



Improving the Efficiency of *Campylobacter* spp. Isolation from Livestock and Poultry in Ukraine

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Abstract | This article presents research on the spread of *Campylobacteriosis* pathogens in Ukraine, revealing infection rates of 26.3% in broilers, 3.33% in turkeys, 55% in pigs, and 8.33% in cattle. In this research of the study, 33 isolates of *Campylobacter* spp. were identified for the first time, representing 23.9% infection, among which *Campylobacter coli* accounted for 75.8% and *Campylobacter jejuni* for 24.2%. Notably, up to 70% of poultry isolates were identified as *Campylobacter coli*. To improve the indication system, methods were modified by reducing cultivation time, simultaneously using selective nutrient media of various compositions, and employing both commercial materials and those prepared from components. Additionally, a comparative assessment of two isolation methods was conducted using a model of 138 samples of cecal contents from poultry (n=106) and animals (n=32) from farms in Vinnytsia, Volyn, Kyiv, and Cherkasy regions. 97% of the isolates were obtained by direct plating, with 15.6% being pure cultures. The enrichment method with reduced cultivation time yielded 72.7% of *Campylobacter* isolates, with 51.5% being pure cultures. A CRYOBANK for *Campylobacter* spp. was created to preserve genetic material and study foodborne pathogens, their properties, adaptation, resistance, and variability within the “One Health” concept. It was established that the optimal way to restore the metabolic processes of *Campylobacter* from a cryogenic state involves reducing the cultivation time to 24 hours during enrichment and isolation. The validation of *Campylobacter* isolation protocols has simplified the research process, reduced costs by 50%, and decreased laboratory workload, which will enhance the efficiency of routine *Campylobacter* diagnosis and identification in laboratories.

Keywords | Foodborne zoonoses of Ukraine, *Campylobacter* spp. isolates, *Campylobacter* isolation methods, CRYO-BANK, Identification

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Campylobacteriosis is one of the global foodborne zoonoses of priority significance in Food Security and falls within the scope of the “One Health” concept (Kaakoush *et al.*, 2015; Liu *et al.*, 2022).

The causative agents of the infection are bacteria of the genus *Campylobacter* are commensal microorganisms of the intestines of mammals, poultry, reptiles, etc mollusks (Iraola *et al.*, 2020; Kreling *et al.*, 2020; Olvera-Ramírez *et al.*, 2023), in which they do not cause signs of the disease, are therefore not diagnosed, and easily enter the food supply chain (Althaus *et al.*, 2017; Amjad, 2023). The risk factors for *Campylobacter* infection are: consumption of insufficiently heat-treated poultry meat and poultry products, unpasteurized (raw) milk and contaminated water (Facciola *et al.*, 2017). Heat-loving species *Campylobacter jejuni* and *Campylobacter coli* are the causative agents of 95% of cases of campylobacteriosis (EFSA, 2023).

Campylobacteriosis in Ukraine is an actual zoonosis, but with a low level of diagnosis. According to the reports of the “Public Health Center of the Ministry of Health of Ukraine” in 2023, 116 cases of human campylobacteriosis were registered, which is 0.28 per 100,000 people of the population. The average yearly incidence rate from 2016 to 2023 is 0.34 cases per 100,000 people. Concern cause acute intestinal infections and food toxic infections of unknown etiology, which formed in 2023, 70 cases per 100,000 population, with the peak number of cases (120.92) occurring in 2017 (PHC of Ukraine, 2023). However, in 2023, 62 isolates of *Campylobacter* spp were isolated in only one of the hospitals in the city of Dnipro from children for acute intestinal infections, which accounted for 6% of lesions (Annual report on the main performance indicators of the bacteriological laboratory). Similar results were obtained from studying the epidemiological process of acute bacterial intestinal infections and determining the role of *Campylobacter* spp. in this pathology (Kyryk, 2013; Kyryk, 2017a). There is a suspicion of inconsistency reality and discovered facts of infections caused by *Campylobacter* which indicates inadequate *Campylobacter* diagnostics in Ukraine related to the difficulties and material costs of conducting research (Kyryk, 2012; Pinchuk and Pustovit, 2018). Lack of mandatory studies for the detection of *Campylobacter* as a microbiological indicator of food safety, the complexity of technical reproduction of cultivation conditions, the high cost of diagnostic work does not allow conducting large-scale research on detection *Campylobacter* spp. in comparison with other intestinal pathogens, such as *Salmonella*, *Escherichia coli*, *Yersinia enterocolitica*, *Shigella* and *Vibrio*.

To control *Campylobacter* in EU countries, a monitoring system for zoonotic pathogens has been implemented

based on Directive 2003/99/EC. Regulatory documentation on *Campylobacter* surveillance in Ukraine is absent, so the prevalence and epidemiology of campylobacteriosis has not been established. In general, campylobacteriosis in animals and poultry is not reported in Ukraine (Drahut, 2013). Therefore, official data on disease incidence are not available, although scientific data indicate the widespread prevalence of the zoonosis (Tsarenko and Korniienko 2021). For example, for period from 2015 to 2023, by specialists of state laboratories of veterinary medicine of the State Production and Consumer Service of Ukraine, 1208 materials from poultry were examined for campylobacteriosis, however, no positive results were found (“Report on the Activities of the State Service of Ukraine for Food Safety and Consumer Protection Laboratories” No. 2-Vet). Such data cast doubt on the epizootiological status regarding campylobacteriosis in poultry in Ukraine especially since *Campylobacter* spp. are isolated in research studies (Rodionova, 2017; Kasianenko *et al.*, 2019; Shchur *et al.*, 2023b; Shchur *et al.*, 2023c). For “State monitoring of antimicrobial resistance of zoonotic and commensal bacteria in veterinary medicine”, which was the first time conducted in 2021, 746 samples from broilers were examined and 26 isolates were isolated *Campylobacter* spp. (Mazur *et al.*, 2022; Shchur *et al.*, 2023a).

Therefore, the analysis of official data reveals the shortcomings in campylobacteriosis research in Ukraine both in humans and in animals. It is crucial to understand that reliable data fundamentally depend on the accuracy of pathogen isolation methods and disease diagnosis, whose importance cannot be overstated.

The sensitivity of *Campylobacter* to environments and cultivation conditions, as well as the presence of accompanying microflora, hinders effective isolation and identification of *Campylobacter* (Ricke *et al.*, 2019; Chon *et al.*, 2020). Additionally, the presence of viable but uncultured forms of cells (VBNC) (Santos *et al.*, 2023) and prevalence among accompanying microflora of bacteria resistant to antimicrobial drugs, for example, *E. coli*, which produces extended-spectrum beta-lactamase (ESBL), significantly reduces their isolation from the intestine animals and poultry (Apostolakis *et al.*, 2019; Kim *et al.*, 2019; Apostolakis *et al.*, 2020; Al-Khresieh *et al.*, 2023). Material and technical limitations, especially in low-income countries, often interfere with the diagnosis of *Campylobacter*, so it is important to simplify the methods for cultivation and reduction of research costs (Levican and Hinton, 2022; Levican *et al.*, 2023).

Campylobacter species are microaerophiles and capnophiles. For the ability to grow in the range from 37°C up to 42°C, they are classified as thermophiles. Oxidase-positive, non-enzymatic, non-spore-forming, gram-negative microorganisms of a spirally curved shape with “screw-like” mobility. Catalase-positive, which indicates their pathogenic-

ity. Growth on special blood media, containing hemin, protein hydrolysates, and amino acids (Myintzaw *et al.*, 2023; Soto-Beltrán *et al.*, 2023).

Despite rapid detection methods such as Enzyme-Linked Immunosorbent Assay (ELISA), polymerase chain reaction (PCR) and other, cultural methods are references for isolation of microorganisms and diagnosis (Akkina *et al.*, 2022). In addition, isolation and cultivation are important steps in creating national collections (banks) of isolates for research using modern methods that will appear in the future, as well as for studying the evolution of *Campylobacter*.

Therefore, all are selected for further research isolates were subjected to cryopreservation. This will preserve the genetic material of *Campylobacter* spp. for prospective studies on genomic surveillance of *Campylobacter* infections, sequencing whole genome (WGS), and harmonization of monitoring of antimicrobial resistance in animals, which will contribute development of surveillance measures for *Campylobacter* spp. in the concept of “One Health”, aimed at biosecurity, disease prevention and control.

The aim of our study was to test, compare, and evaluate two basic cultural methods for isolating *Campylobacter* spp. from animal and poultry intestine samples for their subsequent implementation in the diagnostic practice of laboratories in Ukraine.

MATERIALS AND METHODS

Experimental studies were carried out on the basis of research bacteriological Department of the State Scientific Research Institute for Laboratory Diagnostics and Veterinary and Sanitary examination (SSRILDVSE) Kyiv, Ukraine.

SELECTION OF SAMPLES

Samples of blind processes were randomly selected within the framework of the “Planned initiative and search research work on the assessment of the degree of spread of antibiotic resistance of pathogens of bacterial zoonoses in Ukraine”. State registration No. 0118U100599. All selected samples representative.

From May to September 2023, by random sampling at slaughterhouses in Vinnytsia, in the Volyn, Kyiv, and Cherkasy regions, samples of content cecal appendages of the intestine were selected from broiler chickens ($n = 76$), turkeys ($n = 30$), that came from 6 poultry farms; samples cecal contents of pigs ($n = 20$) from one pig farm and cattle ($n = 12$) from one livestock farm. The sample included 8 farms represented by three clusters: in the center - Cherkasy and Vinnytsia, in the West - Volyn and in the North - Kyiv region.

Poultry intestines after slaughter were collected in plastic bags, and the contents of caecums of pigs and cattle - in

plastic containers. The samples were placed in thermal boxes with cold-gens and transported to laboratories. Samples were examined on the day of delivery to the laboratory.

METHODS OF ISOLATION

Nutrient media, components for nutrient media, and selective additives manufactured by HiMedia were used in the work, India. Environment CASA and Columbia agar produced by BioMerieux, France. Cefazolin and Rifampicin manufactured by Borshchagiv Chemical and Pharmaceutical Plant PJSC, Ukraine. Sodium bicarbonate produced in Turkey, citric acid produced in China, and culture of *Pseudomonas aeruginosa* ATCC 27853. Lysed horse blood was prepared in the laboratory: defibrinated horse blood was diluted in a 1:1 ratio with sterile deionized water. It was frozen at -20°C Vestfrost VD 285 FN (Turkey), kept for 8 hours, then thawed at room temperature, refrozen, thawed again, and repeated until complete lysis of blood cells occurred, followed by centrifugation BIOSAN LMS 3000 (Latvia). Lysed horse blood was kept for up to 6 months at a temperature of -20°C .

The growth properties of nutrient media were checked using test cultures *Campylobacter jejuni* ATCC 33291 and *Campylobacter coli* ATCC 33559 obtained from the Museum of Test cultures of microorganisms SSRILDVSE, previously restored and checked for compliance main typical properties.

METHOD NO. 1- DETECTION OF *CAMPYLOBACTER* SPP. BY DIRECT PLATING

The principle of the method is to study samples with a high content of *Campylobacter* spp. And accompanying microflora by direct application to a dense selective medium, bypassing the stage enrichment. This allows for the reduction of the influence of accompanying microflora on the isolation of *Campylobacter* spp. The method is defined by the ISO 10272-1:2017(E) standard.

We used Detection Procedure C as the basis for our method and employed Blood-Free *Campylobacter* Selectivity Agar Base – mCCD agar (M887) with the addition of *Campylobacter* Supplement V (BFCSA) (FD067) containing cefoperazone. At the same time, seeds were planted for food a medium that differs in its composition from mCCD agar. The nutrient substrate was *Campylobacter* Agar Base (M994) with the addition of Yeast Extract Powder (RM027), lysed horse blood, and antibiotics (Cefazolin and Rifampicin), which recommended in Basic Laboratory Procedures in Clinical Bacteriology (Vandepitte *et al.*, 2003).

The preparation of blind appendages for the study was carried out according to the European Union protocol Reference Laboratories (EURL) for *Campylobacter* (EURL-Campylobacter, 2022).

The contents of blind appendages were applied directly to mCCD agar with a bacteriological loop, with a volume of 10 µl, in parallel on Campylobacter Agar. With another loop, a previously applied drop was scattered with a stroke of caecum that can be accommodated over the entire surface of the medium so that the sowing strokes are from the edge to edges of the cup, to obtain clearly isolated colonies. Cultivated in an exsiccator under microaerobic conditions, created using a burning candle, a mixture of sodium bicarbonate with citric acid in a 1:1 ratio, and a cup of Columbia agar seeded with *Pseudomonas aeruginosa* culture was placed at the bottom of the exsiccator. This allowed us to increase the carbon dioxide CO₂ content and decrease the oxygen O₂ concentration. Cultivated at a temperature of 41.5°C in the incubator JOUAN SA EB 115 (France) for 48 hours. In 24 hours checked for the presence of growth and, upon detection, selected from each medium characteristic for *Campylobacter* spp. colony, performed microscopy followed by checking for compliance with the main typical properties. In case of absence of growth, cultivation was continued, restoring microaerobic conditions. If no growth was observed, microaerophilic conditions were reinstated, and cultivation was extended for up to 48 hours.

METHOD NO. 2- DETECTION OF *CAMPYLOBACTER* SPP. BY ENRICHMENT

At the same time, the material was sown by the enrichment method according to DSTU ISO 10272-1:2007. The principle of the method is based on the accumulation of *Campylobacter* spp. in samples with a low level. Previous period incubation (resuscitation) lasts 4 - 6 hours at a temperature of 37°C before increasing to 41.5°C and with subsequent cultivation for up to 24 - 48 hours.

The technique consists in adding 1 g of cecum contents to 9 ml of Bolton Broth Base (M1592) with the addition of 5% horse blood lysed and Bolton Selective Supplement (FD231) containing Amphotericin B, Vancomycin hydrochloride, and Cefoperazone sodium. Cultivation was carried out according to the scheme described above. Isolation was done on mCCD agar and Campylobacter Agar. Cultivated at a temperature of 41.5°C for 24 - 48 hours. At the end of the cultivation, we performed a check for morphological type by microscopic examination of the characteristic colonies and conducted testing for biochemical and growth properties.

PURIFICATION OF CULTURES

Pure cultures of microorganisms are necessary to establish their morphological and biochemical characteristics, as well as to determine the species affiliation of bacteria. To isolate microorganisms in a pure culture, their complete isolation from other species is required.

After obtaining characteristic colonies on dense selective nutrient media, microbiological preparations were pre-

pared, and stained according to Gram, and purity was determined by microscopy colonies (homogeneity, homotypicity, and monotinctoriality). When other types of microorganisms besides *Campylobacter* were detected, the culture was purified by the method of chromogenic screening CASA medium and cultivated at a temperature of 41.5°C for 48-96 hours.

IDENTIFICATION OF *CAMPYLOBACTER*

A VITEK analyzer with Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) technology, manufactured by BioMerieux, France, was used to identify the selected isolates to the species level. The method is based on the extraction of proteins and peptides from bacterial cells with subsequent identification based on the principle of molecular "fingerprint".

Isolates were sown on Columbia agar to obtain a satisfactory spectral output. Cultivated for 48 hours at a temperature of 41.5°C. Calibration of the mass spectrometer was carried out using the standard species mass spectrum of *Escherichia coli* ATCC 25922. Cultures from Columbia agar were applied to spots of the target slide and covered on top with 1 µl of the matrix for MALDI-TOF MS. After crystallization of the samples, the target slide with the samples was placed in the mass spectrometer chamber where desorption/ionization took place. Ionized proteins under the influence of an electric field move away from the source of ionization to the detector with an acceleration inversely proportional to their atomic masses. Using the software MYLA v. 4.7 (BioMerieux, France) carried out a procedure for comparing the protein of the studied analyte with standard molecular weights contained in the VITEK MS database.

CRYOPRESERVATION

For further testing and research of selected *Campylobacter* species, their storage in a cryogenic state in cryogenic tubes (CRYO - BEADS) (BioMerieux, France) was used at a temperature of -70.0 ± 5.0°C in the low-temperature freezer "Arctiko LF 100" (Denmark).

Bacterial suspension of *Campylobacter* was prepared by introducing colonies of 48-hour culture from Columbia agar into the Nutrient Broth (M002) medium with a bacteriological loop. Thoroughly mixed to homogeneity and density was adjusted by adding nutrient broth Nutrient Broth (M002) or bacterial mass up to 4 according to R092E McFarland standard to increase the reproduction percentage of *Campylobacter* isolates. Following aseptic conditions, the prepared *Campylobacter* suspension was introduced into test tubes (CRYO - BEADS). The tube was closed and secured uniform distribution of the bacterial suspension. Using a pipette, the solution was removed from the test tube above the cryogranules, the tube was closed with a lid and placed in a tripod, which was placed in the low-temperature refrigerator for storage at a temperature of -70.0 ± 5.0°C.

ENRICHMENT METHOD

To compare the effectiveness of methods for isolating thermophilic *Campylobacter* spp., both direct plating and an enrichment method with reduced cultivation time in Bolton Broth (to 24 hours) were applied to samples from 30 broiler chickens and 20 pigs.

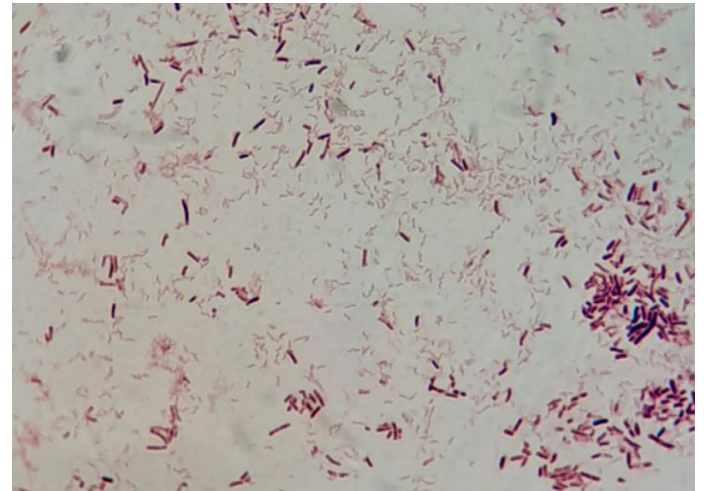


Figure 1: *Campylobacter* spp. in mixed culture. Gram-negative, thin, spirally curved bacteria that have one complete curl or resemble a 'comma' or 'gull wings'. Gram stain. View under a Leica DM 5000 light microscope (immersion objective 100).

Samples were collected from broiler chickens, turkeys, pigs, and cattle from different regions with the aim of detecting the spread of *Campylobacter* spp. in Ukraine, investigating the species circulation of the pathogen, and analyzing the sensitivity of the isolation methods of *Campylobacter* spp. regarding various species of animals and poultry. The isolation of the pathogen, along with the sensitivity of the methods used, is influenced by the peculiarities of the digestive system, the composition of the intestinal microbiota, the maintenance and diet of the feed, the geographical location of farms, and seasonality.

During the work, two microbiological methods of isolation of *Campylobacter* spp were tested from samples of contents of caecum/cecum: detection of *Campylobacter* by direct plating and detection of *Campylobacter* by enrichment. These two methods are developed and described by the International Organization for Standardization (ISO 10272-1:2017(E) and State Consumer Standard of Ukraine (DSTU ISO 10272-1:2007). We performed parallel isolation on solid nutrient media with varying compositions. Also, the cultivation time at the enrichment stage in Bolton Broth Base according to DSTU ISO 10272-1:2007 was reduced and materials prepared from components were used.

DIRECT APPLICATION METHOD

The direct plating method was applied to all samples (n=138). After incubation, the cultures were examined for the presence of *Campylobacter* spp. The growth of colonies of regular round shape with even edges, shiny convex surface, grayish color with a metallic sheen on mCCD agar and flat with uneven edges of cream-colored colonies with a specific smell of "melted milk" and the ability to fuse on *Campylobacter* Agar was established. Microscopic examination of Gram-stained microbiological preparations revealed Gram-negative, thin, spiral-shaped bacteria with a single complete turn, resembling "comma" or "seagull wings," consistent with the descriptions. (Perez-Perez and Blaser, 1996; Silva *et al.*, 2011; Ammar *et al.*, 2021). Such samples were preliminarily considered positive for *Campylobacter*, even with mixed cultures (Figure 1). After purification of cultures, efficiency of removal of isolates of *Campylobacter* spp. from the accompanying microflora were confirmed visually (all colonies on CASA agar had a brick-red color with a darkened center) and morphologically (bent S-shaped rods were found in smears). All tested isolates showed a positive test for catalase and oxidase.

As a result of using the direct application method, 11 isolates of *Campylobacter* spp. were isolated from pigs, 19 from broilers, 1 isolate from cattle, and 1 isolate from turkey. Among the isolated *Campylobacter* spp. 5 isolates were free from accompanying intestinal microflora, 4 of them from broilers and 1 from cattle.

As a result of using the enrichment method, 24 isolates of *Campylobacter* spp. were isolated, of which 11 isolates were from pigs and 13 from broilers (Table 2).

Using the direct plating method, 12 isolates were obtained from broilers and 11 from pigs (Table 1), indicating the sensitivity of the methods.

Among the selected isolates, free from accompanying microflora were 6 *Campylobacter* spp. isolated from pigs and 11 from broilers. We found all these isolates in cultures on plates with *Campylobacter* Agar Base, cultivated for 24 hours (Table 2). Therefore, we note that *Campylobacter* Agar Base in combination with Bolton Broth Base works as an elective medium on which thermophilic *Campylobacter* spp. grow faster and more intensively, ahead of other types of bacteria of the intestinal microbiota. Our results are consistent with those obtained by (Sakur *et al.*, 2024), who used Bolton Broth, which contains components that accelerate the growth of *Campylobacter* by providing an additional energy source, while the blood content maintains the microaerophilic environment. This explains the improved growth of *Campylobacter* in media with added blood.

OBTAINING PURE CULTURES OF *CAMPYLOBACTER* SPP

As an alternative to the CASA chromogenic medium, to reduce the level of accompanying microflora, we performed purification by the method of screening on selective dense

Table 1: The results of isolation of Campylobacter spp. by the direct application method.

Methods	Environment	Cultivation time	Broilers		Pigs	
			Growth is noted Campylobacter spp. / Number of tested samples	Pure cultures / from detected ones	Growth is noted Campylobacter spp. / Number of tested samples	Pure cultures / from detected ones
Direct (sowing in parallel on two mediums)	mCCD agar.	cultivation	12/30 40/100	4/12 13.3/40	11/20 55/100	- -
	Campylobacter gar	24 hours	10/30 33.3/100	1/10 3.3/33.3	9/20 45/100	- -
Isolates were isolated using the direct application method / Pure cultures			12 / 4 11 / -			

Table 2: The results of isolation of Campylobacter spp. according to the enrichment method.

Methods	Environment	Cultivation time	Broilers		Pigs	
			Growth is noted Campylobacter spp. / Number of tested samples	Pure cultures / from detected ones	Growth is noted Campylobacter spp. / Number of tested samples	Pure cultures / from detected ones
Enrichment (Broth cultivation for 4 hours at 37°C and 24 hours at 41.5°C. Isolation in parallel for two environments)	mCCD agar	cultivation 24	5/30 16.7/100	3/5 10/16.7	4/20 20/100	1/4 5/20
	Campylobacter agar	hours	13/30 43.3/100	11/13 36.6/43.3	11/20 55/100	6/11 30/55
Isolates were selected for the reduction of cultivation time / Pure cultures			13 / 11 11 / 6			

nutrient medium mCCD agar. A single typical colony was transferred onto agar, streaked across the entire surface of the medium, and the loop was then transferred to another dish, repeating this process several times. In the last dish, clearly isolated colonies were obtained. Microscopy was performed followed by checking for compliance with the main typical properties.

IDENTIFICATION OF *CAMPYLOBACTER*

Traditional biochemical tests are least suitable for the identification of *Campylobacter* species, since these bacteria use amino acids instead of carbohydrates during metabolism (Huang and Garcia, 2022). Molecular methods are more expensive and take longer. The MALDI-TOF MS method allows for direct protein profiling without purification of individual proteins and obtaining unique mass spectra with high reliability, which characterize the analyzed the product of research according to the principle of molecular “fingerprint”. The simplicity of sample preparation and the speed of obtaining reliable results make this method highly productive for clear identification at the species level (Kyryk, 2017b).

To determine *Campylobacter* species, typical colonies from selective dense nutrient media were plated on Columbia agar. After cultivation for 48 hours at a temperature of 41.5°C the colonies researched on a VITEK MS analyzer with MALDI-TOF technology. Among the isolates isolated from broiler chickens, 14 were identified as *Campylobacter coli*, 6 – *Campylobacter jejuni*. All 11 isolates from pigs – *Campylobacter coli*, from cattle and turkey – 2 isolates of *Campylobacter jejuni*. We obtained 70% of isolates identified as *Campylobacter coli* from broilers, which indicates the specificity of the circulation of this species among poultry in these farms. Recently, *Campylobacter coli*, which is more resistant to antibiotics, has been increasingly identified in poultry (Zhang *et al.*, 2022; Schreyer *et al.*, 2022). Since chicken is considered a primary natural source of campylobacteriosis, the spread of antibiotic resistance poses a significant public health issue. The detection of aerotolerant *Campylobacter coli*, which colonizes poultry and displaces other *Campylobacter* species, contributes to the microorganism’s survival in the environment and potential transmission between hosts, thereby increasing its virulence (O’Kane and Connerton, 2017; Guk *et al.*, 2019).

Thus, the high prevalence of *Campylobacter* isolation in poultry and the results obtained necessitate further research in Ukraine.

According to research by scientists, the accuracy of MALDI-TOF mass spectrometry for the identification of isolates of all *Campylobacter* species reached 100% and only 99.4% for the *Campylobacter jejuni* species, compared to real-time PCR (Bessède *et al.*, 2011). This shows that the

method is accurate and reliable for the identification of *Campylobacter* species. A more comprehensive analysis, including the determination of genotypic characteristics, is needed to validate the results regarding identification.

CRYOPRESERVATION OF *CAMPYLOBACTER* AND RESTORATION OF METABOLIC PROCESSES FROM THE CRYOGENIC STATE

Conservation of *Campylobacter* spp. is an important procedure for diagnostic and research work. *Campylobacter* spp. picky about the atmosphere of cultivation, nutrient media, have a long incubation time with a narrow temperature range of growth. Mostly after a short time after isolation, under the influence of light, atmospheric oxygen, desiccation and temperature changes, *Campylobacter* lose their viability, acquiring a non-cultivable cocciform form (Lv *et al.*, 2020). In order to preserve the selected isolates in a state as close as possible to their original form and to restore them quickly after long-term storage, we resorted to cryopreservation. CRYOBANK *Campylobacter* spp. created for the purpose of preserving *Campylobacter* material with the prospect of future research for in-depth study of their phenotypic and genotypic characteristics, genomic surveillance of *Campylobacter* infections, advanced molecular methods and for harmonization of antimicrobial resistance monitoring in animals. For the operation of CRYOBANK *Campylobacter* spp., a freezer with a temperature range of $-70.0 \pm 5.0^{\circ}\text{C}$ was used.

Currently, there is no universal approach to the conservation of microorganisms of different species due to their varying properties (sensitivity to temperature, pH, humidity, and medium composition). Therefore, a method must be tailored to the microorganism, considering the optimal temperature conditions and medium to ensure rapid and reliable recovery of metabolic processes (Nursofiah *et al.*, 2021; Al-Khresieh *et al.*, 2023). Alongside the use of various cryoprotectants (Guo *et al.*, 2020), the thawing procedure also plays a crucial role in recovery. We conducted cryopreservation according to the instructions for using cryogenic tubes (CRYO – BEADS).

The overall technology for cryobank deposition involved:

- Isolating the pathogen from native material;
- Recording informative data in a log (animal species from which *Campylobacter* was isolated, region, geocoordinates, isolate characteristics, storage location);
- Preparing duplicate samples;
- Storing in cryogenic tubes and placing them in a low-temperature chamber.

After 12 months of cryopreservation, 5 isolates of *Campylobacter* isolated from broiler chickens underwent recovery of their metabolic processes from the cryogenic state ac-

according to detection Procedure A according to ISO 10272-1:2017(E). Since the scheme includes a preliminary period of incubation (resuscitation), which promotes the recovery of cells damaged by freezing, we took it as a basis. According to Hunt *et al.* (2021), the enrichment stage may improve recovery. The aim was to obtain a higher percentage of culture reproduction by applying three variations with cultivation time at different stages of the enrichment method. In our opinion, we have discovered the optimal method for thawing *Campylobacter* isolates and their reproduction.

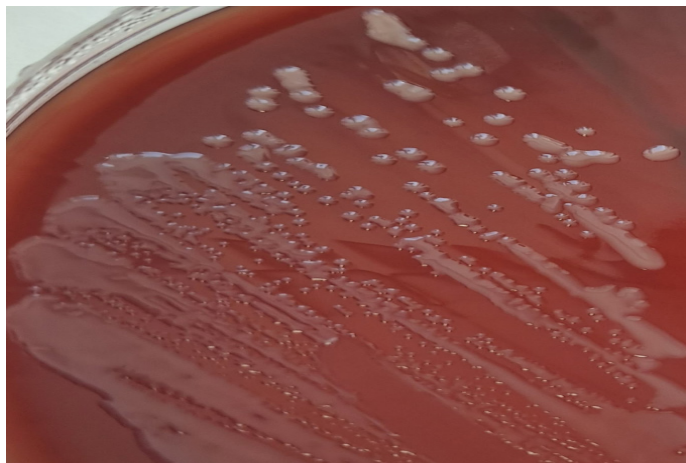


Figure 2: Cream colonies of *Campylobacter* spp. from a round shape with a shiny convex surface to the formation of a plaque on the surface of the medium with the effect of “swarming”. *Campylobacter* Agar, 24-hour cultivation.

After transferring the cryogranules with culture into test tubes with Bolton Broth Base, they were subjected to 4-hour cultivation in microaerobic conditions at a temperature of 37°C. After incubation:

- Made seeds on dense nutrient media mCCD agar and *Campylobacter* Agar Base. Cultivation was continued for another 24 hours in microaerobic conditions already at a temperature of 41.5°C.
- Cultivation in Bolton Broth Base was continued for another 24 hours at a temperature of 41.5°C. After that, *Campylobacter* was isolated on mCCD agar and *Campylobacter* Agar Base and cultivated for 24 hours at a temperature of 41.5°C.
- Continued to be cultivated in Bolton Broth Base for another 48 hours at a temperature of 41.5°C. After enrichment in Bolton Broth Base, *Campylobacter* was isolated on mCCD agar and *Campylobacter* Agar Base and cultivated for 48 hours at a temperature of 41.5°C.

Cultivation scheme A - contributed to the moderate growth of *Campylobacter*, or even its absence. After growing at a temperature of 41.5°C for another 24 hours in microaerobic conditions, additional growth of *Campylobacter* was not observed. In our opinion, the reason for this is insufficient cultivation time in the selective broth for the accumulation

of biomass of microorganisms, which led to the absence or single growth of colonies on selective agars.

The analysis of the obtained data demonstrated the abundant growth of *Campylobacter* after cultivation by method B (Figure 2 and 3).

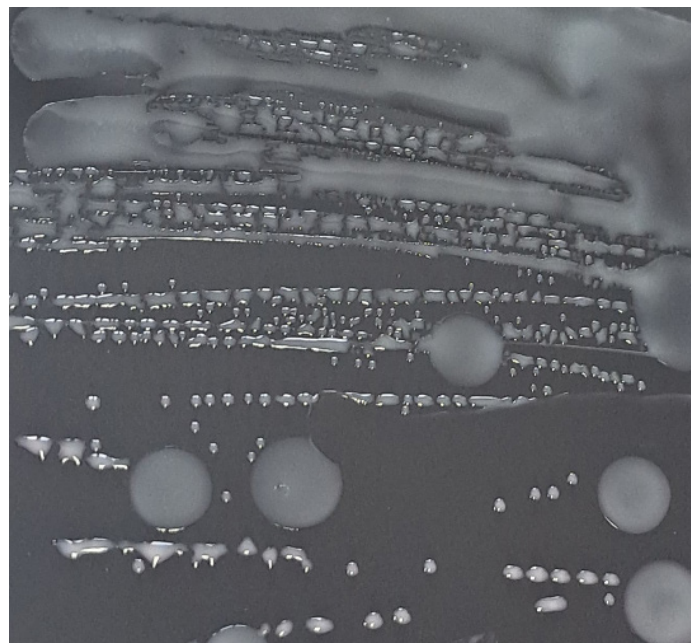


Figure 3: Greyish colonies of *Campylobacter* spp. on mCCD agar from rounded shape with metallic sheen to formation of plaque on the surface of the medium with a “swarming” effect, 24-hour cultivation.

After cultivation by method C, cocci-like forms of *Campylobacter* were detected during colony microscopy (Figure 4) (Kim *et al.*, 2021; Santos *et al.*, 2023). This cryogenic recovery option is not suitable for *Campylobacter* and will not be considered for further use in laboratory practice.

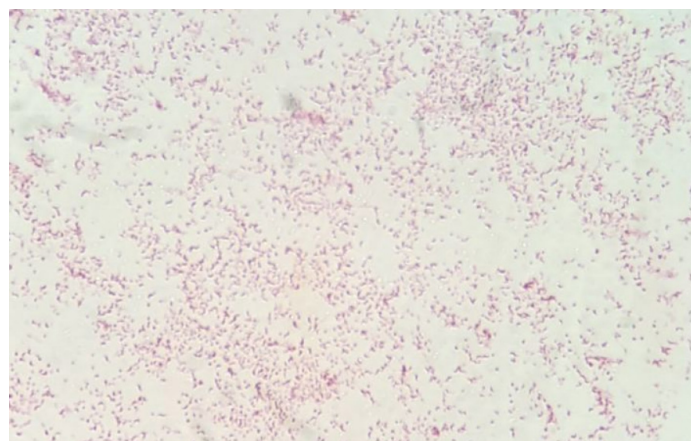


Figure 4: The cocci-like form of *Campylobacter* spp. Fuchsin staining. View under a Leica DM 5000 light microscope (immersion objective 100).

Therefore, the research results indicate that for the recovery of metabolic processes of *Campylobacter* from the cryogenic

state, the optimal method is a method with a reduction of the cultivation time to 24 hours at the stages of enrichment and isolation (option B). For samples that have been exposed to stress factors or contain likely low amounts of *Campylobacter*, this option is best suited for isolating isolates. Also, in the absence of chromogenic media, option B can be used to purify isolated *Campylobacter* from accompanying microflora.

Therefore, to effectively restore the metabolic processes of *Campylobacter* from cryogenic storage, an optimal method would be to limit cultivation time to 24 hours during both enrichment and isolation stages.

COMPARISON OF THE EFFICIENCY OF TWO METHODS OF ISOLATION OF *CAMPYLOBACTER* ACCORDING TO APPROBATION PROTOCOLS

At this stage, a comparative evaluation of two *Campylobacter* isolation methods on two different media (mCCD agar and Campylobacter agar) was performed, along with an optimization of the isolation techniques. The results from using the direct application method for *Campylobacter* are presented in Table 1.

Data from Table 1 show that the direct plating method enabled us to detect *Campylobacter* colonies on selective agar within 24 hours and to determine the morphological type and purity of the culture through microscopy.

As a result, we obtained 12 *Campylobacter* isolates from broilers and 11 from pigs on mCCD agar, with a 13.3% rate of pure culture isolation, indicating the selectivity of the mCCD agar medium.

Using the enrichment method with reduced cultivation time, we obtained pure cultures, 11 from broilers and 6 from pigs (Table 2).

The data in Table 2 indicate that using the enrichment method, we obtained one additional isolate from broilers and a total of 17 pure *Campylobacter* cultures, which represents 51.5%.

In both methods, two selective nutrient media, which are opposite in their composition, were used for the isolation of *Campylobacter* - mCCD agar and Campylobacter Agar. These environments are recommended by the International Organization for Standardization and The World Health Organization, used in the work of the European Union reference laboratory (EURL) for *Campylobacter* and European laboratories of clinical bacteriology. The media are available on the Ukrainian market, so they can be used in diagnostic studies on the isolation of *Campylobacter*.

With direct plating, both media performed almost equally in terms of the number of positive samples detected. The

identification numbers of the positive samples coincided, indicating the growth qualities of the media.

Pure cultures from broilers were detected on mCCD agar 4 and on Campylobacter agar 1. However, pure cultures were not detected in samples from pigs on either medium, indicating the specificity of the intestinal microbiota, which depends on factors such as feeding ration, housing conditions, age of the animals, and breed (Hu *et al.*, 2023). As a result of purifying mixed cultures isolated by the direct method, 12 *Campylobacter* isolates were obtained from broilers, and 11 from pigs.

Using the enrichment method on mCCD agar, 16.7% of samples from broilers and 20% from pigs tested positive.

On the contrary, the Campylobacter Agar medium by the enrichment method not only increased its sensitivity to the isolation of *Campylobacter* (from broilers - 43.3%, from pigs - 55%), but also performed the function of purifying isolates from extraneous microflora (36.6% from broilers and 30% from pigs). The content of horse blood lysed in selective broth and in Campylobacter Agar protects *Campylobacter* from toxic oxygen radicals and accelerates its growth by providing nutrients (vitamins, minerals, triglycerides, amino acids, lipids). The Campylobacter Agar medium, combined with Bolton Broth, functions as a selective medium on which thermophilic *Campylobacter* spp. grow faster than other bacterial species, facilitating the isolation of pure *Campylobacter* isolates.

Therefore, to detect *Campylobacter* spp. in samples from animals and poultry, the direct application method with reduced incubation time and the enrichment method with reduced cultivation time at the "enrichment" and isolation stages are acceptable. By reducing the cultivation time, we contributed to the timely detection of *Campylobacter* spp. and preservation of culture from the influence of accompanying microflora. The combination of two media, Bolton Broth and Campylobacter Agar, worked as a selective medium, resulting in purified isolates of *Campylobacter* spp.

As a result, we obtained 33 isolates of *Campylobacter*, which is 23.9% of the total number of studied samples, of which 32 isolates were isolated by the direct application method. Using the enrichment method, we confirmed the positive samples (identification numbers matched) and obtained an additional, twentieth, isolate from broilers. A total of 20 isolates were isolated from broilers, which is 26.3%, from turkeys - 1, which is 3.3%, from pigs - 11, which is 55%, and from cattle - 1, which is 8.3%.

From the analysis of our conducted research, it should be noted that the percentage of positive samples for *Campylobacter* from poultry was higher in September (43.3%) than

in May (15.2%). It is known that the prevalence of *Campylobacter* in poultry is influenced by seasonality, the reasons for which are still not fully understood (Sibanda *et al.*, 2018).

ECONOMIC ASPECT OF RESEARCH

In this study, we improved the detection of *Campylobacter* from animal and poultry samples by reducing the cultivation time during the enrichment stage and using *Campylobacter* Agar with added Cefazolin and Rifampicin as selective additives. The addition of antibiotics significantly reduced the growth of accompanying microflora and increased the detection of *Campylobacter*. Results from Kim *et al.* (2016) demonstrated improved isolation efficiency of *Campylobacter* from wastewater with the additional use of Rifampicin. Reducing cultivation time during the enrichment stage yielded better results compared to (Kim *et al.*, 2019), who combined sample-to-Bolton broth ratios and used various selective agars for isolation.

To minimize the economic burden of pathogen detection and identification, we optimized the research protocols. It was found that for the direct plating method, with a positive probability according to ISO 10272-1:2017 and considering material costs (commercially available gas-generating packages GENbox microaer, selective additives, lysed horse blood), labor costs, utility expenses, equipment depreciation, etc., the cost of the study is approximately \$35. According to our research protocol (using laboratory-prepared materials), the cost with all expenses is \$17.

On the other hand, for the enrichment method as per ISO 10272-1:2017, with all expenses included, the cost is \$43. According to our research protocol (with reduced cultivation time and using laboratory-prepared materials), the cost for obtaining pure *Campylobacter* cultures is \$22.

The enrichment method's advantage lies in obtaining pure cultures and recovering isolates after long-term storage at low temperatures or freeze-drying. Enrichment broth accelerates *Campylobacter* growth, provides additional energy sources, and accumulates biomass on selective agars for further studies. The direct plating method is suitable for isolating *Campylobacter* from animal and poultry intestine samples, yielding results within 24 hours.

CONCLUSIONS AND RECOMMENDATIONS

As a result of the research, despite the current shortcomings in *Campylobacteriosis* studies in Ukraine for both humans and animals, it was found that *Campylobacter*, as a commensal of the intestines of animals and poultry, is widespread in Ukraine. The infection rates were determined to be 26.3% in broilers, 3.33% in turkeys, 55% in pigs, and 8.33% in cattle. We isolated 33 *Campylobacter* strains, which constitutes

23.9% of the total samples studied. According to MALDI-TOF-MS identification, the isolated strains were classified into two thermophilic species: *Campylobacter coli* (75.8%) and *Campylobacter jejuni* (24.2%), with 70% of poultry isolates identified as *Campylobacter coli*.

It was established that the direct plating method with reduced cultivation time facilitated timely detection of *Campylobacter* in samples, preserved cultures from contamination by other microorganisms, and decreased laboratory diagnostic time. Over 97% of isolates were obtained using the direct plating method, with 15.6% being pure cultures. The enrichment method with reduced cultivation time during the enrichment and isolation stages yielded 72.7% of *Campylobacter* spp. isolates, with 51.5% being pure cultures, and there was a match in identification numbers for positive samples by both methods.

Additionally, a CRYOBANK for *Campylobacter* spp. has been established at the laboratory to support the preservation of genetic material and the study of foodborne pathogens, their properties, adaptation, resistance, and variability within the "One Health" framework. It was determined that to restore metabolic processes of *Campylobacter* from the cryogenic state, the optimal method involves reducing cultivation time to 24 hours during enrichment and isolation stages.

Thus, the validation of isolation protocols for *Campylobacter* has simplified the research, reduced the cost of testing by 50%, and decreased the laboratory workload. We believe that the developed protocols for direct plating and enrichment methods for routine *Campylobacter* detection in laboratories will enhance the effectiveness of *Campylobacteriosis* diagnosis and identification.

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NOVELTY STATEMENTS

For the first time, isolation methods with reduced cultivation time were applied both at the enrichment and isolation stages, which facilitated the timely detection of *Campylobacter* isolates and improved the production of pure cultures. The prevalence of *Campylobacter* spp. among

animals and poultry in Ukraine was found to be 23.9% of infections. Isolates were first identified as *Campylobacter coli* (75.8%) and *Campylobacter jejuni* (24.2%), with 70% of poultry isolates identified as *Campylobacter coli*. For the first time in Ukraine, a *Campylobacter* spp. CRYOBANK was created to preserve cultures for further research using new methods to be developed. The cost-effectiveness of the tested methods was calculated, which showed a 50% reduction in costs and a reduction in the workload of the laboratory.

AUTHOR'S CONTRIBUTIONS

All authors contributed equally to the manuscript.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

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