The Main Properties of Diphtheriae Causative Microorganism Circulated in Postepidemic Period from Biofilm Culture

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Abstract: Task – to study the main properties toxigenic strain of *Corynebacterium diphtheriae* circulated in postepidemic period from biofilm culture. It was shown that Diphtheriae causative agent has the ability to biofilm formation that is accompanied by exopolysaccharide matrix formation. This combines with changes of morphologic properties (decrease of bacteria sizes), cultures properties (decrease of colonies sizes, R-S-dissociation) and antibiotic susceptibility. The most effective towards Diphtheriae infection causative microorganism are cefotaxime, gentamycin, linkomycin, kanamycin and cefazolin, because they have no changes in susceptibility of *Corynebacterium diphtheriae* in the biofilm structure.

Keywords: *Corynebacterium diphtheriae tox*+, biofilm, antibiotic susceptibility.

INTRODUCTION

Microbe biofilms are responsible on etiology and pathogenesis of many acute and, especially, chronic bacterial infections of the individual [1, 8, 9]. Biofilm contain causative agents of such infection diseases as tonsillitis, bronchitis, pneumonia [8, 10] and diphtheria [2].

Polymicrobe fix associations during living synthesize polymer matrix consisting from exopolysaccharides [7]. Microorganisms embedded into matrix change their biologic properties that protect them from the action of congenital immunity factors and from cell and humoral factors of adaptive immunity [9, 10]. Investigation of ability of Diphtheriae causative agent to biofilm formation get particular actuality nowadays due to the fact of lengthened circulated strains of toxigenic *Corynebacterium diphtheriae* among bacteriocarriness in postepidemic period [2].

The task of investigation – to study the main properties of biofilm culture of Corynebacterium diphtheriae toxigenic strain circulated in postepidemic period.

MATERIALS AND METHODS

Circulated strain of *Corynebacterium diphtheriae gravis tox*+ taken in 2000 year from patient with diagnosis “localize form of diphtheria” in Rostov-on-Don bacteriologic laboratory of central city hospital.

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Strain testing on ability to biofilm formation was done on P.L. Watnick method [11]. 720-hours biofilm culture of *Corynebacterium diphtheriae gravis tox*+ was chosen for investigation.

Morphologic, cultural, biochemical, toxigenic properties of type and biofilm cultures of circulated strains of *Corynebacterium diphtheriae tox*+ was determined after 48 hours of cultivation according to recommendations [6].

Biofilm culture of *Corynebacterium diphtheriae gravis tox*+ sample preparation adhesed to base was exposed to chemical fixation during scanning electron microscopy. Then fixed samples of the biofilm were put on scanning electron microscope stage and aurum was evaporated in evaporation vacuum plant EicoIB-3 ion coater (“Eico” firm, Japan) at 6-8 mA ionic floor. Such sample of circulated strain biofilm culture was investigated in scanning electron microscope S-450 («Hitachi» firm, Japan) at accelerating potential in 30 kV.

Antibiotic susceptibility of toxigenic diphtheria causative agent of biofilm culture was determined by serial dilution method (micromethod) in culture fluid according to methodic instructions [3].

Statistical evaluation of the results was done with help of statistical packages «Microsoft Office 2007» and «Statistica 6.0» for Windows XP. Authenticity of results was evaluated at significance level $P \leq 0.05$.

RESULTS OF INVESTIGATION

Experimental model of biofilm circulated toxigenic strain of *Corynebacterium diphtheriae gravis tox*+ gave

E-ISSN: 2311-8687/14

us the possibility to estimate changes of biologic properties its type culture. Comparison of type and biofilm cultures of diphtheria bacteria was made on such criteria as bacteria morphology, character of grown colonies, ability to split saccharides, toxigenity and ability to antibiotics action.

Scanning electron microscopy (Figure **1**) gave us figures which reflect special aspects of morphology of diphtheria bacteria biofilm cultures. Biofilm formed by diphtheria causative agent was like cell pellet embedded into exopolysaccharides matrix and varied on density, created open spaces that probable are water channels. Exopolysaccharide was like many mucous joint together as “web cords” which coat microbe cells by common layer.

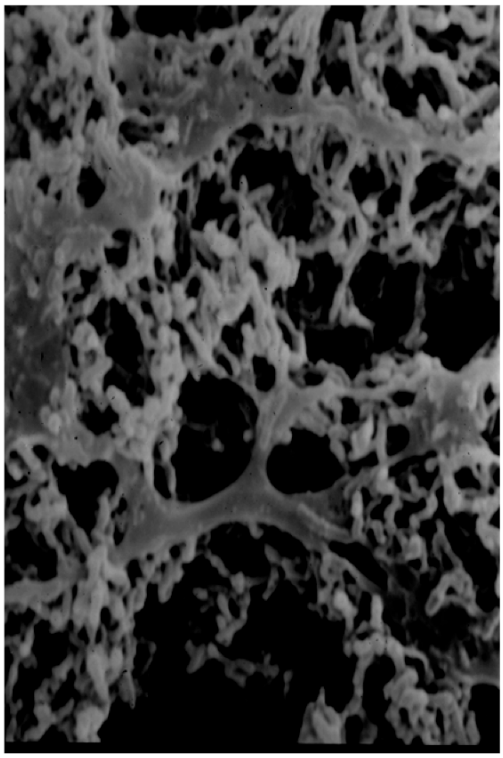

Figure 1: Scanning electron microscopy of fragment of *Corynebacterium diphtheriae gravis tox*+ biofilm culture (circulating) at cultivation during 720 hours. 4000 zoom.

There was difference in morphologic properties between type and biofilm cultures of circulated *Corynebacterium diphtheriae* strain (Figure **1**). It is known that size of microbe cells of *Corynebacterium diphtheriae* type culture vary in width from 0.3 to 0.8 mkm, in length from 1.5 to 8.0 mkm [2]. The width of *Corynebacterium diphtheriae gravis tox*+ circulated strain from biofilm culture after 720 hours of cultivation was the same (0.4±0.001 mkm), and length decrease ($p \leq 0.05$) to 1.3±0.05 mkm (Table **1**).

The process of biofilm formation had no effect on the ability of C. diphtheriae gravis *tox*+ diphtheriae exotoxin produce. Both standard and biofilm (720-hour) culture circulating strain C. diphtheriae gravis *tox*+ were positive in immunoprecipitation assay Elek, indicating that their ability to produce diphtheriae exotoxin. Presence *tox*+ - gene was detected by PCR in a typical and biofilm cultures diphtheria.

Comparative examination of cultural properties has revealed that sizes of biofilm colonies were lower (2-4 mm) then type culture (3-4 mm). Thereby colonies of Corynebacterium diphtheriae type culture has had convex, rough and shiny surface with granular, easy crumble structure, but colonies of biofilm cultures has had smooth and matte surface and creamy consistence.

Studying of biochemical properties found no difference between type and biofilm cultures of corybebacterias.

Estimation of antibiotic susceptibility (recommended in treatment of Diphtheriae) to circulated toxigenic strain of *Corynebacterium diphtheriae gravis* was found that type culture of that strain was susceptible to all samples that had MIC from 0.4±0.05 mcg/ml to 7.8±1.0 mcg/ml. 720 hours biofilm culture of *Corynebacterium diphtheriae gravis* circulated strain had decrease antibiotic susceptibility ($p \leq 0.05$) to ceftriaxone (MIC = 9.0±1.2 mcg/ml) and benzylpenicillin (MIC = 4.1±1.0 mcg/ml). Towards anaerocef (cefoxitine) was reverse characteristic: 720 hours biofilm culture of circulated strain had more high susceptibility to anaerocef ($p \leq 0.05$) then type culture of the same strain (MIC = 0.3±0.04 mcg/ml and 0.5±0.04 mcg/ml respectively).

DISCUSSION

It was established that Diphtheriae causative agent had ability to biofilm formation. It is known that process of biofilm formation is accompanied by exopolysaccharide matrix formation – product of microbe cells vital activity [4, 9]. Formation of exopolysaccharide by microbe cells of *Corynebacterium diphtheriae gravis tox*+ circulated strain was accompanied by changes of its size and spatial organization. Concentration of exopolysaccharide was maximal at 720 hour of cultivation, at this time there was decreasing of corynebacterias cells in size. Scanning electron microscopy revealed that bacterial cells of *Corynebacterium diphtheriae* circulated strain in biofilm structure had shapes as sticks with readily visible metachromatic bodies, the sticks exposed at random, and were gathering together

Table 1: Properties of Type and Biofilm Cultures of Circulated Strain of *Corynebacterium Diphtheriae Gravis tox+*

Biologic Properties	Type culture of *Corynebacterium diphtheriae gravis tox+* (mkm)	Biofilm culture of circulated strain of *Corynebacterium diphtheriae gravis tox+* (mkm)
Morphology (width× length of cells, mkm)	0.3-0.8×1.5-8.0	0.4±0.001 ×1.3±0.05
Cultural properties:		
Size of colonies (mm)	3-4	2-4
Shape of colonies	round	round
Color of colonies	milky (blood agar),	milky (blood agar),
	black (blood-tellurite agar)	black (blood-tellurite agar)
Character of the surface	convex, rough, shiny	convex, smooth, matte
Character of boarder	fringy	flame-shaped
consistence	crumbly	creamy
Enzymatic properties:		
glucose	+	+
maltose	+	+
sucrose	-	-
starch	+	+
Reduction of the nitrates	+	+
urease	-	-
cistinase	+	+
Toxigenity	+	+
Antibiotic susceptibility (minimal inhibitory concentration: M±m):		
vankomycin	1.3±0.3	1.7±0.6
cefotaxime	7.8±1.0	6.2±0.7
anaerocef	0.5±0.04	0.3±0.04*
gentamycin	1.0±0.7	1.8±0.6
ceftriaxone	4.5±1.0	9.0±1.2*
linkomycin	6.1±0.9	7.7±1.3
kanamycin	1.0±0.2	1.8±0.6
cefazolin	1.0±0.2	1.1±0.3
benzylpenicillin	0.4±0.05	4.1±1.0 *

Conventions: * - significant difference (p≤0.05) between MIC (minimal inhibitory concentration) type and biofilm cultures of *Corynebacterium diphtheriae gravis tox+* circulated strain.

into close connected clusters. There was free space between clusters that probably was water channels. Exopolysaccharides matrix as “web cords” totally covered microbe cells. Such morphologic properties of Diphtheriae agent bacterial biofilms can be explained by dampened growth of cells in biofilm in compare with planktonic cells, and, as result, this lead to slowing of metabolic processes, dissociation of R-form of colony into S-form, and lead to increase stability of biofilms in compare with planktonic form of cells. This can initiate mecha-nism of adaptation which is antibiotic susceptibility [5].

Against the background of the existing persistence strains C. diphtheriae gravis *tox+* in the treatment of various inflammatory diseases using broad-spectrum antibiotics, which can serve as the development of multiple antibiotic resistance exciter.

It was established that decreasing of antibiotic susceptibility of *Corynebacterium diphtheriae* circulated strain is connected with ability to microbe biofilm formation in the human body. Biofilms have getero-genic exopolysaccharide matrix which form total structure and fill up intercellular spaces with three dimension filter system formation [8]. Biofilms quench

peptidoglycane in result and inhibit antibiotic carrying to bacterial cells. We used those antibiotics that both as total or partially inhibit peptidoglycane synthesis (cefazolin, cefotaxime, benzylpenicillin), as inhibit enzyme transpeptidase, which take part in muco-peptide synthesis of cell wall (vankomycin, ceftriaxone, anaerocef). As a result MIC of antibiotic increase and susceptibility of biofilm culture decrease [5]. We suppose that using of these antibiotics in diphtheria treatment can lead to antibiotic resistance formation in biofilm of Corynebacterium diphtheriae bacteria. This makes good media for long persistence of diphtheria causative agent in bacteriocarriness body that compli-cate antibiotics treatment.

Thus, the post-epidemic period in the circulating strains of C. diphtheriae gravis *tox+* has the ability to biofilm formation by forming exopolysaccharide matrix. Corynebacterium in the biofilm changed their morpho-logy (cell size, their spatial organization) and cultural properties, keeping toxigenicity and acquiring resistance to antibiotics.

All of this, apparently, helps to create the conditions for long-term persistence of exciter of the diphtheria in the population and complicate the ongoing antibiotic therapy.

FINDINGS

1) Diphtheriae causative agent has the ability to biofilm formation that facilitate it ability to colonization of upper airway epithelium, increase defense against immune system and increase persistence in the body.

2) *Corynebacterium diphtheriae gravis tox+* circulated strain change its morphologic and cultural properties after long period of cultivation (720 hours).

3) The most effective towards Diphtheriae causative agent are cefotaxime, gentamycin, linkomycin, kanamycin and cefazolin, because *Coryne-bacterium diphtheriae* in biofilm structure have no changes in antibiotic susceptibility to these antibiotics.

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Received on 01-08-2014 Accepted on 18-08-2014 Published on 24-12-2014

DOI: http://dx.doi.org/10.12974/2311-8687.2014.02.01.4