International Journal of Engineering and Applied Sciences

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DETECTION OF TOXIC *ESCHERICHIA COLI* STRAINS BY PCR TECHNIQUE

HARIKRISHNA YADAV. NANGANURU[1], ENZO POLAMBO[2]

SWINBURNE UNIVERSITY OF TECHNOLOGY, MELBOURNE, AUSTRALIA[1]
ASSOCIATE PROFESSOR, SWINBURNE UNIVERSITY OF TECHNOLOGY, MELBOURNE, AUSTRALIA[2]
harikrishyadav@gmail.com

ABSTRACT

Milk products are inhabitant complex system of bacteria. The samples which were obtained from cheese contained 27A, 9F, K12, 22B and 29C strains of bacteria. With the help of biochemical tests like indole, mrvp, Mc. agar, Motility tests identified the type of bacteria that is whether these belong to *E. coli* or not, and by using the genetic testing with help of Eric primers the different strains of *E. coli* could be identified, and using the toxic primers like STX, EAE and LT primers the toxic strain of *E. coli* could be identified by running the samples on gel electrophoresis and comparing the product size length. And finally the toxic strains were identified. All the samples were strains of E. coli bacteria and 22B was the best strain and it had shown high growth and motility. From the gel image of Eric primers, 9F, K12 and 22B are the different strains of E. coli. From the STX toxic primer, K12 and 22B are toxic strains of E. coli. So the samples collected from the soft cheese are all not toxic, only some strains like K12 and 22B are toxic. So the cheese, which has these toxic strains, is harmful to human beings.

KEYWORDS: *Escherichia coli*, Polymerase chain reaction, Virulent

1. INTRODUCTION

Milk products constitute a complex ecosystem for bacteria. Contamination of milk products with pathogenic bacteria is mainly due to processing, handling and unhygienic conditions. This soft, white cheese is made of pasteurized or raw milk. It is characterized by a high water content (43.0%) and low pH (5.1-5.6) (1). Many enteropathogenic microorganisms have been found in milk and dairy products such as cheese, which is usually stored under inadequate temperatures and consumed without any prior thermal treatment. They are frequently associated to outbreaks of food borne diseases (2). Domestic animals play important role in causing *E. coli* infections mainly cattle and sheep that can be asymptomatic vectors of virulent strains (3).

Two main kinds of sheep milk cheeses are made in Slovakia: ‘‘solid’’ sheep cheese and Bryndza cheese which is made also in Poland and Romania. Bryndza as traditional Slovak product which has been produced for centuries and is made from ‘‘solid’’ sheep cheese, prepared at sheep farms from unpasteurised milk. The ‘‘solid’’ sheep cheese is further transported to dairies, where in industrial conditions after possible adding of cow cheese (mainly in winter), Bryndza is produced. Because of the poor hygiene associated with sheep farms the production process holds a large risk of a microbial contamination. Such traditionally produced Bryndza cheese has a short durability and does not satisfy the current hygiene requirements of the European Union (4). Presence of *E. coli* in milk products indicates the presence of enteropathogenic microorganisms, which constitutes with major symptoms. *E. coli* is a normal inhabitant of the intestinal tract of humans and warm-blooded animals.

Although usually harmless, various *E. coli* strains have acquired genetic determinants (virulence genes) rendering them pathogenic for both humans and animals. These pathogens are responsible for three main clinical infections: enteric and diarrheal diseases, urinary tract infections (UTI), and sepsis and meningitis. On the basis on their distinct virulence properties and the clinical symptoms of the host, pathogenic *E. coli* strains were divided into numerous categories or pathotypes (5). *E. coli* frequently contaminates food organism and it is a good indicator of fecal pollution (6, 7 and 8).

Presence of *E. coli* in milk products indicates the presence of enteropathogenic microorganisms,

International Journal of Engineering and Applied Sciences

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which constitute a public health hazard. Enteropathogenic *E. coli* can cause severe diarrhoea and vomiting in infants, and young children (9). Toxic *E. coli* bovine origin has been categorised as enterotoxic *E. coli*. *E. coli* is the head of the large bacterial family, Enterobacteriaceae, the enteric bacteria, which are facultative anaerobic Gram-negative rods that live in the intestinal tracts of animals in health and disease.

The Enterobacteriaceae are among the most important bacteria medically. A number of genera within the family are human intestinal pathogens (e.g. Salmonella, Shigella, and Yersinia). Several others are normal colonists of the human gastrointestinal tract (e.g. Escherichia, Enterobacter, Klebsiella), but these bacteria, as well, may occasionally be associated with diseases of humans. The commensally *E. coli* strains that inhabit the large intestine of all humans and warm-blooded animals comprise no more than 1% of the total bacterial biomass. Pathogenic strains of *E. coli* are responsible for three types of infections in humans.

They are urinary tract infections (UTI), neonatal meningitis, and intestinal diseases (gastroenteritis). The diseases caused by a particular strain of *E. coli* depend on distribution and expression of an array of virulence determinants, including adhesins, invasins, toxins, and abilities to withstand host defences. These are summarized in and applied to the discussion of pathogenic strains *E. coli* Toxins: LT toxin, ST toxin, Shiga toxin, cytotoxins and endotoxin (2008 Kenneth Todar, PhD. Todar's online textbook of bacteriology). Strains of the same pathotype are genetically similar and carry the same virulence determinants involved in the infection. These virulence genes are ideal targets for the determination of the pathogenic potential of any given E. coli isolate (10). After an outbreak of food borne disease caused by enteropathogenic *E. coli* (11), the presence of these microorganisms in cheese acquired additional significance. Toxigenic *E. coli* of bovine origin has been classified into three categories: enterotoxigenic *E. coli* (ETEC), verotoxigenic *E. coli* (VTEC), and necrotoxigenic *E. coli* (NTEC). Coli forms could be found in cheeses and are used as a hygienic indicator for such products. The presence of coli forms in cheese and their relation to entropathogenic *E. coli* in soft cheeses has received considerable attention in previous studies (10 and 11).

The relation between pathogenicity and different serotypes of *E. coli* has been suggested and proved (12). Contamination of dairy products by EPEC strains has been investigated and O126, O128, O25 and O125 were isolated (13 and 14). The first group constitutes one of the most important vectors of *E. coli* diarrhea, and is considered the major cause of diarrhea in children in developing countries; it is also the most frequently etiological agent responsible for traveller's ' diarrhea (15). ETEC causes diarrhea by adhering to the intestinal mucosa by their unique colonization factors, producing either heat-labile enterotoxins (LT-I and LT-II), heat-stable enterotoxins (STa and STb), or both (15).

A few repetitive sequences have been reported in bacteria genome include enterobacterial repetitive intergenic consensus (ERIC) sequences, repetitive extragenic palindromic (REP) sequences, and BOX elements. The enterobacterial repetitive intergenic consensus (ERIC) sequences, also known as intergenic repeat units (IRUs) are present in many copies in the genomes of *Escherichia coli*, *Salmonella typhimurium*, and other enterobacteria (16). These elements are 126 bp long, highly conserved at the nucleotide level, and include a central core inverted repeat. The position of ERIC elements in enterobacterial genomes varies between different species and has been used as a genetic marker to characterize isolates within a bacterial species (17 and 18). The main objective of this paper is to characterize the pathogenic *E. coli* strains from given samples of cheese by using biochemical and genetic testing.

2. MATERIALS AND METHODS

2.1. Media preparation

Mc agar, Tryptone water, MRVP (Methyl Red & Vogues - Proskauer) medium and Motility mediums were prepared for different biochemical tests in respective compositions. For Mc agar medium, 10.4 gm of Mc agar was added in 200ml of distilled water and boiled it to dissolve. The surface of the gel was dried before inoculating. For Tryptone water, 1.5gm of tryptone was dissolved it in 100ml of water. For MRVP medium, 1.7gm of MRVP medium was dissolved it in 100ml of water. For Motility medium, 5gm of nutrient broth and 0.6gm of oxoid agar was added to this nutrient

International Journal of Engineering and Applied Sciences

www.eaas-journal.org

agar. This mixer was dissolved in 200ml of water. These all were sterilized by autoclaving at 121oC for 15min.

2.2. Microorganism samples

27A, 9F, K12, 22B and 29C microorganism samples were given by Enzo Polambo research group in Swinburne University of Technology. Those were 27A, 9F, K12, 22B and 29C. These samples were grown in all above media individually for different tests.

2.3. Biochemical tests

Biochemical tests (Indole, MRVP) were performed for the given microorganism samples to identify the bacteria and growth of the microorganisms were observed in Mc agar and Motility medium. The results were recorded.

2.4. DNA extraction

Each microorganism isolates were grown in 5ml of Luria-Bertani broth. From this 1.5ml was taken and centrifuged at 10000 rpm for 2min. The cell pellet was resuspended in 100µl of sterile double distilled water and held it in boiling water for 10min. Bacteria were then rapidly cooled by placing it on ice for 5min. This mixture was centrifuged at 12000rpm for 2min. The supernatant was removed and stored at -20oC. These samples were used as DNA templates for Polymerase chain reaction (PCR) analysis.

2.5. PCR sample preparation using Toxic primers

Eric, STX, LT and EAE forward and reverse primers were taken. For each toxic primer following tubes were prepared by adding 12.5 µl of Mango mix, 0.5 µl of forward and reverse primer, 1 µl of template and 10.5 µl of water to all microorganisms like following table:

	27A	9F	K12	22B	29C	Control
Mango mix	12.5µl	12.5µl	12.5µl	12.5µl	12.5µl	12.5µl
Forward Primer	0.5 µl	0.5 µl	0.5 µl	0.5 µl	0.5 µl	0.5 µl
Reverse Primer	0.5 µl	0.5 µl	0.5 µl	0.5 µl	0.5 µl	0.5 µl
Template	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
Water	10.5 µl	10.5 µl	10.5 µl	10.5 µl	10.5 µl	10.5 µl
Total	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl

(Table 1. Quantity and order of loading samples into tubes for PCR)

All tubes were prepared above serial like above table and made up to 25 µl. All samples were transferred into micro tubes. These tubes were loaded into PCR. Set all parameters including denaturation, annealing and extension temperatures in PCR. Time kept for these steps 6, 1 and 24 min. But annealing temperatures of Eric, STX, LT and EAE are 42, 45, 45 and 70^0C respectively. Control sample was also prepared and used this as a special template in a special tube.

2.6. Agarose gel electrophoresis of PCR samples

250 ml of 1X TBE buffer was prepared by adding 225ml of water to 25ml of 10X TBE buffer. 25 ml of 1% agarose gel was prepared by adding 25ml of 1X TBE buffer to 0.25gm agarose. Gel red added after dissolving agarose in TBE buffer. Agarose gel was casted in electrophoresis tray with sufficient

International Journal of Engineering and Applied Sciences

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number of wells. 1XTBE buffer loaded into electrophoresis tank until gel was submerged. Like this three sets of electrophoresis trays were filled with gel and buffer solution. 5µl of each PCR sample Eric 27A, Eric 9F, Eric K12, Eric 22B, Eric 29C, Eric control and 1kb ladder were loaded in separate wells of gel 1. In second gel electrophoresis set, gel wells were filled with STX and LT primer of bacterial strains. Third one was also filled up its wells with EAE primer of all sample strains. All These samples were run for 25 min at 110V with constant current supply. After running samples gel was kept in UV illumination chamber to observe DNA bands.

1.1. Details of Toxic primers from NCBI:

Primers	Forward and Reverse	Sequence of Forward and Reverse	Temperature	Product length expected
STX	F primer R primer	TGGTTGCGAAGGAATTTACC CGCCCTTCCTCTGGATCTAT	50.57 52.47	196
LT	F primer R primer	TGTTTCCACTTCTCTCTTAG TATTCCCTGTTACGATGT	42.59 42.59	258
EAE	F primer R primer	TGCGGGCACAGGCGGCGA CGGTCGCGGCACCAGGATTC	64.61 60.45	629

3. RESULTS

3.1. Biochemical Tests

Test	27A	9F	K12	22B	29C
Indole	+ve	+ve	+ve	+ve	+ve
MRVP	+ve	+ve	+ve	+ve	+ve
Mc agar	+3	+1	+2	+3	+1
Motility	+2	+1	+1	+3	+2

(Table 2. Biochemical test results, +3 = more growth, +2 = normal growth and +1 = Less growth)

International Journal of Engineering and Applied Sciences

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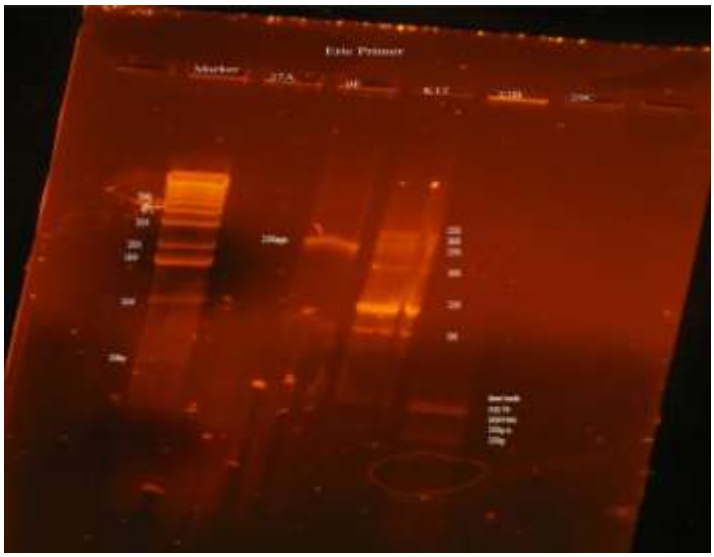
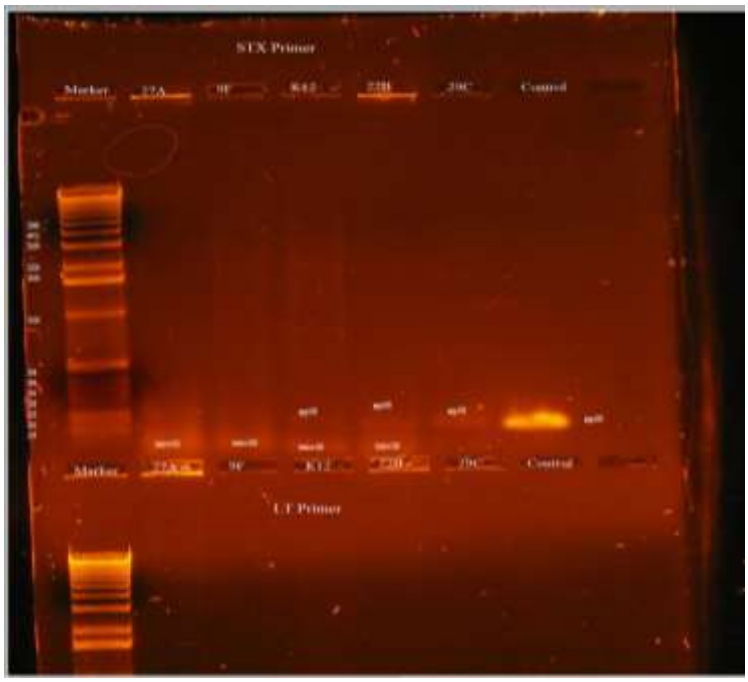
3.2. Gel images



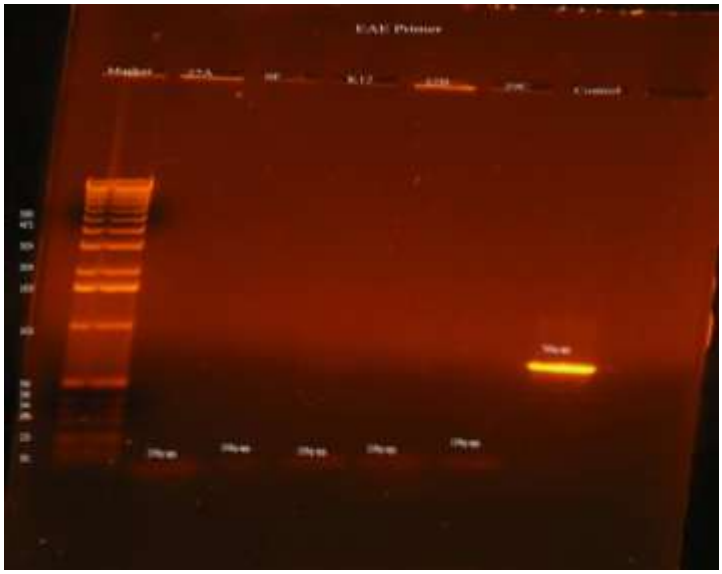
(Gel Image 1 →**PCR detection of Eric Primer on E.coli strains)**



(Gel Image→ 2 PCR detection of STX and LT toxic primer on E.coli strains)

International Journal of Engineering and Applied Sciences

www.eaas-journal.org



(Gel Image 3→ PCR detection of EAE toxic Primer on E.coli strains)

4. DISCUSSION

From the table 2, all biochemical tests results were positive. So the given samples were identified as E. coli bacteria. Mc agar test results for all samples such as 27A, 9F, K12, 22B and 29C were +3, +1, +2, +3 and +1 respectively. From this Mc agar test, 27A and 22B strains had been grown highly and K12 had been grown average and 9F had been grown lower. So the growth of the each strain in this medium clearly observed from this test. Motility test results for all samples such as 27A, 9F, K12, 22B and 29C were +2, +1, +1, +3 and +2 respectively. From this Motility test, Motility was high in 22B and average in 27A and 29C and Low motility in 9F and K12. From all the tests 22B had shown best results in all samples. From the gel image of the Eric primer bands were appeared for only three samples such as 9F, K12 and 22B. In those K12 got 6 bands at 3500, 2800, 2300, 1800, 1200 and 800bp locations. 9F got only one band at 2500bp and 22b got only one band between 500 to 200bp. Based on these bands, these three strains are completely different strains of E. coli. Actually the main aspect to use the Eric primers is to differentiate the E. coli strains clearly. From the observation of the gel image of the STX toxic primer we got bands for only three samples such as K12 and 22B. According to the NCBI data STX primer should have the product length expected as 200bp. But we got bands of both K12 and 22B in between 200 and 100bp. So it was nearly to the theoretical value. Based on these values these two strains are toxic. Cross contamination might be the main reason for could not get bands in rest samples. From the observation of the gel images of the both LT and EAE primers we could not get any bands for all samples. Due to the cross contamination or improper loading of samples into gel we could not get any bands.

5. CONCLUSION

From the biochemical tests concluded that all the samples were strains of E. coli bacteria and 22B was the best strain and it had shown high growth and motility. From the gel image of Eric primers concluded that, 9F, K12 and 22B are the different strains of E. coli. From the STX toxic primer concluded that, K12 and 22B are toxic strains of E. coli. So the samples collected from the soft cheese are all not toxic, but some strains like K12 and 22B are toxic. So the cheese, which has these toxic strains, is harmful to human beings.

International Journal of Engineering and Applied Sciences

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