Bacillus Spp. isolated from the conjunctiva and their potential antimicrobial activity against other eye pathogens

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Abstract

Background: In this study, we attempted to screen and investigate antibacterial activity of *Bacillus* species, which were isolated from conjunctiva, against other eyes pathogens.

Methods: To examine predominant isolates of *Bacillus subtilis, B. pumilus, B. cereus* and *B. mojevensis,* isolated from conjunctiva for their antimicrobial activity against indicator microorganisms as *Micrococcus luteus, Staphyloccocus aureus, S. epidermidis, S.hominis, S. lugdunensis, S.warneri, S. haemolyticus, B. cereus, Listeria monocytogenes,* and *Proteus mirabilis*. Growth inhibitions of indicator microorganisms were tested using agar diffusion tests by cells and supernatants of five *B. mojevensis,* one *B. subtilis*, four *B. cereus* and five *B. pumilus* strains which were isolated from conjunctiva.

Results: The *Bacillus* isolates showed variable ability of inhibition against the tested microorganisms. Two strains of *B. pumillus*, 1 strain of *B. subtilis*, 5 strains of *B. mojevensis*, 1 strain of *B. cereus* were efficacious against the tested microorganisms. Most resistant microorganism to these bacteria was *Proteus mirabilis*. Two of Gram positive bacteria, S. *lugdenensis* (K15-9) and *S. aureus* (SDA48), were also found as resistant.

Conclusions: In this study, *Bacillus* spp isolated from conjunctiva showed antimicrobial activity against Gram-positive bacteria. Human eye-derived microorganisms and their antimicrobial effects might be a useful source of natural products for the future.

Keywords: *Bacillus* spp, antibacterial activity, eyes pathogens, conjunctiva.

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Introduction

Application of antibiotics in the treatment of bacterial disease hasbeen a noticeable medical success in this century. However, gradual emergence and spread of antibiotic resistance among bacterial population due to wrong or excessive use of antibiotics has led to the development of public health problems.

Bacillus genus is made up of Gram positive aerobic or facultative endospore forming rod shaped bacteria. Bacteria of the genus *Bacillus* are known to produce natural products (1). They possess antagonistic activities against many bacterial and fungal pathogens and are often used as agents for the treatment and/or prevention of different plant and animal infections.

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Their antimicrobial activities have mainly been attributed to the production of antibiotic peptide derivatives as bacteriocins and bacteriocin-like inhibitory substance, lipopeptides, which have powerful surfactant like properties with numerous biotechnological applications including deemulsification, health care, and food industry (1) *Bacillus* spp. can produce antibiotics which are in peptid structure, such as bacitracin, polymyxin, tyrosidin, grmysidin, subtilin and sirkulin. For this reason, they have an important role in drug industry. Their antimicrobial activities have mainly been attributed to the production of antibiotic peptide derivatives and lipopeptides (1-3).

Coagulase- negative *Staphylococcus* (CNS) causes the vast majority of post- operative endophtalmitis cases. Intraocular infections with *S. aureus*, enterococci, *Bacillus* or Gram negative species are often intractable. Because of these, blindness or loss of the eye itself is not uncommon (4,5). Resistance to antibiotics in CNS is major concern. Penicillin resistance in CNS is very high (6,7). Methicillin-resistant *Staphylococcus* species, especially *S. aureus* strains, appeared in the hospital environment and acquired resistance not only to ß-lactam antibiotics but also to flouroquinolones,

chloramphenicol, clindamycin, tetracycline, and aminoglycosides (8). Recently, a decrease in the susceptibility of methicillin-resistant *Staphylococcus* species to vancomycin and teicoplanin has also been reported in several hospitals around the world (9-10).

The need for antibiotics and antimicrobials continues to be a major challenge for the treatment of infectious disease which affect millions of people worldwide. Furthermore, antimicrobial resistance is a growing concern. Also, the number of resistant bacteria and the geographic distribution of these organisms are both rising. We will need new products against these organisms. Human eye-derived microorganisms might be a useful source of natural products.

In this study, we screened *Bacillus* spp. strains, which were isolated from human eyes, for their antibacterial activity against human eye pathogens.

Material and methods

Bacterial cultures

Several *Bacillus* spp. were used as the producer strain. Strains of *Bacillus* spp. which were isolated previously from healthy eyes and stored as pure state were chosen. The nutrient agar was used for maintenance of the strain with 20% (v/v) glycerol at -86°C.

In this study, eye pathogens (*Staphylococcus aureus* SDA 40.2, SDA 48, *Staphylococcus epidermidis* KA 11.1, KA 14.1, KA 17.1, SDA 44, *Staphylococcus warneri* PCA 9.5, KA 11.2, *Staphylococcus hominis* PCA 6.3, PCA 9.2, PCA 9.3, *Staphylococcus lugdunensis* PCA 7.2, KA 15.9, *Micrococcus luteus* PCA 7.1, *Enterococcus faecalis* PCA 39.1.1, *Bacillus cereus* 13.2 PCA, *Listeria monocytogenes* 47 PCA and *Proteus mirabilis* KA 44.1) were used as test bacteria.

Screening for antimicrobial activity by cross-streak method

In primary screening, all *Bacillus* spp. isolates were streaked as a straight line at the centre of agar plates. These plates were incubated at 37°C for 24 hours. On the incubation, tested bacteria were inoculated using a single streak that was perpendicular to the *Bacillus* growth streaked at single straight line at the centre of the plate followed by incubation at 37°C for 24 hours. Inhibition zones formed were measured in millimeter (11).

Antimicrobial activity of cell free supernatant by well diffusion method

To extract bioactive compound from culture supernatant during the growth cycle, 28 producer strains *Bacillus* were separately inoculated using 200 ml sterile nutrient broth (NB) and incubated on a shaker at 37°C overnight 120 rpm for 48 hours. Cells were collected from a 48 hours culture by centrifugation (6000 rpm for 20 min, at 4°C) and the supernatant recovered and passed through a 0.22 µm filter.

The determination of the inhibitory effect of cell free supernatant of isolates on test bacteria was carried out according to the well diffusion method. Pre-poured agar media plates equilibrated were spread with 10^6 cfu/ml of respective test organism and allowed to dry. In the agar plates, wells of 6 mm diameter were cut using a cork borer. The wells were filled with 80 µl of cell free culture supernatant and incubated overnight at 37°C. The plates were then examined for clear zones of inhibition surrounding each well and inhibition zones were measured.

The test was duplicated for each *Bacillus* isolate.

Partial purification bioactive compound

The isolates were inoculated into flasks containing 100 ml nutrient broth and incubated at 37°C in a shaker at 120 rpm for 48 hours. After growth, culture media were centrifuged at 6000 rpm for 20 min, at 4°C and the supernatant recovered and passed through a 0.22 µm filter. Cell-free culture was extracted 3 times with an equal volume of ethyl acetate according to the method of Han et al. (12). Ethyl acetate was added to the supernatant in ratio of 1:1(v/v). The mixture was shaken vigorously for 10 min and separated. Subsequently, the ethyl acetate extract was pooled and evaporated to dryness under vacuum at 60°C for 20 min in water bath (13).

After extraction, bioactive compound, which were antibacterial activity, were chosen using disc diffusion methods. A 20-ml aliquot of partial purification bioactive compound was applied to disks (6 mm) placed on agar plates previously inoculated with a suspension of each eye pathogens. The plates were incubated at the 37°C for 24 hours. The diameter of inhibition zones was measured.

MIC test was applied to the partial purification bioactive compound, which had most antimicrobial activity. Therefore, Minimum inhibitory (MIC) values were determined using the method of two fold serial dilution (14).

Antibiotic Susceptibility Testing

Antimicrobial resistance patterns of test bacteria were determined by the agar disk diffusion method. Disks containing the following antibacterial agents were used: gatifloxacin (5 μg), cefuroxime (30 μg), ceftazdime (30 μg), vancomycin (30 μg), gentamicin (10 μg), amikacin (30 μg), ciprofloxacin (5 μg), lomefloxacin (10 μg), moxifloxacin (5 μg), methicillin (10 U). Characterization of strains as sensitive, intermediate or resistant was based on the size of the inhibition zones around each disk according to the National Committee for Clinical Laboratory Standards (CLSI) criteria (15).

Results

Screening for antimicrobial activity using cross-streak method

Twenty eight strains of *Bacillus* sp. which were isolated previously from healthy eyes were included in the study. These isolates were screened for antimicrobial activity against eye pathogens using cross-streak method. Therefore, it was noticed that 11 of 28 strains had antimicrobial activity against one or more eye pathogens.

Antimicrobial activity of cell free supernatant by well diffusion method

Cell free supernatant (CFS) of culture of 11 isolates were screened for antimicrobial activity against eye pathogens. The antimicrobial spectrum of cell free supernatant was given in Table 1.

Table 1. Antibacterial activity of cell free supernatant against tested bacteria by the well diffusion test (mm)

Test bacteria	*B. pumilus*PCA 4.2	*B.subtilis*PCA 11.2	*B. cereus*PCA 15.3	*B. mojavensis*3 PCA	*B. mojavensis*3M17	*B. mojavensis*KA 39.3
*S. hominis*PCA 9.2	12	10	12	14	14	12
*S. hominis*PCA 9.3						
*S. warneri*PCA 9.5	10	12	10	10	12	12
*B. cereus*13.2 PCA				16		
*S. epidermidis*KA 11.1	12			8		
*S. epidermidis*KA 14. 1				14		10
S. lugdunensis KA 15.9				12		
*P. mirabilis*KA 44.1	12	10	10	10	10	10
*S. aureus*SDA 40.2	12	12	12	14	14	12
*S. epidermidis*SDA 44	14	12	14	14	14	14

From this screening, 7 *Bacillus* sp. showed antimicrobial activity against test bacteria. *S. aureus* SDA 48, *S. epidermidis* KA 17.1, *S. warneri* KA 11.2, *S. hominis* PCA 6.3, *S. lugdunensis* PCA 7.2, *M. luteus* PCA 7.1, *E. faecalis* PCA 39.1.1, *B. cereus* 13.2 PCA, *L.monocytogenes* 47 PCA were found as resistant to all the cell free supernatant. While, *S. hominis* PCA 9.2, *S. warneri* PCA 9.5, *P. mirabilis* KA 44.1, *S. aureus* SDA 40.2 and *S. epidermidis* SDA 44 were found the most sensitive to the cell free supernatant.

Antimicrobial activity of partial purification bioactive compound

Partial purification of bioactive compound from liquid culture of *Bacillus spp.* was carried out. The antimicrobial spectrum of partial purification supernatant is given in Table 2. Nevertheless, Inhibitory activity observed on *S. hominis* PCA 9.2, *S. aureus* SDA 40.2, *S. warneri* PCA 9.5, *S. warneri* KA 11.2, *B. cereus* 13.2 PCA, *L.monocytogenes* 47 PCA, *S.epidermidis* KA 11.1, KA 14.1, KA 17.1, *S. lugdunensis* KA 15.9 , *M.luteus* PCA 7.1 *and E. faecalis* PCA 39. 1.1 were not inhibited.

Table 2. Antibacterial activity of partial purification bioactive compound against tested eye pathogens by the disk diffusion test (mm).

Eye Pathogens	S. hominis PCA 6.3	M. luteus PCA 7.1	S. lugdunensis PCA 7.2	S. hominis PCA 9.2	S. hominis PCA 9.3	S. warneri PCA9.5	E. faecalis PCA 39. 1.1	B. cereus13.2 PCA	L.monocytogenes47 PCA	S. epidermidis KA 11.1	S. warneri KA 11.2	S. epidermidis KA 14.1	S. lugdunensis KA15.9	S. epidermidis KA 17.1	P. mirabilis KA 44.1	S. aureus SDA 40.2	S. epidermidis SDA 44	S. aureus SDA 48
B. pumilus PCA 4.2		nt	nt	10					10	10	12	10	10					
B. pumilus PCA 9.4		nt	nt	10		12		12	10	10	10	12	10		10			
B. subtilis PCA 11.2	10	nt	nt	14		12		12	10	10	12	10	10		12	10	12	12
B. cereus PCA 15.3		nt	nt	10					10				10		10			
B. mojavensis PCA 24.1		nt	nt	10		14			10	10	12		10					
B. mojavensis 3 PCA		nt	nt	10		14		12	10	14	14	14	10		12		10	10
B. mojavensis 3M17		nt	nt	12	12	14		10	10			10	10		10	10		
B. mojavensis KA 39.3		nt	nt	10					10		12		10		10			
B. mojavensis 25-2-C2PX		nt	nt	10	12	14			10				10		10			

nt, not tested

The MICs were evaluated for all partial purification of bioactive compound MIC. *B. mojavensis* 3 PCA in Partial purification of bioactive compound showed the lowest MIC value. Although, there is interesting selectivity with good activity against MRSA *S. aureus SDA* 40.2 and *S. epidermidis* SDA 44 (4.31µg/ml), yet no activity against *S.hominis* PCA 9.2*, B. cereus* 13.2 PCA, *L. monocytogenes* 47 PCA and *S. aureus* SDA 40.2. While, these bacteria may need more substance of higher concentration. It was noticed that, MIC values of the other pathogens were found 8.63µg/ml. Notwithstanding, as we could not get enough substance from other strains so we could not find MIC values.

Antibiotic Susceptibility tested eye pathogens

Antibiotic resistance patterns of the eye pathogens were summarized in Table 3. Two *S. aureus*, five CNS cultures showed methicillin resistance. Three culture CNS showed no drug resistance.

Table 3.Sensitivities of eye pathogens to commonly used antibiotics (mm).

Test Bacteria	Cefuroxime (30 µg)	Methicillin (10 U)	Ceftazidim (30 µg)	Ciprofloxacin (5 µg)	Gentamicin (10µg)	Amikacin (30 µg)	Vancomycin (30 µg)	Gatifloxacin (5 µg)	Lomefloxacin (10 µg)	Montifloxacin (5 µg)
E. faecalis**PCA 39. 1.1**	**R**	**R**	**R**	**21**	**14**	**14**	**17**	**23**	**R**	**11**
S. aureus SDA 48	**14**	**R**	**19**	**23**	**20**	**R**	**7**	**R**	**26**	**R**
S. aureus SDA 40.2	**16**	**R**	**20**	**23**	**21**	**24**	**R**	**32**	**22**	**40**
S. epidermidis SDA 44	**30**	**16**	**22**	**24**	**25**	**14**	**19**	**21**	**20**	**26**
S. epidermidis KA 17.1	**R**	**R**	**R**	**R**	**30**	**R**	**17**	**11**	**R**	**20**
S. epidermidis KA 14.1	**30**	**R**	**20**	**R**	**26**	**21**	**23**	**26**	**R**	**23**
S. epidermidis KA 11.1	**16**	**R**	**23**	**35**	**14**	**14**	**R**	**32**	**35**	**26**
S. warneri PCA 9.5	**R**	**R**	**R**	**30**	**19**	**20**	**23**	**30**	**21**	**27**
S. warneriKA 11.2	**35**	**25**	**25**	**27**	**27**	**24**	**22**	**31**	**26**	**21**
S. hominis PCA 9.3	**24**	**21**	**21**	**30**	**20**	**27**	**15**	**11**	**36**	**21**
S. hominis PCA 9.2	**42**	**30**	**34**	**30**	**32**	**40**	**21**	**37**	**34**	**40**
S. hominis PCA 6.3	**46**	**30**	**26**	**37**	**44**	**33**	**11**	**40**	**43**	**36**
S. lugdunensis KA 15.9	**51**	**26**	**21**	**40**	**31**	**30**	**30**	**46**	**56**	**43**
S. lugdunensis 4PCA 7.2	**11**	**R**	**R**	**30**	**22**	**20**	**19**	**37**	**40**	**32**
P. mirabilisKA 44.1	**26**	**16**	**19**	**33**	**21**	**14**	**20**	**52**	**R**	**19**
M. luteus PCA 7.1	**40**	**12**	**26**	**26**	**32**	**21**	**21**	**31**	**22**	**27**
B. cereus**13.2 PCA**	**R**	**R**	**R**	**S**	**21**	**26**	**20**	**35**	**40**	**42**
L.monocytogenes**47 PCA**	**R**	**R**	**12**	**30**	**21**	**26**	**21**	**40**	**26**	**26**

Discussion

There are many species of the genus *Bacillus* which can produce a wide variety of antibiotics including bacitracin, polymyxin, colistin etc. On this note, several bacitracins were characterized; the bacitracin A is the commercial product (16).

The present research work was carried out using bioactive metabolites obtained from *B. pumilus* PCA 4.2, PCA 9.4, *B. subtilis* PCA 11.2, *B. cereus*PCA 15.3, *B. mojavensis* PCA 24.1, 3PCA, 3M17, KA39.3 and 25-2-C2PX.

The antibacterial activity of the *Bacillus* spp. was tested against different eye pathogens. These eye pathogens were Methicillin resistant *S. aureus* SDA 40.2 and *S. aureus* SDA 48, *S. epidermidis* KA 11.1, *S. epidermidis* KA 14.1, *S. epidermidis* KA 17.1, *S. warneri* PCA 9.5, *S. lugdunensis* PCA 7.2 while, other eye pathogens were *S. epidermidis* SDA 44, *S. warneri* KA 11.2 *S. hominis* PCA 6.3, *S. hominis* PCA 9.2, *S. hominis* PCA 9.3, *S. lugdunensis* KA 15.9, *M. luteus* PCA 7.1, *E. faecalis* PCA 39.1.1, *B. cereus* 13.2 PCA, *L.monocytogenes*47 PCA and *P. mirabilis* KA 44.1.

Antimicrobial activity of *Bacillus* spp. was noticed

from the work of other researchers. Oscariz and Pisabarro (17) isolated and identified cerein 7, which was a bacteriocin produced by *B. cereus* Bc7 and inhibits growth of *Listeria* spp. and other gram-positive bacteria. Bizani and Brandelli (18) isolated and identified cerein 8A. That was a bacteriocin produced by *B. cereus* that inhibits growth of *Listeria* spp. and *M. luteus*. Antibiotic production abilities of *B. subtilis, B. polymyxa and B. brevis, B. licheniformis, B. cereus* were showed by Yılmaz and Beyatlı (19). In our study, antimicrobial activity was noticed in 1 strain of *B. subtilis* and 1 strain of *B.cereus*. *B.cereus* partial purification bioactive compound had been effective over *S.hominis* PCA 9.2, *S. lugdunensis* KA 15.9, *L.monocytogenes* 47 PCA and *P. mirabilis* KA 44.1. Also, *B. subtilis*, showed effectiveness over test bacteria except *S.hominis* PCA 9.3, *S. epidermidis* KA 17.1 *and E. faecalis* PCA 39.1.1. *Bacillus cereus* produces several bacteriocin-like inhibitory substances (1). Tabarez et al. (20) reported an antimicrobial activity of substances produced by *B.subtilis*(soil isolate) against multidrug-resistant bacterial pathogen including methicillin-resistant *S. aureus*. El-Bana et al., (21) reported preliminary antimicrobial activity of substances produced by *Bacillus subtilis* NB-6 (air flora isolate), *Bacillus megaterium* NB-3 (air flora isolate) against a number of methicillin-resistant *Staphylococcus aureus* (MRSA). Effective antimicrobial compounds with a broad spectrum of activity against Gram positive and Gram-negative bacteria, and also against methicillin- resistant Staphylococcus clinical isolates (*S. hominis, S. epidermidis, S. aureus, S. haemolyticus, S. warneri, S. cohinii and S. scuiri*), were secreted by B. subtilis B38 strain into the culture medium (9). In this study, any of the biological active matters, which were tested, were not effected over *E. faecalis* PCA 39.1.1 and *S. epidermidis* KA 17.1. Accordingly, these pathogens showed resistance to antibiotics which are commonly used.

In this study, *B. pumilus* PCA 4.2 and *B. pumilus* PCA 9.4 showed antibacterial activity against methicillin resistant *S. epidermidis* KA 11.1 and *S. epidermidis* 14.1. Moreover, in one of the studies, *B.pumilu*s showed surfactin production (22) and in another study *B.subtilis* and *B.pumilus* showed antibacterial activity against many Gram negative and Gram positive bacteria (23). Hence, *B. pumilus* produces plasmid-encoded peptide pumilicins (1). However, pumilicins show remarkable antibacterial activity against MRSA, vancomycin-resistant *E. faecalis* (VRE) and several Gram-positive test bacteria (24). This not with standing, Awais *et al.* (25) studied inhibitory effects of a *Bacillus* sp. isolate on 2 pathogenic strains of *M.luteus* and *S.aureus*. In addition, Hasan et al. (26) have reported a compound produced by *B. pumilus* that inhibits *M.luteus* and *S. aureus*. Ma et al. (27) isolated three lipopeptids from *B. mojavensis* B0621A. One of these lipopeptids, which was identified as iturinic lipopeptid, showed bioactivity. This was named as mojavensis A. We found antimicrobial activity in 4 of 5 strains of *B. mojavensis*. Similar results were found by Kim et al., (28). Reportedly, mersacidin produced by *Bacillus* sp. HIL Y-85, 54728 inhibited the growth and colonization of methicillin resistant *S.aureus* (29).

Therefore, the identity of the bioactive compound produced by the *Bacillus* sp. is still unknown. Thus, further analysis by protein electrophoresis and MS/MS mass spectrometry may help to reveal the identity of the protein.

Conclusions

Bacillus spp. has been considered as a potential agent to control against eye pathogens. The bacteriocin or bacteriocin-like inhibitory substance, lipopeptides produced by the strain *B. mojavensis* 3PCA and *B.subtilis* PCA 11.2 may represent an antimicrobial substance with potential application in the prevention and treatment of eye infection. Although some bacteriocins from *Bacillus* present a narrow antimicrobial spectrum, the antibacterial activity of *B. mojavensis* 3PCA and *B.subtilis* PCA 11.2 were comparable to broad-range bioactive compound associated with *Bacillus* spp.

In this study, we have described the antimicrobial activity of substances produced by *B.subtilis, B. pumulis* and *B. mojavensis* against several methicillin-resistant *S. aureus, S. epidermis and S. warneri* strain which may serve as a promising development for new drugs against microbial pathogens. The inhibition of bacterial strains (methicillin-resistant *S. aureus S. epidermidis and S. warneri)* using B. *subtilis* PCA 11.2 and *B. mojavensis* 3 PCA may represent an antimicrobial substance with potential application in the prevention and treatment of eye infection and may be proposed as an alternative strategy for infection control.

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