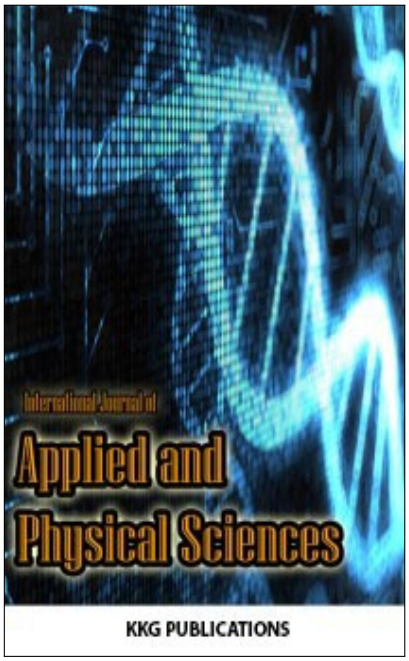

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MILK BORNE PATHOGENS: ISOLATION AND IDENTIFICATION HEALTH RISK TO POPULATION ESPECIALLY YOUNG ONES

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Abstract. Because of their nutritive value, milk and milk-made products are considered a very important food for all consumers, especially young ones. Consumers are at risk because of toxic chemicals and food-borne pathogens in milk, infants, young children, or persons with HIV/AIDS, cancer, diabetes, kidney disease, and transplant patients at greater risk. In the present study for milk-borne pathogens' identification, DNA of bacterial isolates was amplified using a universal set of forwarding primer pA 5'AGA GTT TGA TCC TGG CTC AG 3' (828) and reverse primer pH 5'AAG GAG GTG ATC CAG CCG CA 3'(1542 1522), corresponding to positions 828 and 15421522, respectively from Escherichia coli. 16s rRNA gene sequences were amplified and sequenced. Twelve microorganisms are Salmonella enterica, Citrobacter koseri, Escherichia fergusonii, Klebsiella pneumonia, Serratia liquifaciens, Shigella flexneri, Kosakonia sacchari and Enterobacter species, Klebsiella vericola, Escherichia vulneris, and Escherichia coli strains based on 16s rRNA sequences Submission to NCBI Gene Bank. Some of them have shown pathogenecity and resistance to some antibiotics, which should be properly identified for risk analysis to consumers. In our previous study, we have also reported the presence of carcinogens, phthalates, and hormone disrupters in milk. Food adulterants, chemicals, and pathogens' presence are major causes for developing the disorder in children that needs scientific community attention.

INTRODUCTION

In country like India milk and milk products are considered as center of food for human population especially for infants and children because of its nutritional value [1-2]. Milk and milk products can harbor different types of microorganisms and pathogens.

The presence of pathogens in milk and milk products may lead to a major health concern. There are more than 200 hundred diseases known which are transmitted through food by bacteria, fungi, viruses, and parasites [3].

The consumers are at risk because of these food borne pathogens but infants, young children or the persons suffering from HIV/AIDS, cancer, diabetes, kidney disease, and transplant patients are at greater risk. Individual immunity also is an added factor for diseases.

The presence of food borne pathogens in milk may be due to direct contact with contaminated sources in the dairy farm environment and to excretion from the udder of an infected animal.

There are many reported reasons of these pathogens to be present in milk. Generally Milk is pasteurized but raw milk is also taken by consumers in the form of cheese, ice-creams and curd etc.

Unpasteurized milk may have pathogens in milk or sometimes even pathogens are not destroyed by pasteurization [4-6]. There are microorganisms like Clostridium botulinum, Vibrio vulnificus, listeria, salmonella species and Shiga toxinproducing E.coli in food, which may cause disease. The presence of E.coli indicates lack of hygiene conditions from milk production to consumers at any place [7].

There are six pathotypes of E. coli that are associated with diarrhea and the one is Shiga toxin producing E. coli (STEC) also known as Verocytotoxinproducing E. coli (VTEC) or enterohemorrhagic E. coli (EHEC).

Most Escherichia coli are harmless and an important part of a healthy human intestinal tract but E. coli strains especially serotype O157:H7. E.coli O157:H7 is of major concern in dairy products and food because of causing haemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura [8, 9]. E.coli is considered as the major indicator of fecal pollution in food production because this bacterium usually does not survive food preservation processes [10].

The most commonly identified STEC in North America is E. coli O157: H7 (often shortened to E. coli O157). The

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number of cases of foodborne disease caused by E. coli O157: H7 ranged between 8,000 to 16,000 with 400 deaths (For Salmonella species, the number of cases ranged from 696,000 to 3,840,000 with 3,840 deaths [11]. Salmonellosis is the most common food borne bacterial disease around the world [12], causing diarrhea, cramps, vomiting and fever. The reason for this disease is related to consumption of unpasteurized milk or milk products [13]. Children younger than 5 have higher rates of Salmonella infection than any other age group. For food borne pathogens identification 16s rRNA gene sequences were amplified and sequenced for specific reason that all micro-organisms have this gene as multigene family. This is conserved gene with time with very few changes [14]. 16s rRNA gene is about 1550bp long and genera of bacteria may be. Gen bank has more than twenty million deposited sequences and out of them 90,000 sequences are of 16s rRNA which is large database to compare with the new sequences [15].

For identification of microbes traditional methods as growth on differential media and phenotypic characteristic were being used which may or may not identify correct genera of bacteria, so these days sequence analysis of 16s ribosomal RNA gene is globally used for identification and phylogenic study [16], [17].

Bacterial 16s rRNA have nine hyper variable regions showing significant sequence diversity among different species [18]. These hyper variable regions are flanked by conserved sequences that's why by using universal primer for conserved sequences hyper variable regions can also be amplified and compared [16]. In this study microorganisms are isolated on differential media but identified by 16s rRNA gene amplification by using universal forward and reverse primer from Escherichia Coli.

The aim of the present study was:

- Isolation of microbes from Milk collected from different regions of Delhi and NCR on Luria Bertoni and other differential media.
- Selection of these microorganisms on the basis of their phenotype
- Isolation of genomic DNA
- Amplification of 16srRNA sequences by using universal prokaryotic primer
- Sequencing by Xcelris Labs Ltd., Ahmadabad, India
- Sequence submission to Gene Bank for identification of microorganism genus and species.

METHODOLOGY

Isolation of Microorganism

Milk from different regions of Delhi was collected and homogenized in beaker. The samples were serially diluted with Mili Q sterile water (MilliQ Integral Water Purification System Merck Millipore) by using 10,100,1000,10,000 fold volume of water to 0.1ml milk (v/v). An amount of 0.1ml from each diluted sample was plated on different media like MacConkey (52g/L),XLD(56.68g/L),EMB(37.46),SS agar(63.02g/L)plates. The plates in inverted position were kept at 37[°C] overnight in incubator. Next day colonies were counted and CFU/ml calculated.

Organisms were selected according to their presence on differential media on the basis of their morphology and further purified by streaking again on the same media by picking single colony to get pure culture for further study. These isolated microbes were also tested for their pathogenicity by Hemolysis and Deoxyribonuclease test [19].

Molecular Characterization of Bacteria

DNA was isolated from all the isolated microbes by DNA isolation kit of Qiagen by PCR thermocycler (following the manufacturer's standard protocol). PCR of isolated DNA was performed with PCR Kit from Qaigen. Approximately 1.5Kb internal region of 16srRNA of above purified DNA of bacterial isolates was amplified using universal set of forward primer pA 5'AGA GTT TGA TCC TGG CTC AG 3' (828) and reverse primer pH 5'AAG GAG GTG ATC CAG CCG CA 3' (15421522), corresponding to positions 828 and 15421522, respectively from Escherichia coli [20].

The PCR reaction mixture (100 μl) contains 10ng DNA template, PCR mix 500μL (dNTPs, PCR Buffer, Taq polymerase enzyme) and 20 μL of each primer. The PCR program was as follows: initial incubation at 94[°C] for 5 min followed by 30 cycles at 94[°C] for 50 sec, 57[°C] for 30 sec at 72[°C] for 90 sec) and final extension at 72[°C] for 7 min. PCR products along with DNA 1Kb marker were run on 1.2% agarose gel electrophoresis at 60V with 1x TAE buffer, pH 8.2 to check purity and size of samples. The PCR products were sent to Xcelris Labs Ltd., Ahmedabad, India for sequencing.

The partial isolated sequences were submitted to BLAST search in the national center for Biotechnology Information (NCBI) database [31] for identification. Once identified from BLAST these sequences were submitted to Bankit for accession no. for individual sequences and confirmation of genus and species of micro-organisms.

RESULTS

Milk samples were collected from local dairy and local vendors from different parts of Delhi to analyze the presence of undesirable chemicals and food borne pathogens in milk to determine bio safety of the Milk.

Milk samples were homogenized first and plated on LB media by pour plate method to isolate single colony. XLD, MacConkey and EMB agar were used to identify gram negative bacteria and MRS for gram positive bacteria. SS media was used to identify salmonella and streptococcus. Many different types of colonies were isolated on LB plates. These single colonies were again inoculated in LB broth for getting rich culture for further plating and kept overnight at 37[°C] and 151 rpm. Next day again by streaking method isolated cultures were further purified on differential media. Colonies were identified on the basis of their shape, color and size. Colony forming units were calculated (table 1).

Genomic DNA isolation was done from these isolated cultures for 16s rRNA gene amplification. We identified their genus and species by 16s rRNA PCR by using the universal forward and reverse primers as mentioned in methods section. Twelve microorganisms were identified and 16s rRNA sequences were submitted to NCBI Gene Bank (table 2).

TABLE 1
QUANTIFICATION OF MICRO-ORGANISM FROM MILK SAMPLES ON DIFFERENTIAL MEDIA

SN	Type of media	Phenotype of colony	CFU/ml Mean value	Identification based on morphotypes on differential media
1	L.B	numerous	Not countable	
2	LB	White colonies	3.44×10^3	Microorganism
3	Mac Conkey	Small dark pink	1.25×10^4	E. coli
4	Mac Conkey	Dark center light periphery	25	Proteus species
5	MacConkey	Light pink	5.5×10^3	Enterobacter Shigella
6	MacConkey	Translucent white colony	9.80×10^2	Serratia
7	MacConkey	colorless	6.00×10^2	Salmonella
8	MacConkey	Pinkish sheen	3.00×10^2	Klebsiella
9	XLD	Pinkish red	2.98×10^2	Shigella
10	XLD	yellow	1.780×10^3	Escherichia coli
11	XLD	Red with black center	4.00×10^2	Salmonella
12	XLD	Grey with black center	30	Proteus
13	S. S Agar	colorless	3.980×10^3	Shigella
14	S .S. Agar	Colorless with black center	5.00×10^2	Salmonella
15	S.S. Agar	Pinkish	1.2540×10^4	Escherichia
16	S.S. Agar	Cream pink	7.900×10^3	Enterobacter
17	MRS	Small white opaque colony	3.3×10^2	Lacto bacillus Escherichia
18	EMB	Green metallic sheen	numerous	Enterobacter. Escherichia Klebsiella Serratia Citrobacter

We observed the microbial growth in the milk from 95% sample but the type of bacteria and their prevalence varied. By performing some biochemical tests (like hemolysis and DNase tests) some of these strains were found to be pathogenic which can cause severe diseases.

The isolated microorganisms were identified as Salmonella enterica, Citrobacter koseri, Escherichia fergusonii, Klebsiella pneumonia, Serratia liquifaciens, Shigella flexneri, Kosakonia sacchari and Enterobacter species, Klebsiella vericola, Escherichia vulneris, and Escherichia coli strains. Some of them have shown pathogenecity based on DNAse and hemolysis tests (table 2).

TABLE 2
IDENTIFICATION OF MICRO-ORGANISMS BY 16S RRNA PCR AMPLIFICATION AND SEQUENCE SUBMISSION TO GENE BANK FOR ACCESSION NO. AND FOR PUBLICATION

SN	NCBI Accession No.	Identified genera
1	KP941757	Salmonella enterica
2	KP941758	Klebsiella pneumoniae
3	KP941759	Escherichia coli
4	KP941760	Escherichia vulneris
5	KP941761	Enterobacter aerogenes
6	KP941762	Kosakonia sacchari
7	KP941763	Klebsiella vericola
8	KP 941764	Shigella flexneri
9	KP 941765	Serratia liquifaciens
10	KP 941766	Klebsiella pneumoniae
11	KP941767	Escherichia fergusonii
12	KP941768	Citrobacter koseri

DISCUSSION & CONCLUSION

In this present study Escherichia, Klebsiella, Enterobacter, Serratia, and Citrobacter collectively known as coliform bacilli, which are gram negative bacteria were isolated and identified [21]. E.coli, Serratia species and especially Serratia marcescens cause infections in humans, animals, and insects and show symptoms of sepsis, infection, fever, nausea, chills and vomiting and shock as also reported by [22-24].

Salmonella enterica has shown hemolysis and DNAse test. This was shown to have multiple antibiotic resistance by [25] that's why the major concern and its presence in milk may create health hazards in the consumers. E.coli strains which cause enteric disease like urinary tract infections, including prostatitis and pyelonephritis are classified as enteropathogenic serotypes (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enterohemorrhagic (EHEC), and enteroggregative (EAEC) strains. Klebsiella pneumoniae is a gramnegative, nonmotile, lactose fermenting, rodshape organism. This affects CNS and has symptoms of head pain, nausea, dizziness, and impaired memory [26].

K. pneumonia has two virulent factors, Lipopolysaccharide (LPS) and capsular polysaccharide (CPS) to cause sepsis. LPS contains lipid A, core, and Opolysaccharide antigen. CPS is essentially the outer layer of the pathogen containing polymorphonuclear cells, which create resistance against phagocytosis [27]. Children under 5 are at greater risk of developing hemolytic uremic syndrome (HUS), which causes acute kidney failure because of certain strain of E.coli. Pathogenic effects are reported from Shigella flexineri strain also [28].

Citrobacter species also cause meningitis, septicemia, and pulmonary infections in neonates and young children [29] and one citrobcter diversus is found to cause neonatal meningitis with high frequency of brain abscesses, death, and mental retardation in survivors. The identification of these bacteria is most accurate by 16s rRNA gene sequencing. There might be many reasons for the presence of these microorganisms in milk from fodder to consumers, which need to be recognized scientifically and should be taught to consumers for food safety. These microorganisms are reported to have very toxic effect on humans and especially on young ones. These organisms have also shown resistance to some antibiotics, which should properly be identified further for risk analysis.

Apart from these food borne pathogens, Phthalates, carcinogenic and organo toxic compounds were also present in milk sample as reported earlier by our team [30]. Diseases in the animals like mastitis and other adulterants in their feed may be responsible for many bacterial and chemical contaminants in milk leading to disorder and diseases in human. These problems should be addressed with more advanced scientific methods to make milk and dairy products healthy for consumers.

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