.

Validation was performed according to GOST ISO 16140-2011 "Microbiology of food products and feed for animals. Procedure for validation of alternative methods".

RESULTS AND DISCUSSION

As mentioned above, food products are often contaminated by small amounts of the agent and its isolation requiring its differentiation from concurrent microorganisms is difficult.

A weighed portion of the product sample is additionally inoculated in liquid nutrient media, ITC-broth and PSB-broth, followed by incubation at 4 °C to assess the effect of incubation temperature at the enrichment stage on concurrent microorganism growth and test results. Then, the test was performed according standard testing procedure.

During testing, the sample of known mass was added to enrichment media to prepare the following dilutions: 1:10 – in PSB-broth and 1:100 – in ITC-broth. Suspensions of tested sampled (10 cm^3/tested sample) were collected and divided into 4 groups:

– group I – was inoculated with 0.5 cm^3 *Y. enterocolitica* suspension at concentration of 0.5 McFarland units;

– group II – was infected with *S. typhimurium* suspension at a dose of 0.5 cm^3 and at concentration of 0.5 McFarland units;

– group III – was inoculated with *Y. enterocolitica* suspension and *S. typhimurium* suspension, 0.5 cm^3 per each suspension, at concentration of 0.5 McFarland units;

– IV группа –served as a control group.

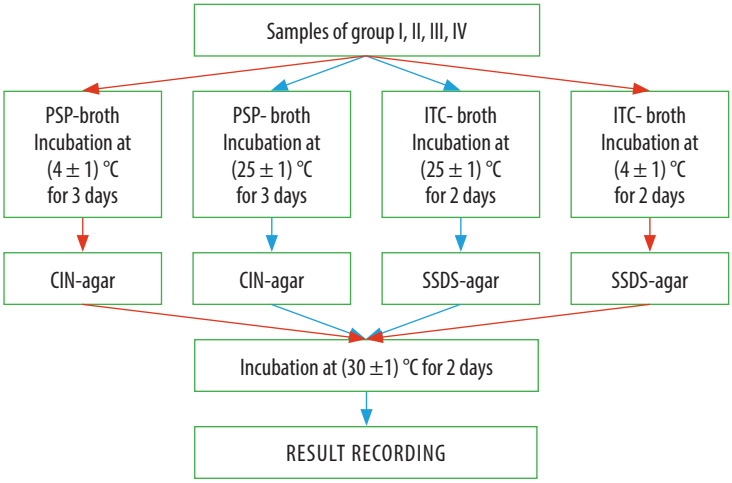

Fig. 2. Test procedure

Red arrows show proposed modified procedure, blue arrows – standard procedure according to GOST ISO 10273-2013.

Then, the test was performed as shown in Figure 2

Samples of each test group were incubated at temperature of (4 ± 1) °C and (25 ± 1) °C for 48 hours for ITC-broth and for 72 hours for PSB-broth. Then, they were reinoculated onto solid nutrient media (CIN- and SSDS-agar) and incubated at temperature of (30 ± 1) °C. Results were recorded 48 hours after re-inoculation.

Test results are given in Table 2.

Table 2
Effect of incubation temperature on *Y. enterocolitica* and *S. typhimurium* growth
n=3

Tested material	Enrichment medium	Incubation temperature, °C	Selective agar	Result
Product sample suspension + *Y. enterocolitica*, group I	ITC	4 ± 1	SSDC	Characteristic colonies, (21 ± 6) CFU
	PSB	4 ± 1	CIN	Characteristic colonies, (29 ± 4) CFU
	ITC	25 ± 1	SSDS	Characteristic colonies, (189 ± 10) CFU
	PSB	25 ± 1	CIN	Characteristic colonies, (156 ± 8) CFU
Product sample suspension + *S. typhimurium*, group II	ITC	4 ± 1	SSDS	Small discrete colonies
	PSB	4 ± 1	CIN	No growth
	ITC	25 ± 1	SSDS	Colorless colonies with dark centre, (148 ± 8) CFU
	PSB	25 ± 1	CIN	Small creamy colonies with even edges and darker centre, (168 ± 10) CFU
Product sample suspension + *Y. enterocolitica* + *S. typhimurium*, group III	ITC	4 ± 1	SSDS	*Y. enterocolitica* – characteristic colonies, (23 ± 3) КОЕ; *S. typhimurium* – no growth
	PSB	4 ± 1	CIN	*Y. enterocolitica* – characteristic colonies, (28 ± 4) CFU; *S. typhimurium* – no growth
	ITC	25 ± 1	SSDS	*Y. enterocolitica* – (144 ± 7) CFU; *S. typhimurium* – (291 ± 8) CFU
	PSB	25 ± 1	CIN	*Y. enterocolitica* – (89 ± 4) CFU; *S. typhimurium* – (198 ± 12) CFU
Product sample suspension not inoculated with tested microorganisms, group IV	ITC	4 ± 1	SSDS	No growth
	PSB	4 ± 1	CIN	No growth
	ITC	25 ± 1	SSDS	No growth
	PSB	25 ± 1	CIN	No growth

Table 3
Results of tests of the method to be validated for its specificity

Sample No.	Description of sample	Obtained result
1	Minced pork + *Y. enterocolitica*	*Y. enterocolitica* bacteria were detected in 25 g of the product
2	Chilled pork + *S. typhimurium*	*Y. enterocolitica* were absent in 25 g of the product
3	Chilled pork + *Y. enterocolitica*	*Y. enterocolitica* bacteria were detected in 25 g of the product
4	Minced pork +*S. typhimurium*	*Y. enterocolitica* bacteria were absent in 25 g of the product
5	Minced pork + *Y. enterocolitica* + *S. typhimurium*	*Y. enterocolitica* bacteria were detected in 25 g of the product
6	Minced pork + *Y. enterocolitica* + *S. typhimurium*	*Y. enterocolitica* bacteria were detected in 25 g of the product
7	Liver sausage + *Y. enterocolitica*	*Y. enterocolitica* bacteria were detected in 25 g of the product
8	Liver sausage + *S. typhimurium*	*Y. enterocolitica* bacteria were absent in 25 g of the product
9	Liver sausage + *S. typhimurium* + *Y. enterocolitica*	*Y. enterocolitica* bacteria were detected in 25 g of the product
10	Liver sausage	*Y. enterocolitica* bacteria were absent 25 g of the product

Table 4
Results of test of the validated method for its sensitivity

Initial concentration of *Y. enterocolitica* suspension, CFU/cm^3	Result
1×10^6	*Y. enterocolitica* bacteria are detected
1×10^5	*Y. enterocolitica* bacteria are detected
1×10^4	*Y. enterocolitica* bacteria are detected
1×10^3	*Y. enterocolitica* bacteria are detected
1×10^2	*Y. enterocolitica* bacteria are detected
1×10^1	*Y. enterocolitica* bacteria are detected
1	No *Y. enterocolitica* bacteria are detected

Table 5
Determination of repeatability and intralaboratory reproducibility of the results of tests using the validated method

Sample No.	Results obtained by the first operator, test replicate 1/test replicate 2			Results obtained by the second operator, test replicate 1/test replicate 2		
	0	1	2	0	1	2
1	–/–	+/+	–/–	–/–	+/+	–/–
2	–/–	+/+	–/–	–/–	+/+	–/–
3	–/–	+/+	–/–	–/–	+/+	–/–
4	–/–	+/+	–/–	–/–	+/+	–/–
5	–/–	+/+	–/–	–/–	+/+	–/–
6	–/–	+/+	–/–	–/–	+/+	–/–
7	–/–	+/+	–/–	–/–	+/+	–/–
8	–/–	+/+	–/–	–/–	+/+	–/–
9	–/–	+/+	–/–	–/–	+/+	–/–
10	–/–	+/+	–/–	–/–	+/+	–/–

0 – matrix;
1 – matrix infected with *Y. enterocolitica* strain;
2 – matrix infected with non-target *S. typhimurium* strain;
«+» – *Y. enterocolitica* bacteria are detected;
«–» – no *Y. enterocolitica* bacteria are detected.

Data given in Table 2 show that in group I Yersinia-characteristic growth was observed in all plates. The number of characteristic colonies (21 ± 6 and 29 ± 4 CFU) was averagely 7 times lower at incubation temperature (4 ± 1) °C, as compared with those (189 ± 10 and 156 ± 8 CFU) at incubation temperature of (25 ± 1) °C.

In group II, small discrete colonies were observed in plates with SSDS-agar at incubation temperature (4 ± 1) °C, and no *Salmonella* growth was observed in plates with CIN-agar. Abundant growth of *Salmonella* (148 ± 8 and 168 ± 10 CFU) was observed in both agars at incubation temperature of (25 ± 1) °C.

In group III growth of *Y. enterocolitica*-characteristic colonies (23 ± 3 and 28 ± 4 CFU), was observed in SSDS- and CIN-agar plates at incubation temperature of (4 ± 1) °C whereas no *Salmonella* growth was observed. Growth characteristic of both strains was observed in SSDS- and CIN-agar plates at incubation temperature of (25 ± 1) °C, in all cases *S. typhimurium* growth (291 ± 8 and 198 ± 12 CFU) was two times higher than growth of *Y. enterocolitica* (144 ± 7 and 89 ± 4 CFU).

Obtained results indicate that additional incubation step at (4 ± 1) °C included in the standard method allows complete inhibition of non-psychrophilic microorganisms' growth.

Tests of the method for its specificity. Microbiological method specificity is its ability to detect target strain in sample matrix in the presence of non-target microorganism strains.

Data given in Table 3 show that the presence of non-target strain and matrix components have no effect on the of *Y. enterocolitica* bacteria detection results. Thus, the validated method is specific.

Tests of the method for its sensitivity. Microbiological method sensitivity is the lowest microorganisms' concentration that can be detected with the said method.

To test the validated method for its sensitivity, 10-fold dilutions of suspension of target *Y. enterocolitica* strain prepared using saline solution were used. Initial concentration of the suspension was 0.5 McFarland units that was equal to 1.0×10^8 CFU/cm^3. Test results are given in Table 4.

Thus, the method sensitivity was 10 CFU/cm^3.

Determination of repeatability and intralaboratory reproducibility. Repeatability is a validation parameter determined by assessment of results of tests of identical samples under repeatability conditions. Intralaboratory reproducibility is determined by analysis of the results of tests of the same samples performed by different laboratory operators (Table 5).

Experimental data evidence that repeatability and intralaboratory reproducibility is 100%.

CONCLUSION

The method validation procedure showed the specificity of the horizontal method for *Y. enterocolitica* bacteria detection in food animal products according to GOST ISO 10273-2013, performed at the microbiological laboratory. The method sensitivity is 10 CFU/cm^3. Its intralaboratory reproducibility and repeatability are confirmed by appropriate tests. Use of additional step of incubation at temperature of (4 ± 1) °C allows complete inhibition of non-psychrophilic microorganisms' growth.

Conflict of interests. The authors claim no conflict of interest.

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