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A SIMPLE METHOD FOR ISOLATION OF SOME *BACILLUS* STRAINS WITH AN EXPRESSED ANTI-CANCER ACTIVITY

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ABSTRACT:
There is now increasing evidence that probiotic bacteria can provide health benefits to humans. In many areas of medicine (gastroenterology, urology, allergology, oncology and others), these sanative microorganisms may be considered as possible and viable alternatives applicable to patient care. Particularly, we have found that oral administration of *Bacillus oligonitrophilus* KU-1 cells can be used for treatment and prevention of some tumors. Here we present a simple method for isolation of bacteria with anticancer properties from soil.

Keywords: probiotics, cancer, *Bacillus oligonitrophilus*, soil.

RESUMEN:
Está aumentando la evidencia de que hay bacterias probióticas que pueden proporcionar beneficios saludables a los seres humanos. En muchas áreas de la medicina (gastroenterología, urología, alergología, oncología y otras), estos microorganismos pueden considerarse como alternativas posibles y viables aplicables al cuidado del paciente. Particularmente, nosotros hemos encontrado que la administración oral de células KU-1 *Bacillus oligonitrophilus* puede ser utilizada para el tratamiento y la prevención de algunos tumores. Aquí presentamos un método simple para aislamiento de suelos, de bacterias con características anticáncer.

Palabras Clave: Probióticos, cáncer, *Bacillus oligonitrophilus*, suelo.

INTRODUCTION

Health benefits of probiotics have been well documented[1, 2]. In the last decade, many investigators have studied the therapeutic and prophylactic effects of some probiotic bacteria and found that oral administration of such microorganisms might modulate pattern of cancer and other diseases[3-14]. Microorganisms most commonly used as probiotics are lactic bacteria (for example, lactobacilli and bifidobacteria). However, it has been showed lately that other non-dairy microorganisms might also be used as probiotics [15-18].

In 2005, we reported that soil bacterium *Bacillus oligonitrophilus* KU-1 was used successfully as a monotherapeutic drug in cancer patients[19]. The theoretic background for use of silicate-degrading bacteria like B. oligonitrophilus KU-1 in medicine in was reported previously by Voronkov and co-workers[20-22] and discussed in our recent publication[23].

Here we present a simple technique for isolation from soil of some Bacillus strains with anticancer activity.

MATERIALS AND METHODS

Media.
All chemicals are reagent grade unless otherwise noted. Contents of the used media are presented in Table 1. The mixtures are brought to 1,000 ml with distilled H2O and autoclaved.

Table 1. Content of microbiological media used for isolation and cultivation of soil *bacilli*

Medium	Ingredients (g/L) and pH
Giss medium	Peptone 3, 65; agar 4.11; mannitol – 2.85; Na_2HPO_4 0.77; NaCl 3.55; aqua blue 0.03; aurin 0.03; pH 7.4±0.2.
Ploscirewi medium	Peptone 16; agar 8.75; salts of bile acids 8.1; lactose 7.6; sodium hydrocitrate disubstituted 8.82; neutral red 0.04; brilliant green 0.02; Na_2HPO_4 2.25; sodium thiosulfate 6.86; metallic iodine (I_2) 0.12; Na_2CO_3 1.42; pH 7.1-7.3.
Alexandrov medium	Na_2HPO_4 10; $(NH_4)_2SO_4$ 2; $MgSO_4$ 0.5; SiO_2 0.15; $CaCO_3$ 0.05; orthoclase 0.5; pH 8.0.
Nutrient agar	Peptone 5; meat extract 3; agar 15; $MnSO_4xH_2O$ 0.01; pH 7.0.

Extraction from soil and identification of bacteria.
Bacteria are extracted from soil samples taken from Samara Luka (Samara Region, Russia) by placing 500 mg of soil into a sterile Erlenmeyer flask with 2 mL of sterile distilled water. The flask is shaken at 200 rpm for 5 minutes and 20 µL of the slurry is serially inoculated to various growth media (Giss, Ploscirewi, Alexandrov media).

After incubation in Giss medium with mannitol at 22 0C for 24 h, single dark blue bacterial colonies should be scrapped by using a microbiological loop and cells should be drifted to Giss media with various carbohydrate supplements. In Giss medium with glucose, bacilli with anticancer activity are permanently dark blue. In Giss media with saccharose and lactose, these bacteria are usually dark blue. In Giss medium with maltose, anticancer bacteria are rarely dark blue.

Then, the selected bacteria should be tested in Ploscirewi medium, which can be used for selection of Zn group (*Bacillus oligonitrophilus* KU-2) as well as other groups with possible anti-cancer activity. These groups are resistant to various disturbing factors (diverse salts, etc) in this medium.

Further, Alexandrov medium with glucose (at 22 0C for 24-48 h without aeration, pH=8.0) may be used for selection: anticancer bacteria shows suspension growth (after 1-2 days) with bubble formation. During bacterial growth, the acidity of the medium decreases usually to a pH value of 4.0-4.5.

Antimicrobial sensitivity.
Finally, typing with antibiotics on the nutrient agar can also be used for selection of anticancer bacteria.

Table 2. Typing with antibiotics in *Bacillus* strains on nutrient agar

Antibiotic (a dose is indicated in brackets)	Diameter of inhibition zone (mm)			
	Bacillus oligonitrophilus		*Bacillus mucilaginosus*	
	Z_n (KU-2)	K_x-53 (KU-1)	D-2 (KU-6)	D_a (KU-5)
Ampicillin (30 µg)	15	15	16	17
Doxycyline (10 µg)	11	12	10	11
Kanamycin (30 µg)	16	17	16	16
Carbenicillin (25 µg)	9	16	18	6
Chloramphenicol (30 µg)	20	23	20	18
Monomycin (30 µg)	0	0	0	0
Neomycin (30 µg)	16	16	15	17
Oleandomycin (15 µg)	0	0	0	0
Polymixin (300 IU)	12	12	11	13
Rifampicin (5 µg)	0	0	0	0
Rystomycin (30 µg)	0	0	0	0
Fuzidin (10 µg)	0	0	0	0
Streptomycin (30 µg)	12	13	14	6
Cefalexin (30 µg)	0	0	0	0
Erythromycin (15 µg)	6	7	9	6

Note: 0 – total tolerance, sensibility – diameter of inhibition zone more than 15 mm.

Table 2 presents data on typing with antibiotics.

RESULTS AND DISCUSSION

Using the above-mentioned techniques, we have isolated four bacterial strains: Zn (KU-2), Kx-53 (KU-1), D-2 (KU-6), Da (KU-5). The first two strains belong to B. oligonitrophilus while the second two are B. mucilaginosus (according to 24). Anticancer activity of B. oligonitrophilus KU-1 has been tested with success in cancer patients [19]. Broad clinical trials are warranted to test the other strains.

It should be noted here that the use Giss media reveals a maximal ability of these bacteria to synthesize organic acids. This is of great significance because organic acids (succinic acid, lactic acid, etc) may inhibit saprogenous bacteria in the gastrointestinal tract reducing formation of carcinogens[25-26].

The subsequent selection in Ploscirewi medium makes possible to identify bacteria (normal growth, weak growth or absence of growth) that are resistant to severe conditions. This medium is considered to be unsuitable for growth of many bacteria because it contains metallic iodine, which is toxic to the bacteria. Moreover, bacteria grown in Ploscirewi medium should be tolerant to bile acids. This is very important for survival of bacteria in the human gastrointestinal tract and for their prolonged and efficient action there. Growth in Alexandrov medium and antibiotic-resistance tests is the final stages of selection.

By our experience, bacteria with anticancer features may be isolated with increased probability from soils in regions where geotectonic ruptures take place. It is very likely to isolate these bacteria from Alpine areas of Pamir, Cordilleras and Andes. However, our suggestions should be checked experimentally.

We hope that the presented material can be used for isolation of bacilli with anticancer activity and for further human benefit maintaining the balance of the gut microflora and favoring the equipoise in healthy and diseased individuals.

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This paper reports a method for isolation of some *Bacillus* strains that might have probiotic properties. There are some publications showing that these bacterias might have anticancer activity. The reported method may be useful for obtaining large amounts of these bacteria that allow the realisation of controlled clinical trials to confirm if they are beneficial for treatment or prevention of cancer.

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Cancer is doubtlessly one of the most difficult diseases to treat. Multiple mechanisms of tumor expression and resistance to medications complicate the search for a cure. New alternatives are always welcomed.

In this article, the authors present a simple method for isolation of bacteria that have shown antitumor properties and have been studied in cancer patients. Further studies are needed, however, and the publication of the method of isolation may be an important first step in that direction.

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