Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 5, Issue, 10, pp.2868-2871, October, 2013

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

A SELECTIVE CULTURE MEDIUM FOR SEPARATING LISTERIA MONOCYTOGENES BY TAKING BENEFIT FROM PROPOLIS (BEE GLUE)

[1]Fatemeh Bitajian, [2]Fatemeh Fallah, [2]Majid Ayatollahi, [1*]Raheleh Sadat Sajadi Nia

[1]Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2]Pediatric Infection Research Center (PIRC), Mofid Childeren`s Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Article History:
Received 07th July, 2013
Received in revised form
11th August, 2013
Accepted 20th September 2013
Published online 10th October, 2013

Key words:
Propolis, listeria monocytogenes, Culture medium, Muller Hinton Broth, Muller Hinton Agar and Brain Heart Infusion Agar.

ABSTRACT

High incidence of food listeriosis, especially listeriosis resulted from consumption of diary products has been reported to be as the second lethal cause resulted from food contamination after salmonella. Due to the importance of rapid diagnosis and treatment of this bacterium, there is a need to provide suitable culture medium for listeria growth and separating it from contaminated food products. The aim of this study was to investigate the possibility of applying a natural substance ;propolis (bee glue); in order to make a selective culture medium for separating listeria.Crude samples of propolis collected from 3 different regions were diluted in 30 ml of 70% ethanol. After performing the Minimal Inhibitory Concentration (MIC) and finding the effective concentration of propolis for listeria monocytogenes to grow and preparation of different dilutions, propolis was added to 170 plates containing 4 various media including Muller Hinton Broth, Blood Agar, Muller Hinton Agar and Brain Heart Infusion Agar and different strains of bacteria were cultured. All bacterial strains growth including: E.coli, Shigella flexeneri, Klebsiella pneumoniae, Staphylococcus auricularis, Shigella sounei, Pseudomonas aeroginosa, Salmonella typhi murium, Enterobacter aeroginosa and Staphylococcus aureus was inhibited except for listeria monocytogenes.
The effect of propolis for separating listeria monocytogenes was proved as the main effective substance in the new media.

INTRODUCTION

High incidence of food listeriosis, especially listeriosis resulted from consumption of diary products has been mentioned in many reports. This bacterium is pathogenic against human and animal and is considered as the second lethal cause resulted from food contamination after salmonella (Banskota *et al*., 2001; Gerald, 2002). 90% of patients suffering from listeriosis shall be bedridden at hospitals and the other 10% are considered as the carriers of this bacterium. Meanwhile, the death rate caused by listeriosis following consumption of contaminated foodstuffs in human has been reported to be 33%, while this figure is considerably higher for pregnant women, children and those suffering from immunodeficiency (Gracieux *et al*., 2003). Due to the importance of rapid diagnosis and treatment of this bacterium, there is a need to provide suitable culture medium for the growth of listeria and separating it from contaminated food products. However, those chemicals and antibiotics which are commonly used in media hinder the desirable growth of listeria and, reversely result in the growth of other microorganisms in the suspicious sample (Fernandes *et al*., 2001; Vaz-Velho *et al*., 2001). Therefore, due to the difficulty of separating listeria using normal media, there is a need for an exclusive culture medium for rapid separating and diagnosis of this bacterium. Propolis is a gum which is made by bees from the sprouts of some special trees. The propolis which can be found in the beehive is a mixture of many adhesive substances which have been collected by the bees from a variety of trees. Science propolis has been considered recently, the chemical compounds of it have not been well realized yet, although roughly 20 elements, of which most are of flavonoid type and indicate the antibiotic features of propolis, have been identified so far (Grang and Davey, 1990). High percentages of flavonoids in propolis are responsible for the major antibacterial characteristics of bee glue. In a study by Fernandes *et al*. (2005), antimicrobial effect of propolis against 25 species of Staphylococcus.aureus and its synergistic effect with 5 antimicrobial drug including Chloramphenical (CLO), gentamycin (GEN) netilmycin (NET), tetracycline (TET) and vancomycin (VAN) have been proved. In another study, the effect of bee glue to treat fungus chronic sinusitis and oral infections has been illustrated (Ban *et al*., 1983; Kovalik, 1979), however, it can be understood from the literature that this substance may not deactivate listeria monocytogenes. In the study of Aspoy *et al*. (1997) it was shown that bee glue has no controlling effect on the growth of listeria and may also be useful in separating this bacterium from other bacterial strains through inhibiting the growth of other bacteria rather than listeria. The objective of our study was to investigate the possibility of applying a natural substance (propolis) to make a selective culture medium for separating listeria, so that in addition to lower costs required for making such kind of medium, may also result in rapid diagnosis of listeriosis.

Corresponding author: Raheleh Sadat Sajadi Nia,
Shahid Beheshti University of Medical Sciences, Tehran, Iran.

MATERIALS AND METHODS

Crude samples of propolis were collected from 3 different regions; Eshgh Abad village, Dasht village and Russian village, Iran. Ethanolic extract of propolis was obtained diluting 5 gr crude propolis in 30 ml of 70% ethanol, and extracted at room temperature. After 3 days the extract was filtered.

Minimal Inhibitory Concentration (MIC): (tube dilution method)
Due to the fact that MIC of propolis to prevent listeria growth has been mentioned to be 7.5 µg/ml (Hsin-Yi *et al*., 2006); different dilutions of propolis are prepared so that the required dilution of 7.5 $^{\mu}$g/ml is achieved; in order to do so, 15 mg of propolis is made into powder and mixed with 85cc of distilled water to reach a volume of 100cc. Then 0.9cc distilled water is added to each of the 4 tubes containing Muller Hinton Broth (MHB). Then, from the tube containing propolis solution, 0.1cc is added to the first tube containing MHB and in the same manner from the first to the second until tube number 4, so that 0.1cc from the last tube is thrown away. After that, listeria monocytogenes is cultured on these media. In order to determine the Minimal Bacteriocidal Concentration (MBC), a loop is taken from the tube in which no opaqueness has been observed and cultivated on blood agar medium.

Preparation of different propolis concentrations from the dry substance

First of all by solving 2gr propolis in 20ml solvent (70% ethanol), the 10% propolis solution is prepared as the stock solution and other solutions are prepared consecutively. In order to do so, 1.5cc of the 10% solution is used with 8.5cc of the solvent to prepare $15x10^{-2}$cc dilution of propolis. After that, 5 pipettes are provided and 0.9cc of alcohol (70% ethanol) is added to each one, and then from the pipe in which $15x10^{-2}$cc dilution has been provided, 0.1cc is added to the first pipette and in the same manner, 0.1cc is poured from the first to the second pipette and the same trend continues until that 0.1cc is poured away from the 5th pipette. Then, three other pipettes, each containing 0.9cc solvent are provided and from the 5th pipette, which had a dilution of $15x10^{-7}$cc (15 µg/ml), 0.5cc is taken away and poured into the first pipette, the same trend is performed from the first to the second pipette and from the second to the third pipette so that the required 7.5 µg concentration from propolis is provided. Then 0.5 McFarland of the bacteria is cultured on each of the 4 pipettes. In order to prepare the dilutions, the contents of the sample shall be solved in certain amount of solvent so that the total becomes a factor of 10, and then the content of the sample is placed in the nominator and the resulted figure is placed in the denominator.

$$0.1cc \quad \frac{1/5}{1/5+8/5}=\frac{1/5}{10}=1/5\times10^{-2}cc$$

After performing the MIC and finding the effective concentration of propolis for listeria monocytogenes to grow and preparation of different dilutions (as mentioned above), propolis is fecundation onto 170 plates and different strains of bacteria are cultivated.

Applied culture media and their preparation methods

Muller Hinton Agar
Ingredients (g/lit)
Infusion from meat 2.0
Casein hydrolysate 17.5
Starch 1.5

Preparation method

34gr of Muller Hinton Agar powder was poured into 1lit of water and boiled until it becomes clear a little, and it was then placed into autoclave under 121 degrees Celsius temperature for 15 minutes, finally the solution is poured into the plates.

Blood Agar
Ingredients (g/lit)
Nutrient substrate (heart extract and peptones) 20.0
Sodium chloride. 5.0
Agar-Agar13.0

Preparation method

40 gr of the agar base powder is poured into 1lit of distilled water and boiled; after becoming clear, autoclaved under 121 degrees Celsius temperature for 15 minutes, after autolaving is finished and when the temperature reaches 40 degrees Celsius, a sheep blood vial equal to 50 mlit of blood is added and then it is poured into the plates.

Brain Heart Infusion Agar
Ingredients (g/lit)
Nutrient substrate (extracts of brain and heart and peptones) 27.5
D(+), Glucose2.0
Sodium chloride 5.0
di-sodium hydrogen phosphate 2.5
Agar-Agar 15.0

Preparation method

52gr of Brain Heart Infusion medium powder was poured into 1lit of water and boiled until it becomes clear, and then placed into autoclave under 121 degrees Celsius temperature for 15 minutes, finally the solution is poured into the plates.

Muller Hinton Broth
Ingredients (g/lit)
Infusion from meat 2.0
Casein hydrolysate 17.5
Starch 1.5

Preparation method

This medium is prepared in the same manner as Brain Heart Infusion medium with 34 gr of Muller Hinton Broth powder. After preparation of the media as mentioned above, the different dilutions of propolis is added by taking benefit from the cotton cap swab into the media and after a certain time that the solvent is evaporated, a little of those bacteria which were taken from laboratory (the standard and clinical samples including listeria monocytogenes obtained from Microbiology Departement of Shahid Beheshti Medical University and Mofid Childeren`s University Hospital respectively; after determination of the quality and species), is taken by loop (near the flame and under hood and the sus equal to 0.5 McFarland) and cultured linearly (2-3 big colonies in 1cc of broth agar or 3-5 small colonies in 1cc broth agar or 5×10^{8} cfu/ml equal to 0.5 McFarland).

Supplement preparation

After preparing the agar-base medium, and when the temperature reaches 45-50 degrees Celsius, 2cc of distilled water is added to it and stirred with a supplement vial and is then added to 1lit of the medium, then the resulted medium is incubated for 24 hours under 37 degrees Celsius temperature and aerobic conditions.

Adding supplement to Muller Hinton Agar medium

When preparing Muller Hinton Agar medium, after it is taken out of the autoclave and reached 40 degrees Celsius temperature, 1/10 of listeria monocytogenes` supplement is added and then the same as the previous media, propolis is added and bacteria are cultured in normal linear manner.,In the next phases, different bacterial strains were cultured solely or together in mixed manner on Muller Hinton Agar, Brain Heart infusion Agar and Blood Agar, and then various samples of propolis and its different dilutions are compared with each other and the control samples.

RESULTS

Effect of different dilutions of propolis on listeria monocytogenes growth

As mentioned earlier, 4 dilutions of propolis were used:

15 x 10-7cc
7.5 x 10-7cc
3.75 x 10-7cc
1.87 x 10-7cc

Meanwhile, these dilutions were added to 1/10 supplement + Muller Hinton Agar medium; the result was so that merely in the dilution of 15 x 10^{-7}cc (15 µg/ml), listeria monocytogenes had not grown (MIC), while growth was seen in all other 3 dilutions. Effect of various propolis dilutions on different bacterial strains growth (E.coli, Shigella flexneri, Shigella sounei, Klebsiella pneumoniae, Staphylococcus auricularis, Pseudomonas aeroginosa, Salmonella typhi murium, Entrobacter aerogenes, Staphylococcus aureu and Enterococcus spp.). First of all, different propolis dilutions (1.87×10^{-7} , 3.75×10^{-7} , 7.5×10^{-7} , 15×10^{-7}) were added to two media: Muller Hinton Agar and Brain Heart Infusion Agar and different bacterial strains were cultured; lack of all bacterial strains growth was seen in all the dilutions on both culture media. (Except for the growth of enterococcus species on Brain Heart Infusion medium).

MIC results

As mentioned earlier, opaqueness was observed in a pipette containing a concentration of 7.5 µg/ml of propolis. Eventually, MIC of propolis to prevent listeria monocytogenes growth was determined as 15µg/ml. Propolis MBC was also determined to be 15 µg/ml and due to the fact that other bacterial strains were not able to grow in such dilution, then the required dilution of propolis to provide an exclusive culture medium for listeria monocytogenes was found to be 7.5 µg/ml. Meanwhile, 7.5 µg/ml of propolis and 1/10 of supplement were added to Muller Hinton Agar medium and all other bacterial strains including E.coli, Shigella flexneri, Shigella sounei, Klebsiella pneumoniae, Staphylococcus auricularis, Pseudomonas aeroginosa, Salmonella typhi murium, Entrobacter aerogenes, Staphylococcus aureus and Enterococcus spp. were cultured on it; in all cases lack of growth was seen. Similarly,7.5 µg/ml of propolis and 1/10 supplement were added to blood agar medium all other bacterial strains which are able to grow in blood agar medium (as mentioned above),were cultured and lack of growth of all cases was observed (except enterococcus).

In a plate containing Muller Hinton Agar+1/10 supplement + 7.5 µg/ml of propolis, listeria monocytogenes together with other bacterial strains including E.coli, Shigella flexneri, Shigella sounei, Klebsiella pneumoniae, Staphylococcus auricularis, Pseudomonas aeroginosa, Salmonella typhi murium, Entrobacter aerogenes, Staphylococcus aureus and Enterococcus spp. were cultured, while eventually, merely listeria grew and other failed to grow.

Control samples

In order to ensure the results, as the controls:
1/10 supplement was added solely (i.e. without propolis) to Muller Hinton Agar medium and listeria monocytogenes was cultured, after which lack of growth was seen. In the same manner, 1/10 supplement was added solely (i.e. without propolis) to blood Agar medium and listeria monocytogenes was cultured, after which lack of growth was seen. Meanwhile, adding 1/10 supplement solely and without propolis to Muller Hinton Agar and Brain Heart Infusion Agar as the culture media for all other bacterial strains including E.coli, Shigella flexneri, Shigella sounei, Klebsiella pneumoniae, Staphylococcus auricularis, Pseudomonas aeroginosa, Salmonella typhi murium, Entrobacter aerogenes, Staphylococcus aureus and Enterococcus spp. resulted in their growth on these two media. Therefore, the effect of propolis for separating listeria monocytogenes was proved as the main effective substance in the new media.

Comparison of the effect of three types of propolis obtained from variousregions

Each time 7.5µg/ml of any type of propolis was added to Muller Hinton Agar + 1/10 supplement separately and listeria monocytogenes and other bacterial strains were cultured in colony count method; however, there was no significant difference between the 3 plates and listeria growth was obsereved in all 3 (Table 1, 2, 3, 4).

CFU results

In order to perform CFU, listeria monocytogenes was cultured in the new medium (containing Muller Hinton Agar + 1/10 supplement

Table 1. Culture results of Listeria monocytogenes and other bacteria on Muller Hinton Agar + 0.1 supplement + propolis from Dasht village

Staph aureus	Enterobacter aerogenes	Salmonella Typhi murium	Pseudomonas aeroginosa	Shigella sounei	Staph auric -ularis	Klebsiella pneum -oniae	Shigella flexeneri	E.coli	Listeria monocy -togenes	Bacteria / Agar
No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	Growth	Muller Hinton Agar + 7.5 µg/ml propolis + 0.1 supplement

Table 2. Culture results of Listeria monocytogenes and other bacteria on Muller Hinton Agar + 0.1 supplement + propolis from Eshgh Abad village

Staph aureus	Enterobacter aerogenes	Salmonella Typhi murium	Pseudomonas aeroginosa	Shigella sounei	Staph auric -ularis	Klebsiella pneum -oniae	Shigella flexeneri	E.coli	Listeria monocy togenes	Bacteria / Agar
No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	Growth	Muller Hinton Agar + 7.5 µg/ml propolis + 0.1 supplement

Table 3. Culture results of Listeria monocytogenes and other bacteria on Muller Hinton Agar + 0.1 supplement + propolis from Russan village

Staph aureus	Enterobacter aerogenes	Salmonella Typhi murium	Pseudomonas aeroginosa	Shigella sounei	Staph auric -ularis	Klebsiella pneum -oniae	Shigella flexeneri	E.coli	Listeria monocy togenes	Bacteria / Agar
No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	No Growth	Growth	Muller Hinton Agar + 7.5 µg/ml propolis + 0.1 supplement

Table 4. Comparing the effect of three types of propolis (7.5µg/ml) on growth of Listeria monocytogenes and other bacteria cultured on Muller Hinton Agar + 0.1 supplement

Propolis from Russian village		Propolis from Eshgh Abad village		Propolis from Dasht village		Propolis / Agar
Other bacteria	Listeria Monocytogenes	Other bacteria	Listeria Monocytogenes	Other bacteria	Listeria Monocytogenes	Muller Hinton Agar + 7.5 µg/ml propolis + 0.1 supplement
No Growth	Growth	No Growth	Growth	No Growth	Growth	

+ 7.5µg/ml of propolis), which resulted in observing 2 colonies in each cm^2 of the medium. Meanwhile, CFU was performed in the medium containing blood Agar + 1/10 supplement + 7.5µg/ml of propolis as well, which resulted in observing 1 colony in each cm^2 of this medium.

DISCUSSION

Concerning the different characteristics of propolis, including but not limited to anti-inflammation, anti-oxidant, anti-tumor and anti-microbial characteristics (Boyanova *et al*., 1989; Rezende *et al*., 2006) which are explainable due to the variability of its ingredients, in this study, the utilization of propolis (bee glue) of the honey beehive was focused to prepare a selective culture medium for the growth of listeria monocytogenes. One of the most important effective materials found in propolis are the flavonoids, which have given considerable therapy features to it, of these features, those focused by us includes the antibacterial effects of this substance. In a study performed by Junior *et al.* the antibacterial effect of ethanolic propolis extract against Staphyloccus aureus, Enterococcus and E.coli was observed (Fernandes *et al*., 2005). Meanwhile, in the study of Aspoy lack of different bacterial strains growth, except for listeria was proved for the media containing ethanolic extract of propolis (Aspoy, 1997). Listeria monocytogenes is considered as one of the highly dangerous bacteria causing meningitis especially in pregnant women, children and those suffering from immunodeficiency (Gerald, 2002); meanwhile, due to the fact that failure to diagnose and final cure of this infection makes it systemic and in some cases results in the appearance of lethal abscesses in brain, the importance of using a selective culture medium for rapid diagnosis of listeriosis is needed. Listeria is a fastidious bacterium, which requires a selective medium for growth; However preparing selective media such as PALCAM (Rezende, 2006) for the listeria to grow is very costly, therefore we decided to use propolis- which is a natural substance and easily available- to prepare an exclusive culture medium for the growth of this bacterium, which is of lower costs and naturally results in rapid diagnosis of listeriosis. We investigated the effect of propolis on other bacteria as well, and after achieving the MIC and MBC of this substance for listeria, we were able to make a suitable concentration of propolis for preparing an exclusive culture medium. In order to improve the growth of listeria and being more ensured concerning the prevention of other bacterial growth in this medium, apart from propolis, 1/10 supplement (with 0.01gr effective substance of Moxalactoms) was added and we then achieved a selective medium, in which merely listeria monocytogenes may grow and lack of growth of other bacteria was observed. The basis of this medium is Muller Hinton Agar in which enterococcus may not grow as well as other bacterial strains, such exclusive medium may be useful for the growth of listeria monocytogenes and lack of enterococcus growth. To sum up, food listeriosis is one of the fequent causes of hospitalization. Chemicals and antibiotics which are commonly used in media hinder the desirable growth of listeria, they reversely, result in the growth of other microorganisms in the suspicious sample. In this study, a new, naturally available, selective culture medium for listeria growth is provided using propolis.

Acknowledgement

We thank the head and honored members of Pediatric Infection Research Center for their general support. All financial supports were provided by Shahid Beheshti University of Medical Sciences.
Authors have no conflict of interest.

References

Aspoy E (1997). Selective effect of propolis in the treatment of patients with chronic fungal sinusitis. Nord Vet Med. 29 (10): 440-445.

Ban J, Popovic S, Maysinger D (1983). Cytostatic effects of propolis in vitro. Acta Pharm Jugosl. 33: 245-255.

Banskota AH, Tezuka Y, Kadota SH (2001). Recent progress in pharmacological research of propolis. Phytother Res.15:561-570.

Boyanova V, Drumeva D, Radoslavova Z (1989). Active prophylactic preporations fro the oral cavity containig propolis extract. Med-Biol Int. 6: 22-25.

Fernandes A, Balestrin EC, Betoni J, Orsi R, Cunha M, Montelli A (2005). Propolis: anti-Staphylococcus aureus activity and synergism with antimicrobial drugs. Mem Inst Oswaldo Cruz. 100 (5): 563-566.

Fernandes JRA, Leomil L, Fernandes H, Sforcin JM (2001). Antibacterial activity of propolis produced by Apis Mellifera and Brazilian stingless bees. J Venom Anim Toxins. 7 (2): 100-122.

Gerald M (2002). Listeria Food Poisoning. Inst of health science. 21: 101-111.

Gracieux P, Roche SM, Pardon P, Velge P (2003). Hypovirulant Listeria Monocytogenes strains are less frequently recovered than virulant strains on PALCAM and Rapid` L. mono media. Int J Food Microbiol. 83 (2) :133-145.

Grang JM, Davey RW (1990). Antibacterial properties of propolis (bee glue). J R Soc Med . 83 (3): 159-160.

Hsin-Yi Y, Chang CM, Chen YW, Chou CC (2006). Inhibitory effects of propolis extract on the growth of listeria monocytogenes. J Sci Food Agric. 86 (6): 937-943.

Kovalik PV (1979). The use of propolis in the treatment of patients with chronic fungal sinusitis. Vestn Otorinolaringol. 6: 60-62.

Rezende G, Pimenta F, Costa L (2006). Antimicrobial activity of two brazilian commercial propolis extracts. Brazilian Journal of Oral Sciences. 5 (16): 967-970.

Vaz-Velho M, Duarte G, Gibbs P (2001). Evaluation of enhanced haemolysis agar for detection of Listeria spp. and Listeria Monocytogenes from production lines of fresh to cold-smoked fish. J Microbiol Methods. 46 (2): 157-163.

****