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ORIGINAL PAPER

Presence of Antibodies Against *Leptospira interrogans* Serovar *hardjo* in Serum Samples from Cattle in Ukraine

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Abstract

The article presents data on serological studies of 573 sera samples of cattle that were collected from the farms affected by leptospirosis in different regions of Ukraine in the period of 2014–2015. Samples were investigated by the microscopic agglutination test (MAT), which was conducted within eight serological groups of *Leptospira* and nine serovars: *Sejroe* (serovars *polonica* and *hardjo*), *Hebdomadis* (serovar *kabura*), *Tarassovi* (serovar *tarassovi*), *Pomona* (serovar *pomona*), *Grippotyphosa* (serovar *grippotyphosa*), *Canicola* (serovar *canicola*), *Icterohaemorrhagiae* (serovar *copenhageni*), and *Australis* (serovar *bratislava*). The circulation of *L. interrogans* serovar *hardjo* among cattle has been observed in all 11 regions of Ukraine investigated within 25.8–60.0% of the leptospirosis-positive serum samples in these regions. Antibodies in the cattle sera against serovar *hardjo* (serogroup *Sejroe*) were detected in 139 of the 370 cows reacting positively in MAT. Overall, they were detected in 24.3% animals out of the total of 573 cows investigated. These are the preliminary results, however, in our opinion, they should allow to include the serovar *hardjo* in a standard panel of strains for MAT in Ukraine.

Key words: antibody, cattle, leptospirosis, microscopic agglutination test, serovar *hardjo*

Introduction

Leptospirosis is a dangerous zoonotic infection with a worldwide distribution that is recognized as an emerging disease (Levett 2001; Sykes et al. 2011). There are no available data about leptospirosis in animals and humans in a number of countries, and thus its global burden remains mostly unknown (Hartskeerl et al. 2011). In general, leptospirosis has been reported in over 150 mammalian species (Ko et al. 2009), but the infectious agent can also be detected in other classes of animals (reptiles, amphibians, etc.) (Levett 2001; Adler and Moctezuma 2010).

To date, there are about 20 species of pathogenic *Leptospira*: *L. kirschneri*, *L. borgpetersenii*, *L. mayottensis*, *L. santarosai*, *L. noguchii*, *L. weilii*, *L. alexanderi*, *L. alstonii* etc. that include serogroup and serovars (Ko et al. 2009). Over 250 pathogenic serovars of *Leptospira* have been recognized (Adler and Moctezuma 2010). Susceptibility to them in species of animals is different. According to Ukrainian and foreign scientific literature, rodents are considered maintenance hosts for leptospires in serogroups *Grippotyphosa* and *Icterohaemorrhagiae*, and dogs are hosts for serogroup *Canicola*. Pigs in most cases are infected by *Pomona*, *Icterohaemorrhagiae*, and *Australis* (serovar *bratislava*). Cattle are the maintenance hosts of serovar *hardjo* (serogroup *Sejroe*) and are often infected by *polonica* (serogroup *Sejroe*) and *kabura* (serogroup *Hebdomadis*) (Levett 2001; Sykes et al. 2011; Ukhovskyi et al. 2014).

In Ukraine, the standard diagnostic panel included all the mentioned above serovars for MAT except serovar *hardjo*.

The wide spectrum of symptoms confuses the clinical diagnosis and makes it unreliable (Sharma et al. 2007). The laboratory diagnosis of leptospirosis in animals, a prerequisite for their treatment, is usually achieved either by isolation of the causative agent with

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further identification (PCR) or by serological analysis revealing the infection (Werts et al. 2001; OIE 2018). Although the serological diagnosis is easier than isolation *Leptospira* by culture from biological specimens it is quite difficult since a wide range of antigens is used to detect infections in different countries where uncommon or previously undetected serovars may occur (Katz et al. 1991). Furthermore, the number of serovars and serogroups constantly increase due to the discovery of new strains, cultured from macroorganisms and the environment (Levett 2001).

Two tests have a role in veterinary diagnosis: the microscopic agglutination test (MAT) and the enzyme-linked immunosorbent assay (ELISA), but serological diagnosis of leptospirosis generally is based on detecting antibodies by MAT which is a referent method for this disease (Sykes et al. 2011; OIE 2018). MAT involves the serial dilutions of patient` sera that react with live *Leptospira* followed by an assessment of organism's agglutination with a dark field microscopy. According to World Organization for Animal Health (OIE) recommendations, a titer 1/100 can be considered as positive, but on practice, it differs from country to country. For example, in Ukraine, it is 1/50. Because of a large amount of *Leptospira* serovars, standard panels for MAT vary in different countries and include generally from five to seven serovars (Sykes et al. 2011). In Ukraine, in accordance with the current instruction the standard panel of *Leptospira* strains for MAT includes eight serovars (*polonica*, *kabura*, *tarassovi*, *pomona*, *grippotyphosa*, *canicola*, *copenhageni*, and *bratislava*).

In addition, leptospirosis may be registered as the incidence of the disease in animals caused by strains that are not included in the standard panel of strains for MAT in certain countries (exotic strains). So, the sensitivity of the assay can be improved by the use of local isolates rather than reference strains, but reference strains assist in the interpretation of results between laboratories (Pinto et al. 2015).

Livestock farming is a major occupational risk factor throughout the world. The highest risk is associated with dairy farming and is linked mainly to serovar *hardjo* (Levett 2001). Nowadays, cattle are the maintenance hosts for this serovar (Ellis et al. 1981; Ryan et al. 2012), and shed *leptospires* both in urine and the discharges from the genital tract (Ryan et al. 2012). The infection with this pathogen occurs worldwide: in Malaysia, Argentina, Chile, India, and the European countries (Myers and Jelambi 1975; Bahaman et al. 1988; Sehgal 2000; Salgado et al. 2015). Ellis et al. reported in 1981 that a combined random survey of both beef and dairy cattle in Northern Ireland resulted in positive antibody titers in 34.7% of the population sampled toward serovar *hardjo* using the MAT assay (Ellis et al. 1981). The serological herd prevalence of serovar *hardjo* in beef herds in England was 72% in 1987 (Pritchard et al. 1987); herd prevalence was 11% among beef herds in 2001 in Spain (Alonso-Andicoberry et al. 2001); and in the USA 42% of suckler herds were infected with *Leptospira hardjo* in 2007 (Wikse et al. 2007).

This strain was isolated in 1938 from a patient in Sumatra, Indonesia, by J.W. Wolff, who called it serovar *hardjo* (Wolff 1953). This serovar was first mentioned in the Wolff and Broom list in 1954 (Wolff and Broom 1954). The strain was subjected to analysis by Kmety in 1977, who based on the results placed it in the subgroup *Wolffi* (Kmety 1977). Since 1999, when the DNA relatedness was determined among *Leptospira* strains, a strain *Hardjoprajitno* serovar *hardjo* belongs to *L. interrogans* spp. (Brenner et al. 1999).

There is no information in the literature about the prevalence of serovar *hardjo* (serogroup *Sejroe*) in animals in Ukraine, and this serovar was not included in the routine panel of strains for MAT in our country. Therefore, the aim of the study was to monitor the circulation of this pathogen among cattle in the farms affected by leptospirosis (previously confirmed cases in cattle by other serovars of *Leptospira*) of 11 regions of Ukraine.

Experimental

Materials and Methods

Cattle sera. Serum samples from 573 cattle were analyzed at the Laboratory of Leptospirosis of the Institute of Veterinary Medicine of National Academy of Agrarian Sciences in Kyiv during 2014–2015. Samples have been selected from the leptospirosis-affected farms (confirmed cases in cattle by other serovars of *Leptospira*) in 11 regions (oblasts) of Ukraine: Khmelnytskyi, Chernihiv, Kyiv, Volyn, Donetsk, Poltava, Kharkiv, Vinnytsia, Odesa, Dnipropetrovsk, and Cherkasy. Samples were collected randomly from cattle in herds where the cases caused by other serovars of *Leptospira* had been confirmed. Serum samples were tested immediately or stored frozen at –20°C. Subsequently, sera were thawed and analyzed for the presence of *Leptospira hardjo* antibodies.

Antigens. Research was conducted with eight reference strains of *Leptospira's* serological groups and eight serovars included in the diagnostic panel for the MAT analysis as the most common causative agents of leptospirosis among animals in Ukraine: *Sejroe* (serovar *polonica*), *Hebdomadis* (serovar *kabura*), *Tarassovi* (serovar *tarassovi*), *Pomona* (serovar *pomona*), *Grippotyphosa* (serovar *grippotyphosa*), *Canicola* (serovar *canicola*), *Icterohaemorrhagiae* (serovar *copenhageni*) and *Australis* (serovar *bratislava*). In addition,

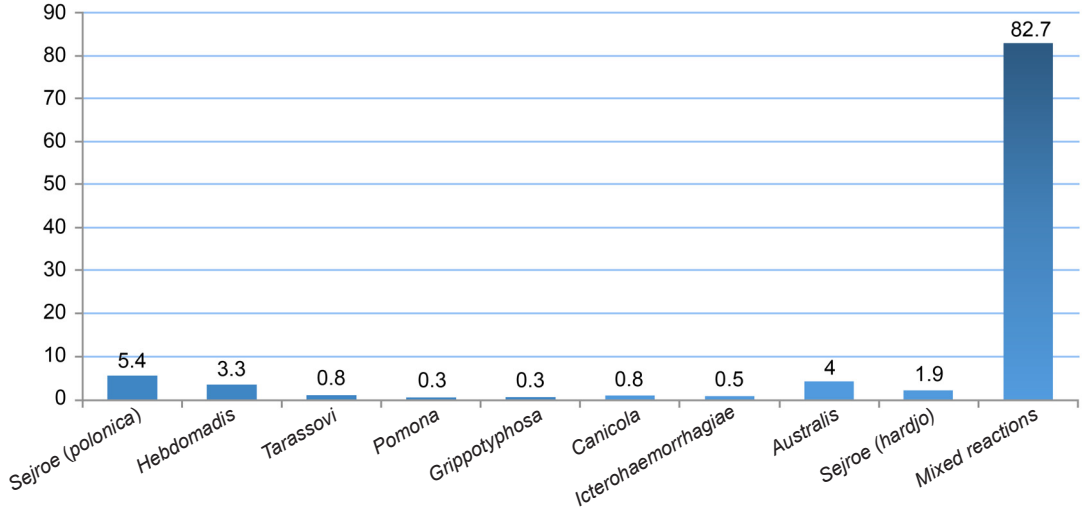

Fig. 1. The etiological structure (%) of *Leptospira* serological groups in the cattle positive sera from the affected farms in different regions of Ukraine (N = 370).

MAT was conducted with serovar *hardjo* (serogroup *Sejroe*). All reference strains were provided by the OIE and National Collaborating Centre for Reference and Research on Leptospirosis (Amsterdam, Netherlands) and cultivated in the Laboratory of Leptospirosis at the Institute of Veterinary Medicine of National Academy of Agrarian Sciences in Kyiv. Each serovar was grown in 10 ml volumes in liquid Korthof medium, incubated at 28–30°C for 6–10 days (depending on the serovar) in aerobic conditions. The concentration of bacteria was approximately $1–2 \times 10^8$ organisms/ml.

Microscopic Agglutination Test. The MAT procedure was carried out according to the Manual of Standards for Diagnostic Tests and Vaccines of the World Organization for Animal Health (OIE). The results were recorded in accordance with the current Ukrainian regulations. The samples of sera diluted 1/25 were mixed with an equal volume of each of the *Leptospira* serovars. Final serum dilution (including the antigen added) 1/50 was used during the preliminary examination. For the positive samples in the preliminary examination that reacted with one or more serovars, the series of two-fold dilutions were prepared to titer endpoint – 50% agglutination. The samples showing titers equal to or higher than 1/50 were recognized as positive.

MAT was conducted using four titers: 1/50, 1/100, 1/500 and 1/2500. The results have been evaluated using a dark field microscope (with magnification × 300).

Results

The etiological structure of leptospirosis in cattle from the affected farms (previously confirmed cases in cattle by other serovars of *Leptospira*) in different regions of Ukraine was studied during 2014–2015. Over the whole period, 573 samples of cattle sera were investigated, and 370 positive reactions have been detected, which constitutes 64.6%.

The etiological structure of MAT-positive *Leptospira* serological groups in cattle is shown in Fig. 1.

Analysis of the data showed that the dominant *Leptospira* serological groups, which were circulating among cattle and recorded as monoreactions, were the follows: *Sejroe* (serovar *polonica*) (5.4%), *Australis* (4.0%), and *Hebdomadis* (3.3%). Other serological groups and serovars were detected in a smaller quantity: *Sejroe* (serovar *hardjo*) in 1.9% of cows, *Tarassovi* and *Canicola* – in 0.8% each, *Icterohaemorrhagiae* – in 0.5%, and *Pomona* and *Grippotyphosa* – in 0.3% each.

Since the antibodies against serovars *hardjo*, *tarassovi*, *canicola*, *copenhageni*, *pomona* and *grippotyphosa* in the serum samples were diagnosed to a lesser extent, one could conclude about their minor role in the etiology of leptospirosis in the cattle studied. However, at the same time, a significant number of mixed reactions (antibodies to more than one serovar) were noticed in 305 cows (82.7% from the total number of the positive-reacting cows). This phenomenon, as shown in Table I, is probably associated with the addition of serovar *hardjo* (serogroup *Sejroe*) to the panel of reference strains used in MAT, because antibodies to this pathogen were recorded in a large percentage of positive mixed reactions (23.2%).

As shown in Table I, 570 positive reactions to different serovars in mixed reactions were observed. The dominant, as previously, were serological groups *Sejroe* (serovar *polonica*) and *Australis* (serovar *bratislava*). They consisted of 24.9% and 18.4%, respectively, of positive mixed reactions for several serogroups of *Leptospira*. Simultaneously, antibodies to *L. interrogans* serovar *hardjo* were diagnosed by MAT almost at the same

Table I
The prevalence of antibodies to different *Leptospira* serogroups in mixed reactions diagnosed with MAT.

Total number of positive reactions to serovars in mixed reactions (%)			570 (100%)
The average number of serovars per mixed reaction			2
Specific combinations of multiple serovars that were commonly seen in samples			Serovars *polonica* and *kabura*, *polonica* and *hardjo*, *hardjo* and *bratislava*
Number of positive reactions in the samples with antibodies against *Leptospira* serogroups	*Sejroe* (serovar *polonica*)	number	142
		%	24.9
	Hebdomadis	number	84
		%	14.7
	Tarassovi	number	31
		%	5.4
	Pomona	number	37
		%	6.5
	Grippotyphosa	number	14
		%	2.5
	Canicola	number	10
		%	1.8
	Icterohaemorrhagiae	number	15
		%	2.6
	Australis (serovar *bratislava*)	number	105
		%	18.4
	Sejroe (serovar *hardjo*)	number	132
		%	23.2

level as *Sejroe* (serovar *polonica*) and accounted for 23.2%. Antibodies to serogroups *Hebdomadis* (14.7%), *Pomona* (6.5%) and *Tarassovi* (5.4%) were noted less frequently. Positive reactions to serological groups *Icterohaemorrhagiae* (2.6%), *Grippotyphosa* (2.5%) and *Canicola* (1.8%) were recorded at lower levels.

For 132 sera a positive reaction with serovar *hardjo* and for 142 with serovar *polonica* was shown out of 305 animal's sera with mixed positive reactions. Of those, 97 samples were obtained from the same animals, therefore it may be assumed that there are cross-reactions between both serovars, which is probably due to their belonging to the same serological group *Sejroe*.

Analyzing the data by regions, it has to be noted that antibodies to these pathogens were registered in a different number of animals. The least difference between rates of infection by these serovars was detected in Cherkasy (positive reactions to serovars *hardjo* and *polonica* were observed, respectively, for four and five cows sera), Poltava (three and five) and Donetsk (one and four) Regions. In Dnipropetrovsk Region, there were no cases of mono- or mixed-positive reaction in which the serovar *hardjo* was diagnosed separately from *polonica*. Antibodies to serovar *polonica* were also noted in four cows and together both pathogens with other serogroups, in three cows. In other regions of Ukraine, from which the serum samples were collected, the rates of infection with these serovars differed significantly from seven (in Volyn Region) to 22 (in Kharkiv Region) cows (Table II).

As shown in Table II, antibodies to serovars *hardjo* and *polonica* (serogroup *Sejroe*), were detected in the cattle from all 11 regions of Ukraine from where the serum samples were collected. Of 370 cows, which sera that reacted positively, 100 (27.0%) cows were infected by both serovars. Positive reactions to *Sejroe* (serovar *hardjo*) and other serogroups (excluding serovar *polonica*) were detected in 39 cows' sera, which accounted for 10.5%. In total, antibodies to *L. interrogans* serovar *hardjo* (serogroup *Serjoe*) were diagnosed in 139 (37.5%) of 370 animals, which sera reacted positively. That shows that a significant portion of leptospirosis observed in this study were caused by this serovar. This pathogen was found in 24.3% of the total number of investigated cattle (139 out of 573 animals).

Figure 2 illustrates the positive reaction to serovar *hardjo* in the serum samples from the MAT-positive cows with leptospirosis in different regions of Ukraine during 2014–2015.

As shown in Fig. 2, antibodies to serovar *hardjo* (serogroup *Sejroe*) were diagnosed by MAT in 25.8% of the infected cattle in Poltava Region. In the cattle

Table II
The indicators of cattle infection with serovars *hardjo* and *polonica* from leptospirosis-affected farms in different regions of Ukraine.

Regions	Number of the cattle investigated	Number of the positively reacting cattle sera (% from total sera investigated)	Number of the positively reacting cattle sera to serovars *hardjo* and *polonica* (%)			Number of the sera positively reacting with leptospirosis antigens that did not react to *hardjo* and *polonica* (% from the positive reacting sera)
			Sejroe (*hardjo*) and other serogroups without *Sejroe* (*polonica*) (% from the positive reacting sera)	*Sejroe* (*polonica*) and other sero-groups without *Sejroe* (*hardjo*) (% from total positive reacting)	Two serovars together and other serogroups (% from the positive reacting sera)	
Khmelnytskyi	38	31 (81.6)	3 (9.7)	13 (41.9)	14 (45.2)	1 (3.2)
Chernihiv	86	41 (47.7)	6 (14.6)	14 (34.1)	9 (22.0)	12 (29.3)
Kyiv	73	45 (61.6)	4 (8.9)	23 (51.1)	13 (28.9)	5 (11.1)
Volyn	33	29 (87.9)	1 (3.4)	8 (27.6)	7 (24.1)	13 (44.9)
Donetsk	12	10 (83.3)	1 (10.0)	4 (40.0)	5 (50.0)	–
Poltava	53	31 (58.5)	3 (9.7)	5 (16.1)	5 (16.1)	18 (58.1)
Kharkiv	106	71 (67)	11 (15.5)	33 (46.5)	26 (36.6)	1 (1.4)
Vinnytsia	65	57 (87.7)	8 (14.0)	18 (31.6)	9 (15.8)	22 (38.6)
Odesa	40	25 (62.5)	2 (8.0)	16 (64.0)	5 (20.0)	2 (8.0)
Dnipro	32	10 (31.3)	–	4 (40.0)	3 (30.0)	3 (30.0)
Cherkasy	35	20 (57.1)	4 (20.0)	5 (25.0)	4 (20.0)	7 (35.0)
Total	**573**	**370 (64.6)**	**39 (10.5)**	**143 (38.7)**	**100 (27.0)**	**88 (23.8)**

from Volyn, Odesa, Vinnytsia, and Dnipro Regions, antibodies to this serovar were found in 27.6–30.0% of cattle. In Chernihiv, Kyiv and Cherkasy Regions they were detected in 36.6–40.0% of animals. The high rate of infection caused by *hardjo* was detected in Kharkiv and Khmelnytskyi Regions. In these areas, the infection

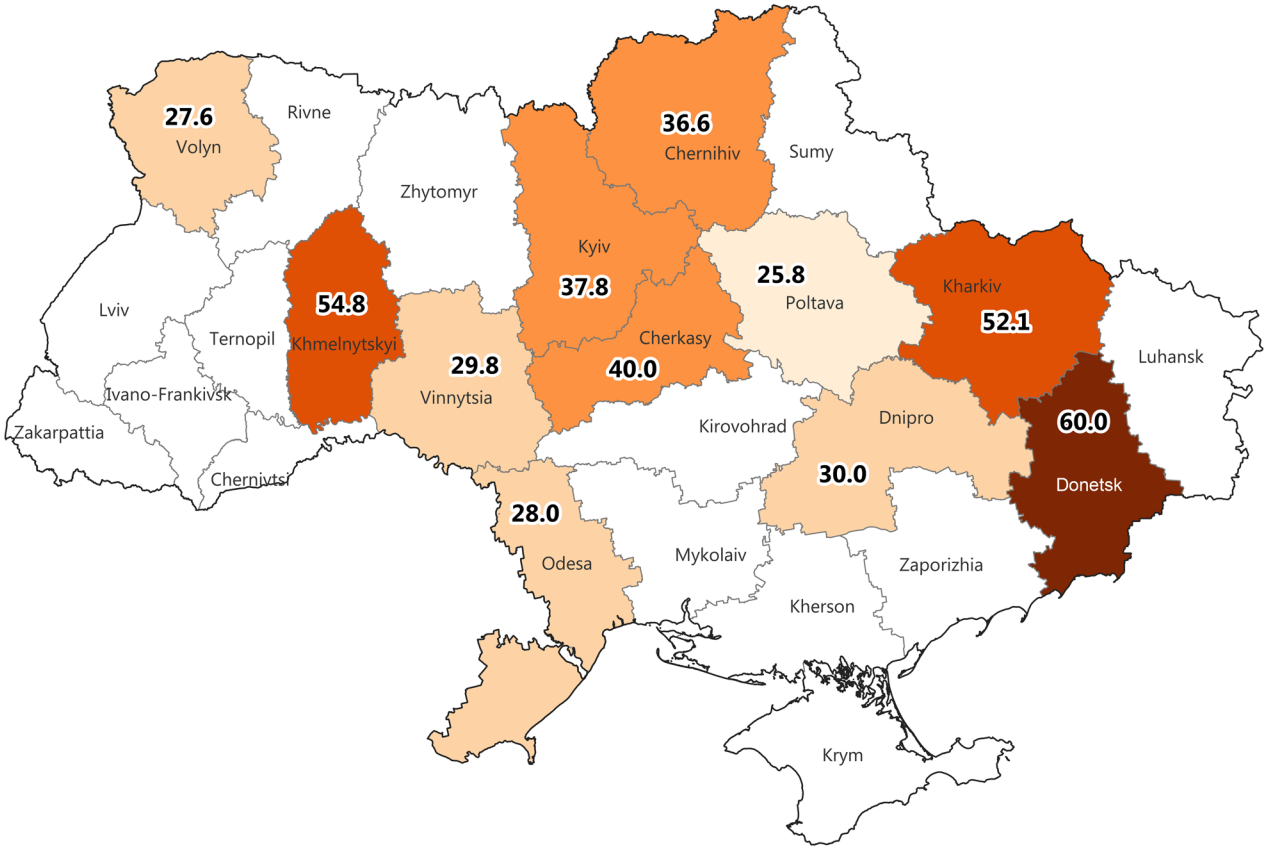

Fig. 2. The percentage of antibodies to serovar *hardjo* among the serum samples from cows with leptospirosis in the regions investigated during 2014–2015 (N = 370).

by *L. interrogans* serovar *hardjo* (serogroup *Sejroe*) was recorded in 52.1–54.8% of the MAT-positive cows. The highest levels of antibodies to this serovar were diagnosed in Donetsk Region and reached 60.0%.

In general, the highest level of antibodies against serovar *hardjo* was in western regions of the country (52.1–60.0%), the lower – in the east (27.6–54.8%) and the north (36.6–40.0%) regions. The lower incidence of positive samples was in the central part of the country (25.8–30.0%) when compared to the east and west regions. In all cases, antibodies to serovar *hardjo* were detected by MAT in titers of 1/50–1/100.

Discussion

Leptospirosis is the most widespread zoonosis worldwide, which is present on all continents except Antarctica and evidence for the carriage of *Leptospira* has been found in virtually all examined mammalian species (Adler and Moctezuma 2010). It is an important zoonotic bacterial infection of livestock that may cause reproductive failure, loss of milk production, economic losses and can result in human infection (Carole and Bolin 2001).

Different species of animals are hosts for various serovars of *Leptospira*. Thus, cattle are maintenance host for serovar *hardjo* (Ellis et al. 1981; Ryan et al. 2012) but the scientific literature from different countries describes cases of infection by this pathogen in other species too (red deer in Italy, brown bears in Croatia, wild boars in Poland, etc.) (Slavica et al. 2010; Andreoli et al. 2014; Żmudzki et al. 2015). The infections with this pathogen are registered at the present time in many countries including Andaman and Nicobar Islands (Sehgal 2000; Sharma et al. 2003; Salgado et al. 2015; Chideroli et al. 2016; Balamurugan et al. 2018; Miyama et al. 2018; Sunder et al. 2018).

Leptospira serovar *hardjo* mainly causes reproductive diseases and failures, such as abortion, mummification, stillbirth, premature and term birth of weak calves, as well as the loss of milk production in dairy herds (Carole and Bolin 2001, Ryan et al. 2012). Moreover, serovar *hardjo* has been isolated from physiologically normal fetuses, the genital tracts of pregnant cattle (Ellis et al. 1982), and vaginal discharge after calving (Levett 2001).

Before our study, there was no information in Ukrainian and foreign literature regarding the circulation of serovar *hardjo* (serogroup *Sejroe*) in animals in Ukraine and this serovar was not included to the standard panel of strains for MAT in our country. In the neighboring countries of eastern Europe, the infections caused by the serovars from the *Sejroe* serogroup were reported in Hungary (Fuzi et al. 1957), Romania (Combiesco et al. 1958.), Moldavia (Matveeva et al. 1977) and Russia (Bondarenko et al. 2002) but these infections were mainly caused by serovar *polonica*. In the south-western region of Poland, during 2010–2011 the seroprevalence of antibodies against serovar *hardjo* was 4.6 and 4.1%, respectively, in herds of 51–100 and 101–500 animals (Rypuła et al. 2014). At the same time, the infections with this serovar occurred among humans in Georgia (Mamuchishvili et al. 2015).

Therefore, we investigated the circulation of this pathogen among cattle from farms affected by leptospirosis and compared the percentage of MAT-positive samples to serovar *hardjo* with a total number of cattle blood sera seropositive to leptospirosis.

The results of our serological studies indicated that infection among cattle caused by *L. interrogans* serovar *hardjo* was detected in 37.5% of the total number of cattle that reacted positively in MAT in the farms affected with leptospirosis in different regions of Ukraine. It is a high number but similar to the numbers reported in other countries such as Northern Ireland in 1981 – 34.7% (Ellis et al. 1981), England in 1987 – 72% (Pritchard et al. 1987), Spain in 2001 – 11% (Alonso-Andicoberry et al. 2001), and the USA in 2007 – 42% (Wikse et al. 2007).

In the majority of the samples investigated, antibodies against serovar *hardjo* were detected together with antibodies to other serogroups of *Leptospira* (mixed reactions) and accounted for 23.2% of these.

Regarding recent cases of leptospirosis caused by serovar *hardjo*, in 2018 the scientists from India published the results that are similar to our results. They investigated 373 cattle serum samples by MAT from 45 farms in 11 states in India. Samples were collected from animals with a history of reproductive disorders like abortion, repeated breeding, anoestrus, and endometritis, and also from apparently healthy animals. The *Leptospira* antibodies against the serovar *hardjo* were shown in 27.76% of cattle (Balamurugan et al. 2018).

Analyzing the results in different regions of Ukraine, least frequently leptospirosis caused by serovar *hardjo* (serogroup *Sejroe*) was detected in Poltava Region, where it was in 25.8% of MAT-positive cattle. The highest number of *hardjo* positive-sera (60%) were registered in the Donetsk Region. In general, infection by *L. interrogans* serovar *hardjo* was detected in all regions of Ukraine, from which samples of sera were received.

Antibodies in titers of 1/50–1/100 can be interpreted as the early stage of infection or chronic leptospirosis. The results obtained in this study that titer of 1/100 or greater should be taken as significant in the cattle infected with serovar *hardjo* were supported by others (Carole and Bolin 2001; OIE 2018).

In our opinion, further research is needed to perform a more meaningful analysis of the epizootic situation of this pathogen in different regions. However,

the data obtained here show that cattle in 11 regions of Ukraine were affected by serovar *hardjo*. Perhaps this serovar has already been circulating in the territory of Ukraine constantly or it entered the country due to the import of animals from other countries. This hypothesis is supported by the fact that serovar *hardjo* is not a component of the typical panel of strains for MAT used to control the sera of imported animals to Ukraine.

For the first time, we investigated the possible circulation of serovar *hardjo* in Ukraine. At the same time, in the world, the highest risk of leptospirosis infection is associated with dairy farming and linked to serovar *hardjo*. In addition, cattle are the maintenance hosts of this pathogen. In our opinion, the results of our research indicate the circulation of this pathogen among cattle in Ukraine. The differences in the detection of positive samples between the regions can be related to the fact that samples were randomly collected. For further improvement of this monitoring and the definition of infection indicators, we need to investigate more samples from each region and cover all territory of Ukraine.

In conclusion, there is a need of conducting more meaningful analysis (to investigate more samples from each region and cover all territory of Ukraine) of the epizootic situation regarding the serovar *hardjo* in different regions of Ukraine and incorporating of this serovar as obligatory into a routine diagnostic panel of *Leptospira* strains used for MAT in the country.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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