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Original Research Article

An Observational Study to Examine the Potentiality of Escherichia Coli as Probiotic Against Shigella

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Abstract

Aim: Isolation, identification and functional characterization of Escherichia coli as probiotic against Shigella in Bihar, India.

Materials and Methods: This observational study was carried out in the Department of Microbiology, Madhubani Medical College Madhubani, Bihar, India, for 1 year. This study focused on poor hygienic regions firstly for the isolation of E. coli as probiotic strains against common pathogenic organism and secondly to evaluate misuse of antibiotics in a vastly populated city of Bihar. One of the hypothesis of this study presume that in the unhygienic areas E. coli from human sources might has the capacity to inhibit pathogenic organism like Shigella by which most of the people remain protected though their living and sanitation condition is very poor.

Results: First Gram staining experiment were done for each selected isolate. Gram staining result revealed that all the isolates were Gram negative. Five biochemical tests were carried out for the identification of selected E. coli. All of them found positive to Indole test, Methyl red test, Catalase test and negative to Voges-Proskauer test, Citrate utilization test. Molecular identification E. coli as a bacteria, coliform and faecal coliform was done by amplifying 16srDNA, lacZ and A gene, respectively. The PCR analysis of these genes resulted in 100% positive for all the eight selected isolates. These were identified by observing the band size with respect to DNA marker on 1.5% agarose gel on the basis of 800 bp, 874 bp and 147bp for the genes 16srDNA, LacZ and uidA. The antagonism of E. coli for Shigella was perceived by co- culturing Shigella with E. coli and observing their growth on the same plate. The result showed that in the co-culture E. coli effectively decreased the number of Shigella colony. On MacConkey agar plates, all the isolates of E. coli except Ec-AKS6 inhibited Shigella growth in co-culture. Shigella and E. coli colonies were also counted separately on MacConkey agar plates for comparison as control to evaluate culture condition.

Conclusion: This study demonstrated that E. coli strains from environmental source can act as a potent antagonist against enteric pathogen Shigella.

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Introduction

Antibiotics include a range of powerful medications that are used to treat diseases caused by bacteria as they destroy or slowdown the growth of bacteria. For decades, the marked increase in antibiotic usage has accelerated the natural phenomena ‘antibiotic resistance’. Antibiotic resistance happens when

bacteria develop the ability to defeat the drug that is designed to destroy them. In fact, antibiotic resistance has been named as 1 of the 3 most important health risks of 21st century by the World Health Organization.[1] Health complications caused by resistant microbes includes increased mortality rate, treatment difficulties and prolonged time of infection. Moreover, antibiotic resistant organism causes deaths which are of around 23 000 people annually in the United States.[2]

The impendence of antibiotic resistance is greater in developing countries because of the comprehensive misuse of antibiotics, lacking of inspection, poor quality of drugs etc.[3,4] Irrational prescribing by doctors, a habit of self-medication among patients[5] and the indiscriminate use of antibiotics in agriculture and farming in many areas of the country have also been found recently.[6]

Antibiotic resistance emerges not only in pathogenic and disease causing organisms but also commensal strains like Escherichia coli (E. coli) that is a member of the normal flora in the gastrointestinal tract of human and warm blood animals.[7]

E. coli belongs to the family Enterobacteriaceae, is a Gram- negative, rod shaped, non-sporulating, a non-fastidious, motile, and facultative anaerobic bacterium. E. coli is widely used as an indicator organism for the microbiological quality of water and food.[8] E. coli is widely dispersed in the natural environment (water, soil, sometimes plants used as food) through human or animal excretion. It is transmitted via fecal–oral route.[9] The existence of E. coli in nature is diverse, that range from exhibiting commensalism to those causing diseases on human or animal hosts.[10] The commensal E. coli when are exposed to antibiotics, are forced to develop different strategies to survive and grow in the toxic environment. Antibiotic resistant E. coli was found in healthy human stool, street food and drinks and surface.[11] If E. coli, especially the pathogenic ones are present in open environment and factors that influence their survival rate are very troublesome issues in case of disease occurrence.[10]

E. coli can develop resistance mechanism mainly by both the efflux pumps interruption and the resistance genes located on plasmids.[12] Plasmids are considered as the main vector in the procurement and propagation of multi-resistant either phenotypically or genotypically.[13] Horizontal gene transfer of plasmid encoded resistant genes is the prevalent mechanism at the origin of acquisition of antibiotic resistance and plasmid-encoded antibiotic resistance encompasses most currently used clinically relevant classes of antibiotics.[14]

This research will try to understand and hypothesize that some people who live in poor hygienic environmental condition may remain unaffected from shigellosis because they might contain E. coli in their gut which inhibits Shigella infection. In addition, in poor sanitation condition there should be competition among microorganisms.

Material and methods

This observational study was carried out in the Department of Microbiology, Madhubani medical college Madhubani, Bihar, India for 1 year. This study focused on poor hygienic regions firstly for the isolation of E. coli as probiotic strains against common pathogenic organism and secondly to evaluate misuse of antibiotics in a vastly populated city of bihar. One of the hypothesis of this study presume that in the unhygienic areas E. coli from human sources might has the capacity to inhibit pathogenic organism like Shigella by which most of the people remain protected though their living and sanitation condition is very poor. So, sample that is associated with human gut such human stool was our

primary target. The locations were visited and the situation was observed. These locations are very crowded. Huge number of people lives in these small areas. Most of them are day laborer. Most of the people use common latrines and the sanitation system is not developed.

Methodology

Aseptic condition was maintained while collecting sewage samples and after collection the samples were brought to molecular biology lab of the Department of Genetic Engineering and Biotechnology. The EMB agar media was prepared into a sterile conical flask and sterilized by autoclaving. After that the media was cooled to 45ºC and then was poured into sterile petridishes. The dishes were allowed to solidify. After solidification, a sterile micro wire loop for the semi- quantitative method was used for the plating and it has a 4.0 mm diameter designed to deliver 0.01 ml. A loopful of the uniformly mixed stool sample was inoculated into EMB agar plate (EMB contains dyes that are toxic to gram positive bacteria. It is a specialized media for gram negative bacteria. In EMB agar plate typical E. coli colony is usually characterized by green metallic sheen). The loop was sterilized using bunsen burner. After inoculation, all the plates were kept in the incubator in an inverted position at 37ºC for 24 hours. All the steps were done in laminar air flow that was previously swiped with 70% ethanol. There is always a bunsen burner light up while working in the laminar air flow. To maintain aseptic condition, hands were washed with 70% ethanol. From each sample spot 3 stool samples were collected (with in close proximity) in 3 separate tubes. Three stool samples instead of one stool sample were collected from same position to avoid experimental error as well as to increase the probability getting positive result for the target organism. After collection, samples carried to the lab in ice bag on the same day and perform streak plating (as explain above) in 3 separate EMB plate to get the single colonies of E. coli. The next day EMB agar plates were prepared and the previous plates were observed for bacterial growth. From the previous 3 plates the plate that shown perfect streaking pattern with characteristics single E. coli colony was selected for next step. Only one single colony that showed green metallic sheen were picked up with sterile inoculating loop and further inoculated in EMB agar plates. After inoculation, the plates were kept in the incubator in an inverted position at 37ºC for 24 hours. At 24 hours after incubation the plates were observed. On the same day, MacConkey agar media was prepared and sterilized and cooled to 45ºC-50ºC and poured into sterile petri plates. After solidification, these plates were also streaked by single colonies from previous EMB agar plates and then kept in the incubator for 24 hours. MacConkey is an indicator, a selective and differential culture medium that is used for the isolation of gram negative enteric bacteria and the differentiation of lactose fermenting gram negative bacteria. Lactose fermenting strains grow as pink colony and lactose non fermenting strains are colorless and transparent. The sample that showed positive results E. coli both in EMB and MacConkey agar was selected, coded and stored in slant for biochemical and molecular identification. According to above protocol, Nine single colonies were picked from 27 stool samples ((3 samples x 9 sample spots = 27 samples).of different sample spots and coded as Ec-CRS1 (E. coli Chittagong Railway Station 1), Ec-RS2 (E. coli Chittagong Railway Station 2), Ec-JHT3 (E. coli Jhautola station 3), Ec- AKS4 (E. coli Akbarshah 4),Ec-AKS5(E. coli Akbarshah 5). Ec-AKS6 (E. coli Akbarshah 6), Ec-KPM7(E. coli Karnaphuli market 7), Ec-KPM8 (E. coli Karnaphuli market 8).

Biochemical tests

Primarily selected E.coli colonies were confirmed by performing Catalase test, Indole test, Methyle red test, Vogues-praskeur test and citrate utilization test according to the procedure describe in Cowan and steel, 2004 and also by gram staining. [15,16]

Molecular identification

DNA extraction

Extraction of the genomic DNA from the isolated E.coli strains were conducted by boiling method.[17] DNA concentration was measured using Nano drop 2000 spectrophotometer.

PCR assay for the identification of bacteria, coliform and faecal coliform

In this study, molecular detection of organism was carried out by PCR using the previously published primers and targeted gene.[18] Primer specificity was determined by searching for similar sequences in microbial genome using the Basic Local Alignment Search Tool (BLAST). A PCR thermal cycler (NyxTechnik) was used for amplification and the PCR products were analyzed by 1.5% agarose gel electrophoresis. In each experiment, positive control (Previously identified E. coli,[16] was carried out as the standard genomic DNA along with negative control (PCR mixtures except genomic DNA). Target gene, primer sequence, cycling parameters, amplicon size is shown in the supplementary Table 1.

Probiotic activity test

Probiotic activity test was performed by co culturing Shigella and E. coli on the same plate. Shigella strain was provided from Microbiology lab of Department of Microbiology. Nutrient broth was taken in eight tubes and was inoculated with freshly prepared E. coli culture. Eight test tubes were inoculated with both Shigella and E. coli culture. One test tube was inoculated with Shigella culture. The tubes were incubated at 37ºC for overnight at shaking condition. The next day, each culture was serially diluted with in test tubes containing sterile distilled water up to 10−7 times. From each diluted solution, 1ml of solution was transferred into plate containing MacConkey agar media. The plates were stirred with hand gently clockwise and anti-clockwise so that sample was mixed thoroughly with the media. The plates were allowed to stand steady to solidify the media. After solidification, the plates were incubated in inverted position for 24 hours at 37ºC. The next day, colony counting was done by total viable count (TVC) method.

Statistical analysis

Statistical significance was evaluated with Student's t-test for repeated measurements. All values are represented as the means standard deviation for three-independent experiments.

Results

As mention in the methodology part, all the stool samples from different sample location were streaked on EMB agar plates for characteristic E. coli colony (metallic green sheen). EMB positive E. coli were further culture on MacConkey agar to observe characteristic E. coli colony (pink) on MacConkey agar. Finally, 9 single colonies from 27 stool samples that were collected from eight sample points of four different locations around Chattogram city were selected and coded (explained in detail in the methodology section) as E. coli according to selective plating result Table 2.

Biochemical test

First Gram staining experiment were done for each selected isolate. Gram staining result revealed that all the isolates were Gram negative. Five biochemical tests were carried out for the identification of selected E. coli. All of them found positive to Indole test, Methyl red test, Catalase test and negative to Voges-Proskauer test, Citrate utilization test. The biochemical test results are summarized in the Table 2. The biochemical test results confirmed the identification of all the primarily selected isolates as E. coli Table 2.

Molecular identification

Molecular identification E. coli as a bacteria, coliform and faecal coliform was done by amplifying 16srDNA, lacZ and uidA gene, respectively. The PCR analysis of these genes resulted in 100% positive for all the eight selected isolates. These were identified by observing the band size with respect to DNA marker on 1.5% agarose gel on the basis of 800 bp, 874 bp and 147bp for the genes 16srDNA, LacZ and uidA.

Probiotic activity test

The antagonism of E. coli for Shigella was perceived by co- culturing Shigella with E. coli and observing their growth on the same plate. The result showed that in the co-culture E. coli effectively decreased the number of Shigella colony. On MacConkey agar plates, all the isolates of E. coli except Ec-AKS6 inhibited Shigella growth in co-culture. Shigella and E. coli colonies were also counted separately on MacConkey agar plates for comparison as control to evaluate culture condition. The test was done in triplicate for statistical analysis

Table 1: Target genes, primers, cyclic condition, composition of PCR mixture and amplicon size

Target genes	Primer sequence 5´-3´	Cycling parameters	Composition of PCR mixtures	Amplicon Size (bp)
Bacterial: 16srDNA	AGAGTTGATCCTGGCTCAGa GACTACCAGGGTATCTAATb	5 min at 95◦C, 35 cycles of 95◦C for 40s, 57◦C for 72◦C for 1 min	For 20 µl: 10 µl master mix, 4µl template, 2 µla, 2µlb, 3µl water	803
Coliform: lacZ	ATGAAAGGCTGGCTACAGGAAGGCCa CACCATGCCGTGGGTTTCAATATTb	5 min at 95◦C, 25 cycles of 95◦C for 1 min and 72◦C for 1 min	For 20 µl: 10 µl master mix, 4µl template, 2 µla, 2µlb, 3µl water	877
Faecalcoliform: uidA	TGGTAATTACCGACGAAAACGGa ACGCGTGGTTACAGTCTTGCGb	5 min at 95◦C,30 cycles of 95◦C for 50s, 62◦C for 50s and 72◦C for 1 min	For 20 µl: 10 µl master mix, 4µl template, 2 µla, 2µlb, 3µl water	150

Table 2: Summarized results of microbiological analysis

ID	EMB	MCK staining	IT	MRT	CUT	VPT	CT	
Ec-CRS1	GMS	Pink G−	+	+	-	-	+	E. coli
Ec-CRS2	GMS	Pink G−	+	+	-	-	+	E. coli
Ec-JHT3	GMS	Pink G−	+	+	-	-	+	E. coli
Ec-AKS4	GMS	Pink G−	+	+	-	-	+	E. coli
Ec-AKS5	GMS	Pink G−	+	+	-	-	+	E. coli

Ec-AKS6	GMS	Pink G−	+	+	-	-	+	E. coli
Ec-KPM7	GMS	Pink G−	+	+	-	-	+	E. coli
Ec-KPM8	GMS	Pink G−	+	+	-	-	+	E. coli

Sample Colony character Gram Biochemical Tests Comments

GMS = Green metallic sheen, G−= Gram negative, IT= Indole test, MRT= Methyl red test, CUT= Citrate utilization test, VPT= Voges-Proskauer Test, CT= Catalase test. Ec-CRS1 (E. coli Chittagong Railway Station 1), Ec-RS2 (E. coli Chittagong Railway Station 2), Ec-JHS3 (E. coli Jhautola station 3), Ec-AKS4 (E. coli Akbarshah 4), EC-AKS5 (E. coli Akbarshah 5). Ec-AKS6 (E. coli Akbarshah 6), Ec-KPM7 (E. coli Karnaphuli market 7), Ec-KPM8 (E. coli Karnaphuli market 8).

Discussion

Our Laboratory has been focusing on antibiotic resistant bacteria since last 10 years. Previously, our lab had identified severe occurrence of antibiotic pollution in and around hospital setting in bihar. In this study we have shown antibiotic resistance profiling of eight E. coli isolates which have been previously isolated from 4 poor hygienic areas of bihar. We have recently reported the probiotic activity of these isolates against diarrheal pathogen Shigella.[20]

In this study, eight E. coli strains were isolated and identified through conventional microbiological analysis in order to examine probiotic activity against Shigella one of the causing agent of diarrhea in Bangladesh. The results of microbiological detection Table 2 of E. coli were similar as[.21,22] Along with culture based detection, molecular identification of the selected isolates were done by PCR amplification of the three genes; 16s rDNA for bacterial identification lacZ gene for coliform identification and uidA gene for fecal coliform identification.[16] All the amplified products showed bands on agarose gel electrophoresis showing positive results for identification. Bands of around 800 bp, 874 bp, and 147 bp were found respectively for the three genes in all sample isolates. Through molecular identification it was confirm that all the samples are coliform bacteria and they are from the intestines of warm blood animal. As the study areas of this research project were mainly the slum areas, it can be assumed that all the isolates are from human gut. [23]

The antagonism of E. coli for Shigella was perceived by co-culturing Shigella with E. coli and observing their growth on same plate. The statistical analysis of the result showed that in the co-culture, all the E. coli samples except Ec-AKS6 (E. coli Akbarshah 6), caused significant inhibition of Shigella. According to this result Ec-CRS1 (E. coli Chittagong Railway Station 1), Ec-RS2 (E. coli Chittagong Railway Station 2), Ec-JHS3 (E. coli Jhautola station 3), Ec-AKS4 (E. coli Akbarshah 4), Ec-AKS5 (E. coli Akbar Shah5), Ec-KPM7(E. coli Karnaphuli Market 7), Ec-KPM8 (E. coli Karnaphuli market 8) are considered as effective probiotic strain. The exact mechanism how E. coli inhibit intestinal pathogen is still not clearly understood. The inhibition activity might be due to the production of specific antimicrobial substances, such as microcins. However, the microcin negative isogenic mutant of E. coli has been shown to be as effective as the wild strain in competing with pathogenic bacteria. In fact, because of the narrow spectrum of

bacteriocin activity, it is unlikely to be responsible for the inhibitory effect of E. coli. 24 Effective adherence of E. coli to intestinal epithelial cells may block necessary receptors for attachment of invasive bacteria thereby inhibiting them.[24]

E. coli adheres strongly to the intestinal cell wall that results in a biofilm formation of nonpathogenic bacteria thus restricts the pathogenic bacteria. According to some other studies, E. coli Nissle 1917 and other probiotic strains may stimulate the synthesis of endogenous epithelial antimicrobial peptides such as human Beta Defensin–2 which helps to exert the beneficial effects of the probiotic strain. The growth and metabolic activity of E. coli may also cause changes in the pH or chemical composition of the colonic lumen that make the surface unfavorable to the pathogenic bacteria.[25] In this study, the sample isolates showed positive results by inhibiting growth of the pathogenic Shigella strain. This inhibition might be the result of competitive exclusion of the pathogen by E. coli by creating hostile micro ecology and competitive reduction of essential nutrients. Production and secretion of antimicrobial substances and selective metabolites can also be the reason of inhibition. To check this, toxicity test has been done to check whether E. coli supernatant has any effect in Shigella inhibition. [26] This experiment was performed but due to lack of proper equipment facilities E. coli supernatant could not properly separated.[27]

Conclusion

This study demonstrated that E. coli strains from environmental source can act as a potent antagonist against enteric pathogen Shigella.

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