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Methodological approach to the study of dynamics of specific concentration of cell wall antigens per cell of *Bacillus* species and examples of its application

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Abstract

Background: Nonpathogenic *Bacillus* strains are used in biotechnology, and pathogenic *Bacillus* strains are cause of food borne disease. It explains the relevance of the methods of detection and quantification of whole cell and cell components of these bacteria.

Aims: Development of methodological approach for investigation of dynamics of specific concentration of cell wall antigens per cell of bacilli without solubilization of cell wall during sample preparation; using of the approach with 6 strains of bacilli as an example.

Method: ELISA.

Results: Methodological approach for investigation of dynamics of specific concentration of bacilli cell wall antigens has been developed. The distinctive features of the approach are rabbit polyclonal antibodies to genera-specific antigens of bacilli as key reagent and lack of need for solubilization of cell wall during sample preparation. It was shown using 6 strains of *Bacilli* as an example that specific concentration of cell wall antigens per cell vary according to bacillus strain, stage of culture growth and media composition. The data will find an application in biotechnology of clinical diagnostics and test-systems for food control including detection of whole bacillus cells.

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Introduction

Bacilli are gram-positive, aerobic, spore-forming bacteria found in soil, water and located in transit in humans and animals (1). The genus *Bacillus* includes 88 species belonging to the *B. cereus* group and the *B. subtilis* group (2). The range of human relations with the members of *B. cereus* group is very wide (2). The extreme positions are occupied by *B. anthracis*, the causative agent of anthrax, and non-pathogenic strains of *B. cereus* itself. The latter are used as probiotic feed additives in agriculture and aquaculture (3, 4, 5) as well as the basis of veterinary drugs (6) and pharmaceuticals for the treatment of dysbiosis (7). Pathogenic strains of *B. cereus* produce a number of enterotoxins that cause diarrhea and vomiting (7, 8). In 2008, 102 confirmed outbreaks of food borne disease caused by *B. cereus* were identified by the European community, corresponding to more than a thousand patients (9).

The members of *B. subtilis* group are not pathogenic and are conventionally used along with nonpathogenic strains of *B. cereus* group in the production of enzymes (10) and secondary metabolites with surfactant properties (11). Synthesis of biosurfactants and a number of enzymes is the basis for the application of these bacilli for bioremediation of hydrocarbon- and nitrogen-polluted soil and water (12, 13), as well as for biodegradation of pesticides (14), polyethylene (15), *etc.*

A number of bacilli are used to protect crops against pests and infectious diseases due ability of these bacteria to produce a substances with the properties of antibiotics and insecticides (16, 17) as well as compounds that enhance systemic plants resistance (18).

The listed above areas of practical use of nonpathogenic *Bacillus* strains, on the one hand, and the need to detect spores, vegetative forms and toxins of pathogenic bacilli strains in foodstuff, on the other hand, explain the relevance of the methods of detection and quantification of whole cell and cell components of these bacteria. The rabbit polyclonal antibodies (PAb) obtained by immunization of animals with a sample containing cells and spores of *B. cereus* BIM 491 ($PAb_{anti\text{-}Bc\ 491}$) are useful as a tool to solve same of the problems. The $PAb_{anti\text{-}Bc\ 491}$ interact with antigens that are shared by members of *B. cereus* group and *B. subtilis* group, and $PAb_{anti\text{-}Bc\ 491}$ ability to form immune complexes with cell wall (CW) antigens (19) is the basis for the detection and quantification of whole cells of bacilli.

Aims - development of methodological approach for investigation of dynamics of specific concentration (C_{sp}) of CW antigens per cell of bacilli without solubilization of CW during sample preparation; using of the approach with 6 strains of bacilli as an example.

Materials and Methods

Bacterial cells. *Bacillus cereus* BIM B-491 (*Bc* 491), BIM B-169 (*Bc* 169), BIM B-205 (*Bc* 170), BIM B-204 (*Bc* 204), BIM B-205 (*Bc* 205), BIM B-206 (*Bc* 206), *Bacillus mycoides* BIM B-179 (*Bm* 179), *Bacillus thuringiensis* BIM B-180 (*Bt* 180), *B. subtilis* BIM B-25 (*Bs* 25), BIM B-182 (*Bs* 182), BIM B-210 (*Bs* 210), BIM B-276 (*Bs* 276), BIM B-277 (*Bs* 277), *Bacillus amyloliquefaciens* BIM B-278 (*Bam* 278), *Bacillus pumilis* BIM B-211 (*Bp* 211), *Bacillus licheniformis* BIM B-175 (*Bl* 175) were used. The physiologically active culture of each stain after the second 24 h incubation was used as inoculum. All strains were incubated 12 h under aerobic conditions at (28 – 30) °C in 50 ml of meat-peptone broth (MPB) (HiMedia, India) (medium 1) for determination of $PAb_{anti\text{-}Bc\ 491}$ cross-reactivity. Selected strains (*Bc* 169, *Bc* 205, *Bc* 491, *Bc* 206, *Bm* 179, *Bs* 25) were incubated 6, 12 and 24 h in 50 ml of medium 1 and 50 ml of MPB 2-fold diluted by distilled water (medium 2) for study of the dynamics of C_{sp}.

Cell fractions for study of the dynamics of C_{sp}. Cells (6 strains × 2 media × 3 times = 36 samples) were separated by centrifugation at 2 000 g for 20 min and washed (3 × 50 mL) with 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.5 (buffer 1). Cells were suspended in buffer 1 (1 mL) to obtain ~ (30 – 50) % suspension and protease inhibitor cocktail (0.3 M benzamidine hydrochloride hydrate; 1344 мМ EDTA, disodium salt; 200 mM imidazole; 115 mM sodium molybdate; 146 μM pepstatin A, and 210 μM leupeptin, all (Sigma, USA)) (50 μL) was added. Cells were manually homogenized (Wheaton, USA) and ultrasonicated on ice using Branson Digital Sonifier 450 (Branson, USA), 50% duty cycle, 4 × 1 min with 1.5 min pauses. The fraction containing cell wall and spores (CW/sp) was sedimented by centrifugation at 13 000 g for 30 min using Eppendorf Mini Spin plus (Eppendorf AG, Germany). Supernatant (cell free fraction, CFF) was collected in separate tube. The value of the optical density at 260 nm in a cuvette with 1 cm optical path length ($A_{260\ nm,\ 1\ cm}$) of each specimen CFF (CFF_6, CFF_{12}, CFF_{24}; the numeral in the lower register corresponds to the duration of culture growth, h) was measured using spectrophotometer Specord (Carl Zeiss, Germany). Each $A_{260\ nm,\ 1\ cm}$ value (AU) was multiplied by the corresponding CFF volume (mL) to obtain a parameter proportional to the amount of total nucleic acids (tNA) in CFF (referred to below as amount of tNA). Each specimen CW/sp (CW/sp_6, CW/sp_{12}, CW/sp_{24}; the numeral in the lower register corresponds to the duration of culture growth, h) was weighted using analytical balance CPA225D (Sartorius, Germany). CFF specimens and CW/sp specimens, a total of 36 pieces of each, were stored at -20 °C.

Cell fractions for determination of $PAb_{anti\text{-}Bc\ 491}$ cross-reactivity. Cells were washed; specimens CFF_{12} (n = 16) were obtained by ultrasonication; values $A_{260\ nm,\ 1\ cm}$ of each specimen CFF_{12} were measured as described above.

Antiserum. Six-month male rabbits were immunized subcutaneously in the area of the scapular as follows: injection - 1, 14, 28, 60, 90-th day, blood sampling - 40, 72, 100-th day from the beginning of the procedure. The drug for the immunization: 2 mg of lyophilized nonviable cells and spores of *Bc* 491 (24 h of culture growth), 0.1 ml of sterile 0.15 M NaCl, 2 ml of complete Freund's adjuvant. Antiserum (referred to below as $PAb_{anti\text{-}Bc\ 491}$) was stored at -70 °C.

Purification of antibodies to *Bc* 491 antigens. Antiserum of rabbit immunized with whole cells/spores of *Bc* 491 was used for isolation of total human immunoglobulins enriched with antibodies to *Bc* 491 antigens ($Ig_{anti\text{-}Bc\ 491}$). The procedure involved sequential precipitation of serum albumin with caprylic acid at pH 4.8 and $Ig_{anti\text{-}Bc\ 491}$ with ammonium sulfate (45% saturation of antiserum) at pH 7.0 – 7.5 (20); the precipitate was dissolved in buffer 1, ammonium sulfate was removed by dialysis against the same buffer and optical density at 280 nm was measured with spectrophotometer Specord (Carl Zeiss, Germany). Concentration of $Ig_{anti\text{-}Bc\ 491}$ was calculated using $A_{280\ nm,\ 1\ cm,\ 1\ mg/\ ml} = 1.35$.

ELISA was performed using 96-well high binding microplates Microlon (Greiner bio-one, Germany). *Buffer solutions.* Buffer 1, buffer 2 (buffer 1 supplemented with 0.2 M NaCl) and either buffer 3 (buffer 2 supplemented with 1 g/L bovine serum albumin (BSA)) or buffer 4 (buffer 2 supplemented with 3 g/L BSA) were used for immobilization of antigens, washing of wells (3 × 200 μL) and as an incubation buffer, respectively.

Determination of $PAb_{anti\text{-}Bc\ 491}$ cross-reactivity. *Immobilization.* CFF_{12} of *Bc* 491 pre-diluted to obtain $A_{260\ nm,\ 1\ cm}$ = 0.1 was immobilized by passive adsorption at 4°C overnight (0.1 mL per well, each well in duplicate) in 16 series of wells.

Preparation for the 1st stage of ELISA. 16 series of samples were prepared for each tested strain (n = 15; the list is above) and *Bc* 491 as control by serial dilution of the appropriate CFF_{12} specimens; samples B_1 - B_6 in each series with $A_{260nm,\ 1\ cm}$ in the range (0.1 - 15) AU. Buffer 4 containing rabbit antiserum (titre 1/3 000) was added (0.1 mL) to each sample B_1 - B_6 (0.2 mL) and sample B_0 (0.2 mL of buffer 2). *1st stage of ELISA*. Each probe was stirred and dispensed into wells of appropriate series and incubated for 1 h at 37 °C. *2nd stage of ELISA*. Conjugate of sheep PAb against rabbit Ig with horseradish peroxidase (ShAR – HRP) (Xema Co. Ltd, Russia) pre-diluted with buffer 3 was dispensed into each well and incubated for 1 h at 37 °C. *The enzymatic peroxidation reaction* was initiated by addition of 3,3′,5,5′-tetramethylbenzidine liquid substrate system (Sigma, USA) (0.1 mL per well). The reaction was stopped by addition of 4.8% H_2SO_4 (0.1 mL per well). The A_{450} values were measured in each well using the automatic multi-channel spectrophotometer (Uniplan, Russia).

Study of the dynamics of C_{sp}. *Immobilization.* CFF specimens (n = 36) pre-diluted to obtain $A_{260\ nm,\ 1\ cm}$ = 0.025 were immobilized in an appropriate series of wells (36 series) as described above. *Preparation for the 1st stage of ELISA*. The same CW/sp specimens (n = 36) were suspended in buffer 2; the volumes of suspensions were in the same proportion as the values of amount of tNA in appropriate CFF specimens. After that 36 series of samples containing the appropriate CW/sp specimen were prepared by serial dilution of these suspensions with buffer 2; sample B_1 - B_5 in each series. Vortex mixer Bio Vortex V-1 plus (BioSan, Latvia) was used to ensure homogeneous suspensions during the sample preparation. Buffer 4 containing 22.5 μg/mL $Ig_{anti\text{-}Bc\ 491}$ (0.1 mL) was added to each sample B_1 - B_5 (0.2 mL) and sample B_0 (0.2 mL of buffer 2); probes were stirred with rotary mixer Multi Bio RS-24 (BioSun, Latvia) for 2 h at room temperature. CW/sp were precipitated by centrifugation at 13 000 g for 10 min with centrifuge Eppendorf Mini Spin plus (Eppendorf AG, Germany). *1st stage of ELISA*. Each supernatant was dispensed into wells of appropriate series (containing immobilized CFF specimen of the same strain cultured in the same medium for the same time) and incubated for 1 h at 37 °C. ELISA was completed as described above.

Results and Discussion

We used lyophilized vegetative cells and spores of *Bc* 491 as an immunogen to produce $PAb_{anti\text{-}Bc\ 491}$ that interacts with genus-specific antigens of bacilli, which was proved by cross-reaction of $PAb_{anti\text{-}Bc\ 491}$ with antigens of 15 strains. Out of 15 strains, 9 strains have values of cross-reactivity in the range of (183 - 9) %; 2 strains – 3%; 4 strains - less than 1%.

Commonly known that vegetative cells and spores of bacilli contain antigens, some of which are sharing between cells and spores (antigens of group 1, Ag_1), while others are present in cells and absent in spores (antigens of group 2, Ag_2) and *vice versa* (antigens of group 3, Ag_3) (21). It is logical to assume that purified $PAb_{anti\text{-}Bc\ 491}$ referred to above as $Ig_{anti\text{-}Bc\ 491}$ contain antibodies to all three groups of antigens.

In this work we were of interest only to CW antigens while used in ELISA CW/sp specimens containing both CW and spores to simplify sample preparation for ELISA. We used competitive ELISA and CFF as source of antigens competing with antigens of CW/spores for $Ig_{anti\text{-}Bc\ 491}$ binding to eliminate the contribution of Ag_3 contained in CW/sp to data of ELISA. We assumed that the contribution of Ag_1 contained in CW/sp has also been minimized. The reason for this is the fact that antibodies obtained by immunizing animals with intact spores, mechanically disrupted spores, spores extracts and purified/recombinant envelope spores proteins capable of reacting with antigens of intact spores in rare cases (22) due to cross-linking of spore coat proteins (23). It is known that immune response to spores includes phagocytosis of spores, their germination in macrophages and killing before any round of cell duplication (24) that makes spore antigens accessible to the immune system. Spore germination could not occur during step of pre-incubation described below, and therefore only Ag_2 and a small part of Ag_1 (exposed to the external environment) contained in CW/sp were able to interact with $Ig_{anti\text{-}Bc\ 491}$.

Considering the above, in this study we really determined the dynamics of C_{sp} of CW antigens, despite the presence of spores in the composition of the CW/sp specimens.

Necessary to note, that CW is a "uncomfortable" specimen for ELISA since there are no universal methods to ensure one-step solubilization/extraction of all CW antigens (proteins, polysaccharides, teichoic acid, peptidoglycan) while retaining their immunoreactivity and natural proportion of the concentrations.

We proposed methodological approach that allows using CW without solubilization and can be applied to whole cells of bacteria, which is important for biotechnological processes that have as effectors *Bacillus* species.

We used 6 strains of bacilli satisfying the criterion of the presence of antigens that are identical/similar to antigens of *Bc* 491. The presence of the antigens was verified by measurement of cross-reactivity with $Ig_{anti\text{-}Bc\ 491}$ that was no less than 40%, *viz.*, 100%, 97%, 183%, 55%, 118%, 40 % for *Bc* 491 (immunogen), *Bc* 169, *Bc* 205, *Bc* 206, *Bm* 179, *Bs* 25, respectively. The possible explanations of values of cross-reaction above 100% are the follows: (*i*) tested strain has antigens that are identical to antigens of *Bc* 491, but their concentration per cell is higher; (*ii*) tested strain has antigens that are similar but not identical to antigens of *Bc* 491 and interact with $Ig_{anti\text{-}Bc\ 491}$ with higher affinity than antigens of *Bc* 491.

Selected strains were grown in the two media, first of which was MPB used for cultivation of bacilli by tradition (medium 1) and the second was MPB 2-fold diluted with distilled water (medium 2) to reduce the concentration of nutrients. The duration of growth was 6, 12 and 24 h. CFF specimens and CW/sp specimens, 36 pieces of each, were used for ELISA tests (**Fig. 1**).

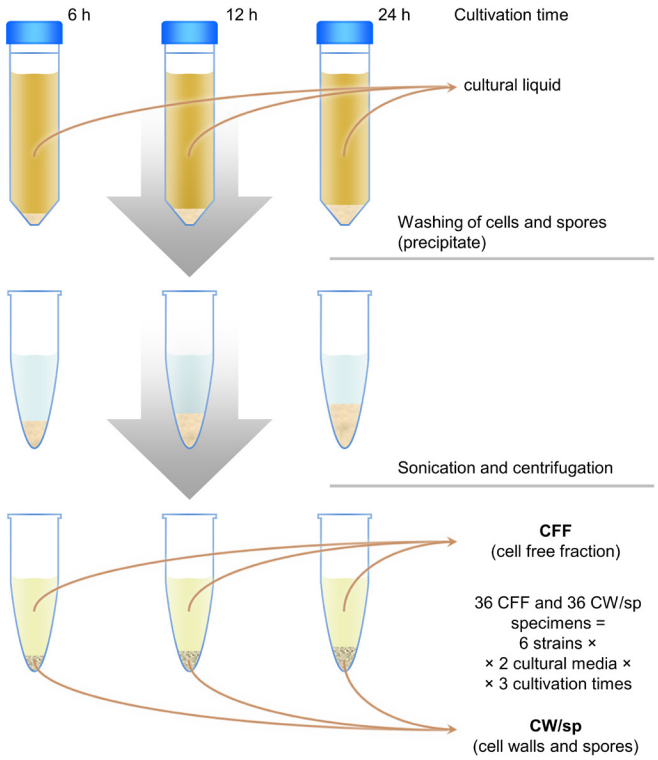

Figure 1. Scheme of obtaining CFF and CW/sp specimens.

The time dependence of tNA amount in CFF and weight of CW/sp were determined for each strain of bacilli (**Fig. 2**). The parameter proportional to tNA amount in CFF was calculated as $A_{260\,nm,\,1\,cm}$, AU, multiplied by the corresponding CFF volume, mL; the parameter is proportional to the number of vegetative cells of bacilli (25). The weight of CW/sp is proportional to total number of vegetative cells and spores. Comparison of the dynamics of two parameters allows estimating the ratio of cells to spores in different stages of culture growth.

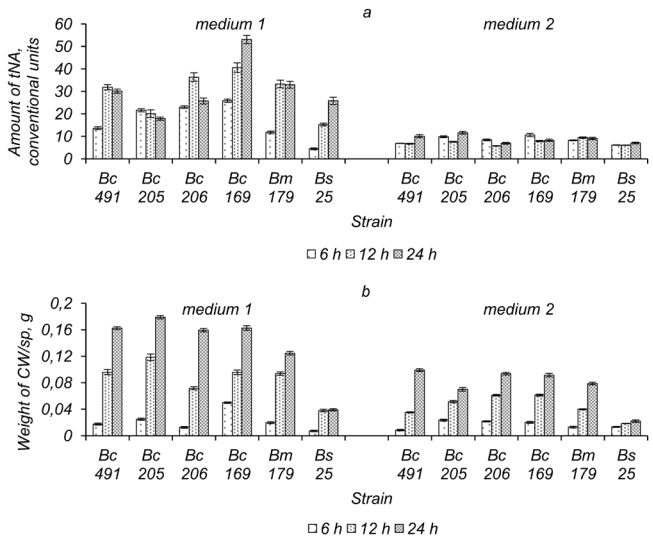

Figure 2. Dynamics of tNA amount in CFF (*a*) and weight of CW/sp (*b*) of 6 strains of bacilli. (6 – 24) h – duration of culture growth in medium 1 and medium 2. Values are means ± SD.

It is obvious that this ratio depends on the medium composition and strain (Figure 2). In particular, CW/spores weight gain in the medium 1 occurs either mainly due to the increase in cell number (strain *Bc* 169, (6 - 24) h) or mainly due to the increase in spores number (strains Bc 491, *Bc* 206 and *Bm* 179, (12 - 24) h; strain *Bc* 205, (6 - 24) h) (**Fig. 2**).

It is also obvious that the decrease in the concentration of nutrients in the medium predictably limit the growth of all tested strains and reduces the duration of logarithmic growth phase (corresponding to a period of pronounced increasing in tNA amount) (**Fig. 2a**). In particular, in the medium 2 the log phase is completed up to 6 hours for each strain; increase in the number of tNA in CFF of *Bc* 491, *Bc* 205, *Bc* 206, *Bs* 25 in the period (12 - 24) h after decrease in values of this parameter in the period (6 - 12) h can be attributed to the spore germination, outgrowth and subsequent vegetative growth (7).

To compare 6 strains of bacilli in terms of the dynamics of C_{sp} of CW antigens we used the following approach (**Fig. 3, 4**).

CW/sp specimens (36 specimens = 6 strains × 2 media × 3 times) were suspended; the volumes of suspensions were in the same proportion as the values of amount of tNA in appropriate CFF specimens (**Fig. 3**), in other words, in the same proportion

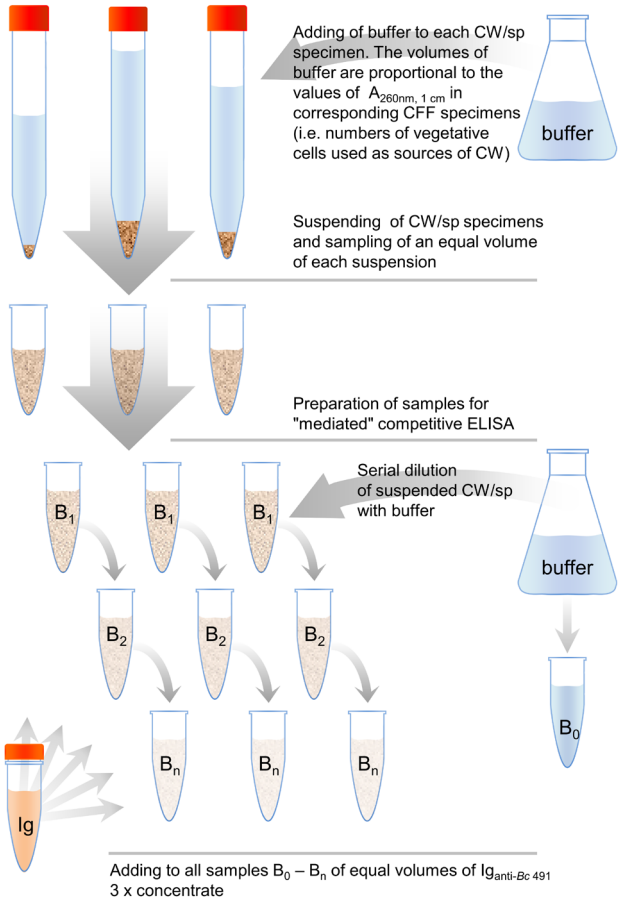

Figure 3. Scheme of samples preparation for study of dynamics of specific concentration (C_{sp}) of cell wall (CW) antigens per cell of bacilli.

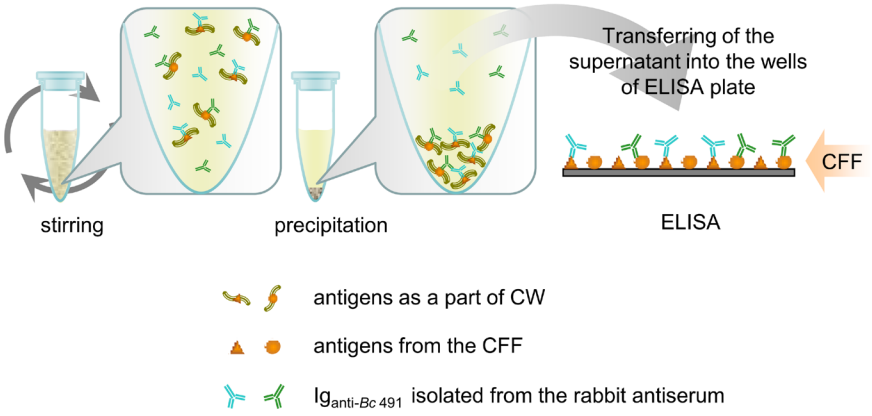

Figure 4. Scheme of "mediated" competitive ELISA. Mediator is the solution containing $Ig_{anti\text{-}Bc\ 491}$. It comes initially into contact with CW/sp antigens and then - with antigens of CFF.

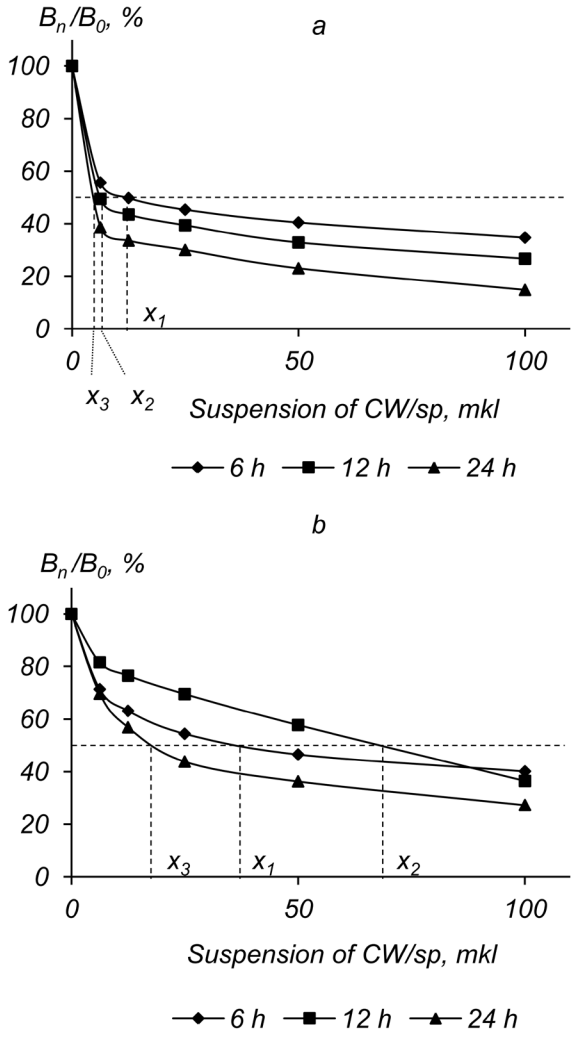

Figure 5. Indirect competition for $Ig_{anti\text{-}Bc\ 491}$ binding between antigens of *Bc* 205 contained in CW/sp and intracellular antigens of the strain contained in CFF. *a* and *b* – strain *Bc* 205 was grown in medium 1 and medium 2, respectively. The mean values of three independent experiments are shown. SD values are in the range ± 5%. The x_n values were used for C_{sp} calculation.

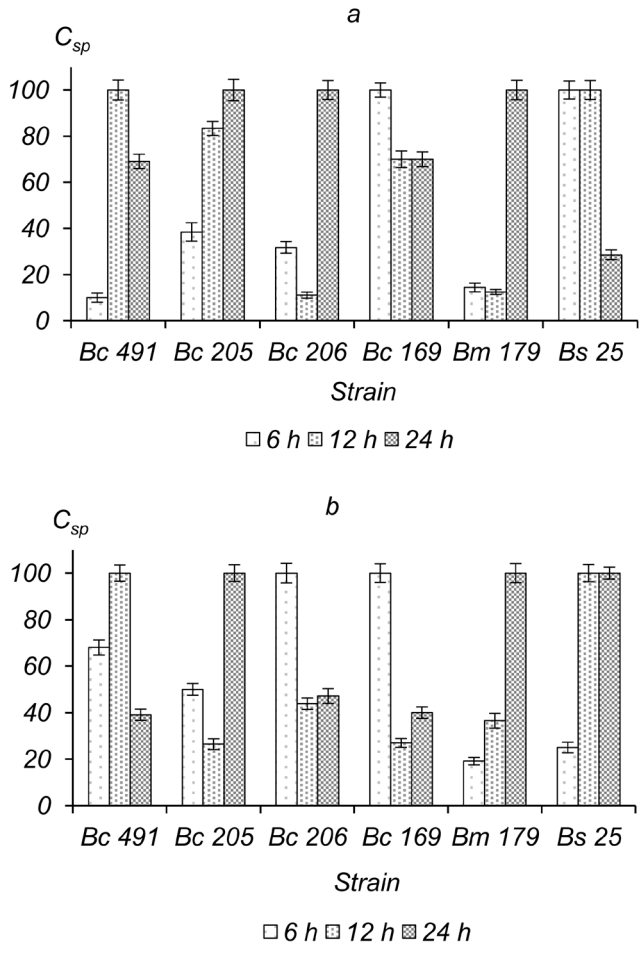

Figure 6. Dependence of specific concentration of CW antigens per cell (C_{sp}) of 6 *Bacillus* strains on duration of culture growth in medium 1 (*a*) and medium 2 (*b*).

Table. The dynamics of specific concentration (C_{sp}) of CW antigens per cell of 6 Bacillus strains: ordering of the results

Strain	medium 1				medium 2			
	group of strains				group of strains			
	1^1	2^1	3^1	4^1	1^2	2^2	3^2	4^2
Bc 491	+				+			
Bc 169				+				+
Bc 205			+			+		
Bc 206		+						+
Bm 179			+				+	
Bs 25				+			+	
C_{sp} dynamics	6 h / 12 h ↑, 12 h / 24 h ↓	6 h / 12 h ↓ insignificantly, 12 h / 24 h ↑	6 h / 12 h ↑ / const, 12 h / 24 h ↑	6 h / 12 h ↓ / const, 12 h / 24 h ↓ / const	6 h / 12 h ↑, 12 h / 24 h ↓	6 h / 12 h ↓ insignificantly, 12 h / 24 h ↑	6 h / 12 h ↑, 12 h / 24 h ↑ / const	6 h / 12 h ↓, 12 h / 24 h const

Note: The groups of strains with similar C_{sp} dynamics were denoted by identical numbers: 1^1 и 1^2, 2^1 и 2^2, 3^1 и 3^2, 4^1 и 4^2. The numeral in the upper register corresponds to the number of the culture medium.

as the number of vegetative cells used as the source of both CFF and CW. Each "equalized" CW/sp suspension was used to prepare the appropriate series of B_1 - B_5 samples (Figure 3), which were incubated with the same amount of $Ig_{anti\text{-}Bc\ 491}$ with continuous stirring to form immune complexes (pre-incubation step). The complexes were removed by CW/sp precipitation, and only free (*i.e.* unbound to antigens) $Ig_{anti\text{-}Bc\ 491}$ remained in the liquid phase.

These preparations (36 × 5 = 180) were incubated with the appropriate CFF specimens (36 items) previously immobilized in wells of plate from solutions with values $A_{260\ nm,\ 1\ cm}$ equal to 0.025 (**Fig. 4**). Value $A_{260\ nm,\ 1\ cm}$ in the solutions for the immobilization of CFF (the same for all CFF specimens), the $Ig_{anti\text{-}Bc\ 491}$ concentration and volumes of CW/sp suspensions used for preparation of B_1 - B_5 samples were selected preliminary; selection criterion was sigmoid shape of curves in coordinates B_n/B_0, % (OY) - volumes of CW/sp suspensions, μL (OX) and the presence along each curve of at least one experimental point with value of B_n/B_0 not more than 50%.

The amount of immune complexes formed on the solid phase was inversely proportional to the quantity of antigens in the CW/sp suspensions. Typical data of "mediated" competitive ELISA (mediator was a solution containing $Ig_{anti\text{-}Bc\ 491}$, being initially in contact with antigens of CW and then - with antigens of CFF) are shown in **Fig. 5** for strain *Bc* 205 as an example. Similar graphs of "mediated" competitive ELISA were obtained for 5 other bacillus strains (data not shown).

The data presented in **Fig. 5** for *Bc* 205, and similar data obtained for 5 other bacillus strains (not shown) were processed as follows. In each graph for each of the three curves we determined volume of CW/sp suspension (μL), corresponding to the experimental point with a value of B_n/B_0, equal to 50%; the values obtained were designated x_1 - x_3, where numerals 1 – 3 in lower register correspond to 6 – 24 h of culture growth. We calculated the values of $1/x_1$ - $1/x_3$. The maximum of three values $1/x_n$ was taken as 100% and other values $1/x_n$ were converted into percent. The calculated values proportional to C_{sp} of CW antigens are shown in **Fig. 6**.

Taking into account data of Figure 6 we grouped tested strains as follows (**Table**). The bacteria belonging to the groups 1^1 и 1^2 (coinciding strain is *Bc* 491), 2^1 и 2^2 (there are not coinciding strains), 3^1 и 3^2 (coinciding strain is *Bm* 179), 4^1 и 4^2 (coinciding strain is *Bc* 169) have similar character of C_{sp} dynamics. The data of Table suggest that character of C_{sp} dynamics of CW antigens depends on the strain and the culture medium, is unpredictable and for each object under study must be determined on a case-by-case basis.

Conclusions

Methodological approach for investigation of dynamics of C_{sp} of CW antigens per cell of bacilli has been developed. The distinctive features of the approach are rabbit polyclonal antibodies to genera-specific antigens of bacilli as key reagent in "mediated" competitive ELISA tests and lack of need for solubilization of CW during sample preparation.

The approach includes the method of selecting the objects of the study; criteria for quantifying of cell fractions (CFF and CW/sp) that are used as a source of bacterial antigens; principles of sample preparation and conditions of ELISA,

methods of experimental data processing and interpretation of results. The methodological approach can be applied to *Bacillus* cells quantification on condition that either whole cells or whole cells and spores simultaneously are used instead of CW/sp specimens in ELISA tests.

It was shown using 6 strains of *Bacillus* as an example that C_{sp} of CW antigens per cell depends on strain, stage of culture growth and media composition. Dynamics of C_{sp} is unpredictable and for each object must be determined on a case-by-case basis.

The data will find an application in biotechnology of clinical diagnostics and test-systems for food control including detection of whole *Bacillus* cells.

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Conflict of interest statement

Authors have not any competing interests/conflicts of interest.

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