UDC 574.34+579.834.115 + 581.573.4

EXPERIMENTAL ECOLOGICAL RESEARCH ON THE RELATIONSHIPS OF PATHOGENIC MICROORGANISMS WITH ALGAE

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Received September 17 , 2019 / Received October 27, 2019 / Accepted November 19, 2019

Aim. The ecological relationships between *Leptospira interrogans* spirochetes and *Chlamydomonas proteus* algae and the response explicitness of individual serological types of leptospires to the allelopathic effect of algae were to be investigated during the experiment. **Methods.** *C. proteus* algae monocultures were cultivated on the Fitzgerald's medium in the Zehnder and Gorham's modification. Sterile filtrates of their cultures were diluted with the nutrient medium in the ratio of 1 : 10, 1 : 100, 1 : 1,000, 1 : 10,000. Leptospires were cultivated on the Terskih and Korthof's medium with the addition of 10 % inactivated sheep blood serum. The test samples contained diluted culture filtrates of algae and leptospires. The control samples were the environment for algae and leptospires cultivation. **Results.** In the samples with the 1 : 10 – 1 : 100 dilution of algae filtrates, the content of leptospires in the test samples was significantly lower than in the control samples, indicating their moderate and weak inhibition. There were no statistically significant differences between spirochete culture densities in the test and control samples with the dilutions of 1 : 1,000–1 : 10,000 algae filtrates. **Conclusions**. In the experiment, a topical type of ecological interspecies relationships is formed between *L. interrogans* and green species of *C. proteus* algae, which is realized through the release of biologically active substances into the habitat by *C. proteus*. According to the increasing sensitivity to the allelopathic effect of *C. proteus*, serological types of leptospires formed a row: *Tarassovi, Icterohaemorrhagiae, Pomona, Grippotyphosa, Australis, Sejroe, Canicola, Hebdomadis.*

Keywords: *Leptospira interrogans, Chlamydomonas proteus, ecological relationships.*

DOI:

INTRODUCTION

One of urgent and relevant challenges, facing humanity, is overcoming the consequences of planet climate changes. At present there are evident changes in the seasonal rainfall distribution in Ukraine's territory, there are more frequent temperature anomalies, experts forecast a rise in the level of the Sea of Azov and the Black Sea, desertification of southern and south-eastern regions of the country. There is also actual threat of considerable decrease in water resources and deterioration of water quality. Due to these conditions, the issues of ensuring the purity of water sources and their safety for economic use become especially relevant.

Water is known to be one of the main paths of dissemination for many dangerous infectious and invasive diseases of humans and animals. A special place in this group of pathogenic microorganisms is taken by *Leptospira interrogans* spirochetes (Stimson 1907) Wenyon (1926). A wide range of hosts [1–6] and considerable ecological flexibility conditions pervasive dissemination of these pathogenic spirochetes. The cases of leptospirosis – a disease, caused by *L. interrogans*, are

currently registered in many countries on all the continents, except for Antarctica [7–16].

The capability of pathogenic leptospires to exist in fresh water for a long time, forming natural sources of infection, makes these microorganisms extremely dangerous, especially in conditions of deficient water resources.

At present there are no effective mechanisms of recovering the territory from *L. interrogans*, except meliorative draining measures. However, such radical actions are not always reasonable both from ecological and economic standpoint. The biological method of combating pathogenic microorganisms in environmental objects opens new perspectives in this direction. However, its elaboration and application require the clarification of many issues, related to the existence of *L. interrogans* in different types of freshwater sources and the place of these microorganisms in a complicated network of biotic relationships between hydrobionts.

A considerable part of primary biological products in freshwater bodies is created by different species of algae. They are also one of the main sources of biologically active substances (BAS) for hydrobiocenoses, ensuring complicated allelopathic interactions between higher plants, algae and bacterial microflora.

Water bodies are an extremely complicated and dynamic environment for *L. interrogans* [15] which creates considerable methodological difficulties in planning, conducting experiments, and interpreting the obtained results *in situ*. The aforementioned and insufficient scientific data create conditions, due to which the study of ecological relationships between pathogenic microorganisms (*L. interrogans*) and freshwater algae should be conducted in *in vitro* experiments under controlled laboratory conditions.

The main aim of this study was to investigate the specificities of ecological relationships between pathogenic leptospires and algae – *Chlamydomonas proteus* Pringsheim 1930, and to compare the response of different serological types of *L. interrogans* to the effect of BAS, produced by this species of algae. Hopefully, the obtained data will facilitate more complete investigation on the specificities of biotic relationships of pathogenic leptospires in natural water bodies and provide sufficient data for further elaboration of efficient methods to decrease the potential of natural leptospirosis sources.

MATERIALS AND METHODS

Unialgal monocultures of green algae, *C. proteus,* were cultivated in Erlenmeyer flasks of 250 cc on the Fitzgerald's medium in the Zehnder and Gorham's modification [18] at 22–25 ºC and 12-hour-long photoperiod of artificial illumination with 25 klx fluorescent lamps.

The Terskih and Korthof's medium, containing 10 % inactivated sheep blood serum, was used to cultivate leptospires.

The cultures of spirochetes of 7–14 days with the accumulation of 50–100 leptospires per vision field, with characteristic morphology, active mobility and no signs of autoagglutination were used in the experiment. The experiments were conducted with cultures of the following strains of leptospires (Table 1), which are most widespread in Ukraine's territory, and used as antigens during laboratory diagnostics of leptospirosis in the serological reaction of microagglutination and lysis.

Culture solutions of algae were passed through sterile cellulose filters with pore diameter of 0.2 μm (Sartorius, Germany). This method of sample preparation allowed removing the symbiotic microflora, notable for *C. proteus* cultures, and preventing the destruction of biologically active substances (BAS), released by algae.

The experiment, studying the allelopathic effect of green algae on pathogenic leptospires, simulated the

Table 1. *L. interrogans* spirochete strains, used in the study

Serological group	Serological variant	Strain	Legend
Australis	*bratislava*	*Yez bratislava*	*Australis*
Canicola	*canicola*	*Hond Utrecht IV*	*Canicola*
Grippotyphosa	*grippotyphosa*	*Moskva V*	*Grippotyphosa*
Hebdomadis	*kabura*	*Kabura*	*Hebdomadis*
Icterohaemorrhagiae	*copenhageni*	*M 20*	*Icterohaemorrhagiae*
Pomona	*pomona*	*Pomona*	*Pomona*
Sejroe	*pollonica*	*493 Poland*	*Sejroe*
Tarassovi	*tarassovi*	*Perepelicyn*	*Tarassovi*

conditions of freshwater bodies on the territory of leptospirosis sources. In particular, the gradient of BAS concentration, released by algae in natural conditions, was presented in experimental samples by the following dilutions of *C. proteus* filtrates – 1 : 10, 1 : 100, 1 : 1,000, and 1 : 10,000. Control samples contained sterile culture Fitzgerald's medium in the Zehnder and Gorham's modification.

The samples were introduced the same volume of pathogenic leptospire cultures, here the inoculates of each serological type were taken from one volume. It ensured the same density of leptospires in the experiment and control at the beginning of the experiment. The study was conducted in five repeats. After 24 h since the beginning of the experiment, the content of spirochetes was determined in the experimental and control samples, using direct calculation in 40 μm chambers.

The character and explicitness of the effect of green algae, *C. proteus,* secretions on pathogenic leptospires was evaluated, comparing the content of spirochetes in the experimental and control samples, here the density of cultures in the control was accepted as 100 % [19].

RESEARCH RESULTS

Explicit inhibition of experimental cultures of *L. interrogans* was observed in the experimental samples, containing cultural filtrates of *C. proteus* in 1:10 dilution (Table 2). For instance, after 24 h since the beginning of the experiment the content of leptospires in the experimental samples was as follows (% from the control): *Tarassovi* –78.0 %, *Pomona* – 70.4 %, *Canicola* – 61.0 %, *Hebdomadis* – 57.8 %, *Sejroe* – 63.8 %, *Icterohaemorrhagiae* – 71.0 %, *Grippotyphosa* – 66.4 %, *Australis* – 64.6 %.

Using the criteria of estimating the effect of ecological factors on populations (cultures) of microorganisms [19], we would like to note that according to the experiment results, the leptospire cultures of serological types *Pomona, Canicola, Hebdomadis, Sejroe, Icterohaemorrhagiae, Grippotyphosa, Australis* were exposed to moderate inhibition due to the allelopathic effect of *C. proteus*. The leptospire cultures of serological type *Tarassovi* had weak inhibition. According to the increasing sensitivity to the allelopathic effect of *C. proteus*, serological types of leptospires formed the following row: *Tarassovi* – 22.0 % (an index of the effect of ecological factor (A)*, Icterohaemorrhagiae* – 29.0 %*, Pomona* – 29.6 %*, Grippotyphosa* – 33.6 %*, Australis* – 35.4 %, *Sejroe* – 36.2 %*, Canicola* – 39.0 %*, Hebdomadis* – 42.2 %.

Lower density of *L. interrogans*, compared to the control, was noted in another group of experimental samples, containing cultural filtrates of *C. proteus* in 1 : 100 dilution. For instance, it was determined that the density of leptospire cells in the tests, taken from the experimental samples, was as follows (% from the control): *Tarassovi* – 89.4 %, *Pomona* – 86.2 %, *Canico-*

Table 2. The density of *L. interrogans* cultures in the experiment on the effect of cultural filtrates of *C. proteus* in 1 : 10 dilution

Density of cultures of different serological variants, ×10⁶/cc															
Sejroe		*Hebdomadis*		*Tarassovi*		*Pomona*		*Grippotyphosa*		*Canicola*		*Icterohae-morrhagiae*		*Australis*	
E*	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C
11.50	16.30	6.70	10.90	6.70	8.40	5.70	7.10	13.50	21.40	7.60	13.30	4.80	6.50	6.50	10.50
10.30	18.20	7.20	12.40	6.10	8.20	4.90	7.60	11.80	20.90	8.00	12.10	5.00	6.70	7.00	9.40
9.80	17.50	6.90	11.70	6.50	7.50	5.30	8.00	13.20	19.50	8.20	12.50	4.70	7.30	6.80	10.70
11.10	15.10	7.30	13.30	5.90	8.60	5.60	7.80	14.40	18.70	8.50	14.30	4.90	6.90	6.40	11.20
10.80	16.70	6.80	12.10	6.30	7.70	5.10	7.30	12.70	18.30	7.90	13.70	5.10	7.10	6.70	9.90
M															
10.70	16.76	6.98	12.08	6.30	8.08	5.32	7.56	13.12	19.76	8.04	13.18	4.90	6.90	6.68	10.34
t															
9.98		12.38		7.07		10.12		8.95		12.08		12.65		11.04	
t_{cr} = 5.04; P = 0.01															

*Note. Hereinafter: E – experiment; C – control; M – mean arithmetic; t – Student's coefficient; t_{cr} – critical value of parameter t; P – probability rate.

la – 78.5 %, *Hebdomadis* – 82.8 %, *Sejroe* – 85.3 %, *Icterohaemorrhagiae* – 83.4 %, *Grippotyphosa* – 80.3 %, *Australis* – 76.8 % (Table 3).

The analysis of the obtained data demonstrated that the content of cultural filtrates of *C. proteus* in the medium in 1 : 100 dilution caused weak inhibition of leptospires of serological types – *Hebdomadis, Icterohaemorrhagiae, Pomona, Grippotyphosa, Canicola, Sejroe, Australis*. Here the index of the effect of ecological factor was as follows: *Pomona* – 13.8 %, *Sejroe* – 14.7 %, *Icterohaemorrhagiae* – 16.5 %, *Hebdomadis – 17.2 %, Grippotyphosa* – 19.7 %, *Canicola* – 21.5 %, *Australis* – 23.2 %. At the same time, no explicit effect of experimental algae secretions was determined in the samples with leptospire cultures of serological type *Tarassovi*.

In the subsequent experiments with an even higher dilution index for cultural filtrates of *C. proteus* – 1 : 1,000, the difference between the density of leptospire cultures in the experiment and control was as follows: *Tarassovi* – 92.3 %, *Pomona* – 88.9 %, *Canicola* – 93.8 %, *Hebdomadis* – 89.4 %, *Sejroe* – 90.6 %, *Icterohaemorrhagiae* – 89.3 %, *Grippotyphosa* – 91.8 %, *Australis* – 87.8 % (Table 4).

The results demonstrated that according to the accepted criterion of estimating the effect of ecological

Table 3. The density of *L. interrogans* cultures in the experiment on the effect of cultural filtrates of *C. proteus* in 1 : 100 dilution

Density of cultures of different serological variants, ×10⁶/cc															
Sejroe		*Hebdomadis*		*Tarassovi*		*Pomona*		*Grippotyphosa*		*Canicola*		*Icterohaemorrhagiae*		*Australis*	
E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C
15.20	16.30	9.60	10.90	7.00	8.40	6.70	7.10	14.20	21.40	10.50	13.30	5.60	6.50	7.70	10.50
15.50	18.20	10.70	12.40	7.40	8.20	6.60	7.60	17.40	20.90	9.90	12.10	5.50	6.70	8.30	9.40
13.30	17.50	9.30	11.70	7.70	7.50	6.90	8.00	15.70	19.50	11.10	12.50	5.90	7.30	7.90	10.70
14.60	15.10	9.90	13.30	7.20	8.60	6.00	7.80	16.10	18.70	10.70	14.30	6.10	6.90	7.60	11.20
12.90	16.70	10.50	12.10	6.80	7.70	6.40	7.30	15.90	18.30	9.50	13.70	5.70	7.10	8.20	9.90
M *															
14.30	16.76	10.00	12.08	7.22	8.08	6.52	7.56	15.86	19.76	10.34	13.18	5.76	6.90	7.94	10.34
t															
3.33		4.37		3.30		4.65		4.93		5.80		6.41		7.01	

t_{cr} = 3.36; P = 0.01

Table 4. The density of *L. interrogans* cultures in the experiment on the effect of cultural filtrates of *C. proteus* in 1 : 1,000 dilution

Density of cultures of different serological variants, ×10⁶/cc															
Sejroe		*Hebdomadis*		*Tarassovi*		*Pomona*		*Grippotyphosa*		*Canicola*		*Icterohaemorrhagiae*		*Australis*	
E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C
13.70	16.30	11.30	10.90	7.90	8.40	7.20	7.10	18.30	21.40	11.70	13.30	6.20	6.50	9.20	10.50
15.90	18.20	9.90	12.40	7.60	8.20	6.90	7.60	17.70	20.90	12.30	12.10	6.10	6.70	9.70	9.40
16.60	17.50	10.50	11.70	6.90	7.50	6.50	8.00	16.20	19.50	11.90	12.50	6.40	7.30	8.80	10.70
15.50	15.10	11.60	13.30	7.40	8.60	6.30	7.80	18.50	18.70	12.50	14.30	6.20	6.90	8.40	11.20
14.20	16.70	10.70	12.10	7.50	7.70	6.70	7.30	20.00	18.30	13.40	13.70	5.90	7.10	9.30	9.90
M *															
15.18	16.76	10.80	12.08	7.46	8.08	6.72	7.56	18.14	19.76	12.36	13.18	6.16	6.90	9.08	10.34
t															
2.09		2.58		2.34		3.72		1.88		1.65		4.54		3.28	

t_{cr} = 3.36; P = 0.01

Table 5. The density of *L. interrogans* cultures in the experiment on the effect of cultural filtrates of *C. proteus* in 1 : 10,000 dilution

Density of cultures of different serological variants, ×10⁶/cc															
Sejroe		*Hebdomadis*		*Tarassovi*		*Pomona*		*Grippotyphosa*		*Canicola*		*Icterohae-morrhagiae*		*Australis*	
E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C
17.20	16.30	12.50	10.90	8.30	8.40	7.50	7.10	22.20	21.40	14.90	13.30	7.30	6.50	9.90	10.50
15.70	18.20	11.30	12.40	7.50	8.20	6.70	7.60	22.50	20.90	15.50	12.10	6.80	6.70	9.30	9.40
16.90	17.50	10.20	11.70	7.70	7.50	7.00	8.00	19.80	19.50	14.80	12.50	7.10	7.30	9.50	10.70
14.40	15.10	11.20	13.30	8.00	8.60	7.80	7.80	21.20	18.70	12.80	14.30	6.70	6.90	10.10	11.20
15.30	16.70	11.40	12.10	7.40	7.70	7.10	7.30	18.60	18.30	13.10	13.70	7.00	7.10	10.60	9.90
M *															
15.90	16.76	11.32	12.08	7.78	8.08	7.22	7.56	20.86	19.76	14.22	13.18	6.98	6.90	9.88	10.34
t															
1.16		1.41		1.13		1.34		0.74		1.56		0.45		0.31	
t_{cr} = 3.36; P = 0.01															

factors, the secretions of *C. proteus* in 1 : 1,000 dilution did not have explicit effect on leptospire cultures of the following serological types: *Hebdomadis, Icterohaemorrhagiae, Pomona, Grippotyphosa, Canicola, Sejroe, Tarassovi*. Only the samples, containing leptospire cultures of the serological type *Australis*, demonstrated weak inhibition effect – 12.2 %.

The data, obtained during the experiments of studying the effect of *C. proteus* filtrates in 1 : 10,000 dilution on the cultures of serological groups of leptospires under investigation, demonstrated that there was no statistically reliable difference between the density of *L. interrogans* cells in the experiment and the control (Table 5). Therefore, at this concentration of BAS, secreted by green algae, in the aqueous medium, leptospires were not affected by the allelopathic impact from them.

DISCUSSION

The results of studies demonstrated that the pathogenic spirochetes of *L. interrogans* responded explicitly to the allelopathic effect of green algae C. proteus only under sufficiently high content of BAS, secreted by the latter during their existence, in the aqueous medium (1 : 10 – 1 : 100). Similar conditions may occur in natural sources of leptospirosis, water bodies, during a warm season in the period of mass propagation of this type of algae, when the content of BAS, secreted by them, is the highest. As green algae C. proteus affect *L. interrogans* spirochetes via the change in characteristics of their existence medium, ecological interspecies relationships between them should be deemed topical. At the same type, it should be noted that leptospire cultures of serological types, used in the studies, demonstrated different sensitivity to the presence of similar concentrations of BAS from algae in the medium. Thus, according to the increasing sensitivity to the allelopathic effect of algae, the investigated serological types formed the following row: *Tarassovi, Icterohaemorrhagiae, Pomona, Grippotyphosa, Australis, Sejroe, Canicola, Hebdomadis*. The mechanisms, conditioning similar differences in the response to ecological factors of different serological groups of leptospires, are yet to be studied in the full detail. However, their adaptive significance was absolutely evident – a complicated intraspecies structure of *L. interrogans* determined wide ecological flexibility of this species, which allows it to exist in different environmental conditions and ensures the resistance to the effect of many ecological factors.

CONCLUSIONS

In the experiment, a topical type of ecological interspecies relationships is formed between *L. interrogans* and green species of *C. proteus* algae, which is realized through the release of biologically active substances into the habitat by *C. proteus*. Explicit inhibition of leptospire cultures under investigation was observed only in the samples with low dilutions of 1 : 10 – 1 : 100 algae filtrates. Pathogenic leptospires practically did not respond to the presence of BAS from algae in the medium under the filtrate dilution indices of 1 : 1,000 – 1 : 10,000. The serological types of leptospires, used in the experiment, demonstrated different sensitivity to the presence of BAS, secreted by *C. proteus*,

in the medium which demonstrates considerable ecological plasticity of *L. interrogans*. The obtained results of investigations give grounds to consider freshwater algae as one of powerful biotic factors of affecting the existence of pathogenic leptospires in freshwater bodies. Relevant epidemic and epizootic significance of *L. interrogans* as a leptospirosis factor requires further subsequent studies.

This study was not financed by any specific grant from financing institutions in the state, commercial or non-commercial sectors.
There was no conflict of interests while conducting the investigations and publishing the results.

Еколого-експериментальне дослідження взаємозв'язків патогенних мікроорганізмів з водоростями

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Мета. З'ясувати в експерименті характер екологічних зв'язків між спірохетами *Leptospira interrogans* та водоростями *Chlamydomonas proteus*, а також виразність реакції окремих серологічних типів лептоспір на алелопатичний вплив водоростей. **Методи.** Монокультури водоростей *C. proteus* вирощували на середовищі Фітцджеральда в модифікації Цендера та Горема. Стерильні фільтрати їх культур розводили поживним середовищем у співвідношенні 1 : 10, 1 : 100, 1 : 1000, 1 : 10000. Лептоспір культивували на середовищі Терських та Кортгофа з додаванням 10 % інактивованої сироватки крові овець. Дослідні зразки містили розведені культуральні фільтрати водоростей та лептоспір. Контрольні – середовище для культивування водоростей та лептоспір. **Результати.** У зразках із розведенням фільтратів водоростей 1 : 10 – 1 : 100 вміст лептоспір у дослідних зразках був достовірно нижчим ніж у контролі, що свідчить про їх помірне та слабке пригнічення. У зразках із розведенням фільтратів водоростей 1 : 1000 – 1 : 10000 статистично достовірної різниці між щільністю культур спірохет у досліді та контролі не було. **Висновки.** В експерименті між патогенними мікроорганізмами *L. interrogans* та зеленим видом водоростей *C. proteus* формується топічний тип екологічних міжвидових зв'язків, що реалізується через виділення останнім у середовище існування біологічно-активних речовин. За зростанням чутливості до алелопатичного впливу *C. proteus* серологічні типи лептоспір утворили наступний ряд: *Tarassovi, Icterohaemorrhagiae, Pomona, Grippotyphosa, Australis, Sejroe, Canicola, Hebdomadis.*

Ключові слова: *Leptospira interrogans, Chlamydomonas proteus*, екологічні взаємозв'язки.

Эколого-экспериментальное исследование взаимосвязей патогенных микроорганизмов и водорослей

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Цель. Выяснить в эксперименте экологические связи между спирохетами *Leptospira interrogans* и водорослями *Chlamydomonas proteus*, а также выраженность реакции отдельных серологических типов лептоспир на аллелопатическое влияние водорослей. **Методы.** Монокультуры водорослей *C. proteus* выращивали на среде Фитцджеральда в модификации Цендера и Горема. Стерильные фильтраты их культур разводили питательной средой в соотношении 1 : 10, 1 : 100, 1 : 1000, 1 : 10000. Лептоспир культивировали на среде Терских и Кортгофа с добавлением 10 % инактивированной сыворотки крови овец. Опытные образцы содержали разведенные культуральные фильтраты водорослей и лептоспир. Контрольные – среду для культивирования водорослей и лептоспир. **Результаты.** В образцах с разведением водорослей 1 : 10 – 1 : 100 содержание лептоспир в опытных образцах было достоверно ниже, чем в контроле, что свидетельствует об их умеренном и слабом угнетении. В образцах с разведением фильтратов водорослей 1 : 1000 – 1 : 10000 статистически достоверной разницы между плотностью культур спирохет в опыте и контроле не было. **Выводы.** В эксперименте между *L. interrogans* и водорослью *C. proteus* формируется топический тип экологических межвидовых связей, который реализуется через выделение последним в среду обитания биологически-активных веществ. За возрастанием чувствительности к аллело-

патическому влиянию *C. proteus* серологические типы лептоспир образовывают ряд: *Tarassovi, Icterohae-morrhagiae, Pomona, Grippotyphosa, Australis, Sejroe, Canicola, Hebdomadis*.

Ключевые слова: *Leptospira interrogans, Chlamydomonas proteus*, экологические взаимосвязи.

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