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# Molecular characterization of *Escherichia coli* isolated from raw cow milk samples collected from district Bahawalpur, Pakistan

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## ABSTRACT

The present study was structured for molecular detection and characterization of *Escherichia. coli (E. coli)* isolated from cow's raw milk samples. A total of 100 milk samples were collected in a way that 10 samples each were collected from markets of Jamalpur, Hasilpur, Khairpur, Qaimpur, Lal Sohanra, Lal Sohanra Park, Yazman mandi, Rajkan, Ahmedpur East and Uch Sharif, district Bahawalpur, Pakistan. Milk samples were then cultured on different culture media for bacterial isolation. Identification of bacteria was done through Gram's staining, properties of bacterial culture on different selective media and biochemical tests including catalase and coagulase tests. Final identification was performed through PCR and resolution of PCR products by Gel electrophoresis. Out of 100 samples, 43 samples were found to be contaminated with *E. coli. E. coli* isolates were then amplified by *16S rRNA* gene-based PCR. Antimicrobial sensitivity test was also performed to confirm the susceptibility of *E. coli* to different antibiotics. Results of antimicrobial sensitivity test showed that the *E. coli* isolates were resistant to amoxicillin and erythromycin but sensitive to azithromycin, ciprofloxacin, gentamicin, norfloxacin and streptomycin.

**KEYWORDS:** *E. coli,* PCR, cow milk, antimicrobial sensitivity, Bahawalpur district.

## INTRODUCTION

Milk is a healthy and nourishing liquid, white in colour and is discharged by the mammary glands of adult female mammals. It is primarily produced for the upbringing of their young ones until they are able to take and digest semi solid or solid food. It is used by human beings as their main source of dietary calcium (Guetouache, Bettache, & Samir, 2014). Milk doesn't contain any infection when it is secreted in the udder but got contaminated with bacteria before it is milked out of the udder. However, bacteria present in milk at this stage are less in number and are unable to cause any disease except in cases of mastitis. Mostly the harmful infection of milk occurs during the process of milking, its improper storage, unhealthy techniques of handling and other activities performed before processing (Oliver, Jayarao, & Almeida, 2005). Contaminated milk may cause diseases such as tuberculosis, brucellosis,

lysteriosis and different kinds of gastrointestinal disorders as well as poisoning (Mortazavi & Gharehyakheh, 2014). It has been found in recent years that most of the illnesses caused by consumption of milk have been related to *Salmonella, Listeria monocytogenes, Campylobacter jejuni* and *Escherichia coli 0157:H7* (Pukančíková, Lipničanová, Kačániová, Chmelová, & Ondrejovič, 2016). Another type of milk contamination is chemical contamination. The use of chemicals in different fields of life especially in agricultural field may result in addition of these chemicals to the animal fodder through the soil, deposition of heavy metals such as lead, copper, zinc, chromium, iron, cadmium and manganese in the body of animals and ultimately their presence in the milk up to the toxic levels. Use of milk contaminated with heavy metals can lead to serious health conditions (Tunegová, Toman, & Tančin, 2016).

*Escherichia coli (E. coli)*, the organism of our concern in this present study, belong to the genus Escherichia and family Enterobacteriaceae (Tenaillon, Skurnik, Picard, & Denamur, 2010). These are rod shaped gram negative bacilli, about 2.0 µm long, 0.25-1.0 µm in diameter having cell volume of 0.6 - 0.7 $\mu m^3$ which live in large intestine of warm blooded animals including human beings (Eckburg et al., 2005). *E. coli* is transmitted to human beings mainly by consuming contaminated foods like raw vegetables and fruits, raw meat and raw milk. *E. coli* along with other facultative anaerobic bacteria makes approximately 0.1% of gut flora and feco-oral route is the main route for the transmission of disease producing strains. Commensal *E. coli* play vital roles in maintaining the gastrointestinal tract. *E. coli* produces vitamin K and helps in breakdown of food, food absorption and prevention of colonization by pathogenic bacteria (Tenaillon et al., 2010). *E. coli* can infect the gastrointestinal tract, urinary tract, enter the blood stream and cause sepsis or cause meningitis, particularly in neonates (Weintraub, 2007). Optimum growth of *E. coli* occurs at 37°C. *E. coli* grows in a variety of defined laboratory media such as lysogeny broth or any medium that contains glucose, ammonium phosphate, monobasic sodium chloride, magnesium sulfate, potassium phosphate dibasic and water (Christensen, Orr, Rao, & Wolfe, 2017).

Traditional methods for bacterial detection and identification include culturing, counting and isolation of required colonies. Subtyping of bacteria was at first carried out by studying their phenotypic characteristics and includes serotyping, biotyping and phage typing (Kretzer et al., 2007). Later, these phenotyping methods were replaced by identification through genotypic characteristics. The genotypic methods are more reliable and include PCR especially multiplex PCR (Paul, Van Hekken, & Brewster, 2013). In PCR, 16s rRNA gene is an amicable PCR amplification target as it is found in all bacteria and have shown much variation among species and strains (Srinivasan et al., 2015). Chromatography, Restriction endonuclease analysis followed by flouresence spectroscopy (Pingoud & Jeltsch, 2001), immunological methods like ELISA especially indirect or Sandwich ELISA (Gan & Patel, 2013), Flow Cytometry (Link, Jeong, & Georgiou, 2007), DNA microarrays (Law, Ab Mutalib, Chan, & Lee, 2015), use of amperometric, potentiometric and impedimetric biosensors are some methods which can be adopted for detection and identification of bacteria (Lazcka, Del Campo, & Munoz, 2007). Electronic Bacteria sensor is said to be a potential future tool which make use of creation of arrays of hundreds of sensors on an electric chip with the ability of each sensor to detect a specific type of bacteria as well as to show effectiveness of a specific antibody in short time (Heo & Hua, 2009). Each method has its own merits and demerits (Tamerat, Muktar, & Shiferaw, 2016).

In the present study standard PCR method had been adopted for the identification and characterization of *E. coli* in raw cow milk samples. Similar studies had been performed by different researchers throughout the world in regarding milk and contamination of milk by different microorganisms because milk is an important nutrient of diet (Mungai, Behravesh, & Gould, 2015) (Yaici et al., 2016) (Tadesse et al., 2018) (Wang et al., 2019) (Dell'Orco et al., 2019). However, very few works had been carried out on isolation, molecular detection and characterization of *E. coli* from raw cow milk, in Pakistan (Soomro, Arain, Khaskheli, & Bhutto, 2002) (Razzaq et al., 2016) (Tahira et al., 2017). By taking into consideration all these facts, the present study was designed with the objectives of molecular detection and characterization of *E. coli* isolated from raw cow milk samples using *16srRNA* gene. The antimicrobial susceptibility patterns of isolated bacteria were also studied.

## 2. Materials and Methods

### Sample collection

In the current research, 100 raw cow milk specimens were obtained randomly in the quantity of 5ml per sample from different areas of Bahawalpur district including Jamalpur, Hasilpur, Khairpur, Qaimpur, Lal Sohanra, Lal Sohanra National Park, Ahmedpur East, Head Rajkan, Uch Sharif and Yazman mandi. Samples were obtained in falcon tubes from January 2018n to February 2018. The collected milk samples were transferred to the ice immediately. Then the samples were transferred to VU laboratory Multan for bacteriological analysis and stored at -20$^{o}$C until the culturing and extraction of DNA. The reference bacterial samples for checking the specificity and sensitivity of PCR using species specific primers regarding *Escherichia coli, Streptococcus pyogenes, Bacillus anthracis*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Staphylococcus epidermidis* were taken from Institute of Molecular Biology and Biotechnology, BZU, Multan.

### Isolation and Identification of *Escherichia coli*

Milk specimen collected, were diluted to the desired concentration by addition of peptone water and inoculation was performed on MacConkey agar by pour plate method. Sub culturing was done on LB agar and MacConkey agar by streaking to get pure culture of *E. coli.* The isolates were then identified by gram staining, colony morphology on MacConkey agar and LB agar; biochemical characterization of the isolates through catalase and coagulase tests. Isolates were then further confirmed by amplification of *E. coli* specific *16S rRNA* gene.

### Bacterial Genomic DNA Extraction

Total DNA was extracted from samples following the protocol for bacterial genomic DNA extraction through phenol/chloroform method. The isolated genomic DNA was then checked on agarose gel.

### Primers design:

A set of primers having 468bp fragment length was used for the purpose of PCR amplification of 16SrRNA gene of *E. coli* as published by Shom et al., 2011 (Table 1). The primers used in

this study were synthesized by Pakistan Hospital and industrial Lab Co, Lahore. To check the specificity of each primer, BLAST program was used as it is in the NCBI website.

**Table 1** Primer sets used for identification of *Escherichia coli*.

| No. | Target bacterial species | Oligonucleotide primers 5'–3' | Fragment Length (bp) | Reference |
|---|---|---|---|---|
| 1 | *Escherichia coli* | F* GGTAACGTTTCTACCGCAGAGTTG<br>R* CAGGGTTGGTACACTGTCATTACG | 468 | Shome et al., 2011 |

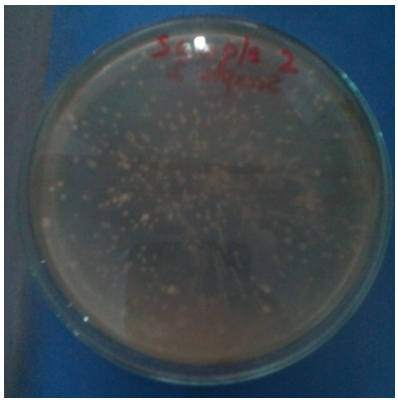
**Identification of *Escherichia coli* by PCR**

*Escherichia* genus specific PCR was performed to amplify 16S rRNA gene of *E. coli.* Each 20 µl reaction mixture comprised of 3 µl genomic DNA, 10 µl PCR master mixtures (Promega, USA), 1 µl of each of the two primers with the final volume up to 20 µl with 5 µl of nuclease free water. Amplification was performed through the process of initial denaturation at 95$^{o}$C for 5 minutes. Then denaturation took place at 94$^{o}$C for 45 sec. Annealing of primers was done at 55$^{o}$C for 45 sec. The extension was performed at 72$^{o}$C for 1 minute. The final extension was done at 72$^{o}$C for 5 min. 30 cycles got completed in this way. Resolution of PCR products was performed through electrophoresis. 2% gel for electrophoresis was made and resolution procedure continued for half an hour at 100 v. Gel was stained by addition of Ethidium bromide. Lastly the results were seen under UV trans-illuminator.

**Antibiotic sensitivity test**

The isolates of *E. coli* were then checked for antimicrobial drug susceptibility by well diffusion method. Sensitivity pattern of the isolates was established against ciprofloxacin, azithromycin, gentamicin, amoxicillin, streptomycin, erythromycin and norfloxacin. The results were then recorded and interpreted accordingly.

**3. Results and Discussion**





**Figures 1 & 2.** White creamy yellowish colonies of *Escherichia coli* grown on LB medium.

100 raw cow milk samples were tested and analyzed in the laboratory through miscellaneous methods including cultural, biochemical, staining and molecular ones in order to detect *E. coli* in these samples. 43 samples were found to be contaminated with *E. coli.* In MacConkey agar medium, *E. coli* colonies are represented by bright pink or red colonies. In LB agar, the colonies of *E. coli* were seen as white creamy yellow colonies (fig 1&2). Gram staining and subsequent microscopic examination of the smears taken from colonies grown on LB agar showed gram negative, pink colored, small rod-shaped organisms arranged in single pairs or small chains. *E. coli* were further confirmed by catalase and coagulase tests which showed negative results in both cases. DNA extracted from *E. coli* isolates was then used in PCR assay. The results revealed that extracted DNA was fit for PCR amplification. The procedure of DNA extraction was acknowledged as adequate. It had the potential to eliminate PCR inhibitors such as was considered satisfactory and was able to remove PCR inhibitors such as proteins etc. causing interference in PCR reaction. The purity and yields of total DNA extract achieved from reference and collected cow milk samples were high. DNA was then extracted from isolated *E. coli* and used in PCR assay. The primer specificity results showed that no cross-reactivity of the primers was observed with other bacterial species including *S. pyogenes, B. anthracis, S. typhimurium, S. aureus, S. agalactiae* & *S. epidermidis.* PCR products were also not obtained for the samples of negative control. Every test was performed four times to ascertain reproducibility of PCR results.

In early phase of this research, PCR was done by using DNA extracted from *E. coli* obtained from cow milk samples. PCR primers targeting 16S rRNA gene of *E. coli* amplified 468 bp fragments of DNA confirmed the identification of *E. coli.* The specificity of PCR assay of DNA from *E. coli* can be seen in Figure 3. It could be observed that negative control and reference samples of *Streptococcus pyogenes, Bacillus anthracis*, *Salmonella typhimurium*, *Staphylococcus aureus, Streptococcus agalactiae* and *Staphylococcus epidermidis* showed no bands while positive control containing *E. coli,* yielded PCR fragments of 468bp which are specific for *E. coli*.

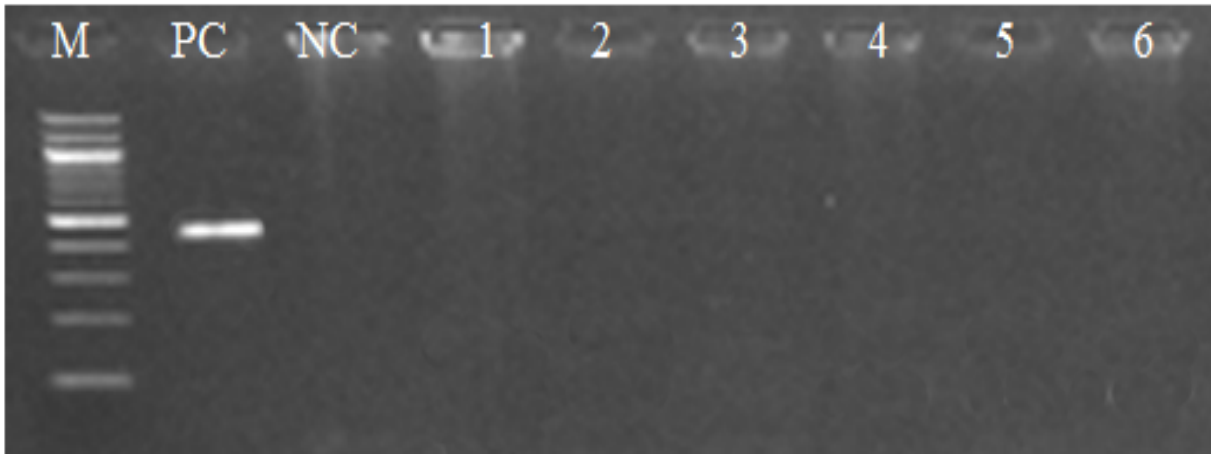



**Figure 3** Specificity of PCR assay of DNA from *Escherichia coli* sample: M: marker, 100 bp, NC: negative control (reagents with primers without DNAs) PC; *Escherichia coli* DNA, (1) *Streptococcus pyogenes*, (2) *Bacillus anthracis*, (3) *Salmonella typhimurium*, (4) *Staphylococcus aureus*, (5), *Streptococcus agalactiae* (6) *Staphylococcus epidermidis*.

To assess sensitivity of the employed method, PCR assay was performed for *E. coli* identification in raw cow milk samples. Evaluation of PCR sensitivity for *E. coli* DNA was performed. The PCR assay for sensitivity was performed with negative control containing reagents with primers but devoid of DNA, positive control containing *E. coli* DNA and its dilutions ranging from 50ng to 0.01ng as shown in Figure 4. The PCR product was resolved on agarose gel in order to check the sensitivity. Sensitivity results for reference samples indicated that sensitivity of this PCR assay was up to 0.01ng.

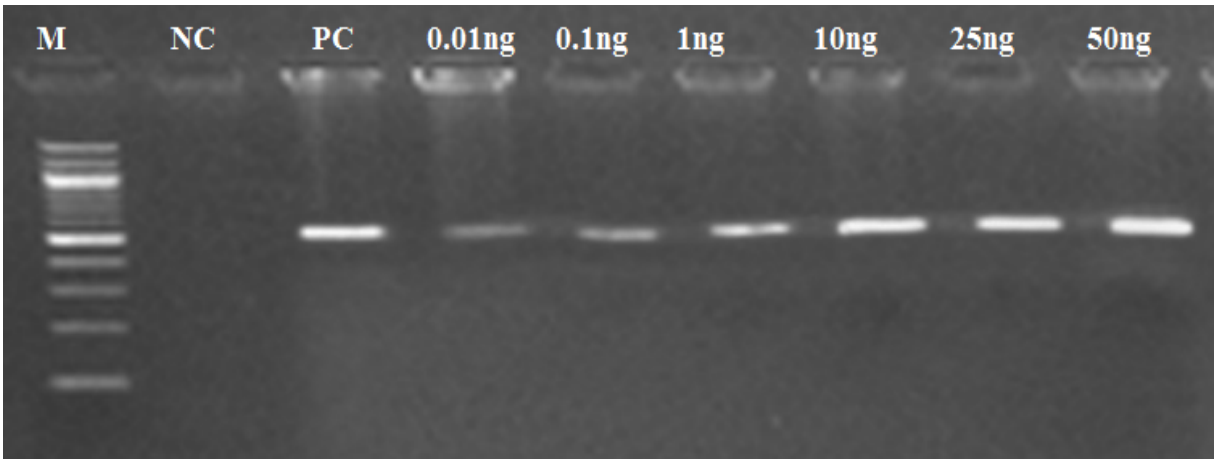



**Figure 4** Evaluation of PCR assay sensitivity for Escherichia coli DNA sample; M: marker 100 bp. NC: negative control (reagents with primers without DNAs) PC; Escherichia coli DNA, (1) 0.01ng (2) 0.1ng, (3) 1ng, (4) 10ng, (5) 25ng, (6) 50ng

**Application of PCR on field cow milk samples**

The application of PCR assay on raw cow milk samples gathered from different tehsils of Bahawalpur district has been represented in Tables 2 & 3which show the accurate percentages of *E. coli* in raw cow milk samples. The results of PCR assay for identification of *E. coli* showed that out of 100 samples collected from various tehsils of Bahawalpur district, samples collected from Jamalpur showed *E. coli* contamination in 4 samples making the percentage upto 40%. Samples collected from Hasilpur showed *E. coli* contamination in 6 samples making the percentage up to 60%. Samples obtained from Khairpur showed *E. coli* contamination in 3 samples making the percentage up to 30%. Samples obtained from Qaimpur showed *E. coli* contamination in 2 samples making the percentage up to 20%. Samples obtained from Badar Sher showed *E. coli* contamination in 4 samples making the percentage up to 40%. Samples which were collected from Badar Sher National Park showed *E. coli* contamination in 5 samples making the percentage up to 50%. Samples taken from Yazman mandi showed *E. coli* contamination in 3 samples making the percentage up to 30%. Samples obtained from Head Rajkan showed *E. coli* contamination in 4 samples thus making the percentage upto 40%. The samples collected from Ahmadpur East have shown *E. coli* contamination in 6 samples making the percentage up to 60%. Samples collected from Uch Sharif showed *E. coli* contamination in 5 samples making the percentage up to 50%. On an average, overall percentage of 43% of contamination of milk samples was detected.

**Table. 2** Results of PCR for identification of *Escherichia coli* from the milk samples collected from cow from various areas of district Bahawalpur

| Sr# | Jamalpur | Hasilpur | Khairpur | Qaimpur | Lal Sohanra |
|---|---|---|---|---|---|
| 1 | - | - | - | - | + |
| 2 | - | + | - | - | - |
| 3 | + | + | - | + | - |
| 4 | + | + | + | - | + |
| 5 | - | + | - | - | - |
| 6 | + | - | - | - | + |
| 7 | - | + | + | + | - |
| 8 | - | + | - | - | + |
| 9 | + | - | - | - | - |
| 10 | - | - | + | - | - |
| | 40% | 60% | 30% | 20% | 40% |

**Table 3** Results of PCR for identification of *Escherichia coli* from the milk samples collected from cow from various areas of district Bahawalpur

| Sr# | Lal sohara Park | Yazman Mandi | Head Rajkan | Ahmadpur East | Uch Sharif |
|---|---|---|---|---|---|
| 1 | - | - | + | + | - |
| 2 | + | + | - | + | + |
| 3 | + | - | - | - | - |
| 4 | + | - | + | - | + |
| 5 | - | + | - | + | + |
| 6 | - | - | - | - | - |
| 7 | - | + | + | + | - |
| 8 | + | - | - | - | - |
| 9 | + | - | - | + | + |
| 10 | - | - | + | + | + |
| | 50% | 30% | 40% | 60% | 50% |

These results showed that cow milk samples from different tehsils of Bahawalpur district have variations in percentages of contamination by *E. coli.* This might be attributed to the differences in environment and milking practices at different farms, handling of utensils, hygienic practices and modes of transportation of milk from remote areas to the markets.

Assessment of *E. coli* isolates was performed regarding antibiotic susceptibility. The results of antimicrobial susceptibility test showed that susceptibility for azithromycin by *E. coli* isolates came out to be 53%. Susceptibility of *E. coli* isolates for Streptomycin came out to be 66%. The susceptibility of *E. coli* isolates regarding gentamicin was determined as 86%. The susceptibility of *E. coli* isolates for norfloxacin came out to be 80%. The susceptibility of *E. coli* isolates for tetracycline came out to be 66%. On the other hand, resistance shown by *E. coli* isolates to amoxicillin came out to be 86%. The resistance shown by *E. coli* isolates to erythromycin was determined as 73%.

The efficiency of PCR technique is influenced by different parameters, the most important of which is composition of primers in relation to each other as well as to the target DNA. The primer specificity in this research was high because no cross-reactivity of the primers was seen with other bacterial species including *S. pyogenes, B. anthracis, S. typhimurium, S. aureus, S. agalactiae* & *S. epidermidis* (Figure 1).

The specificity of PCR was also established as it yielded PCR fragments of 468bp specific for *E. coli* only in positive control containing *E. coli.* The specificity of our employed PCR assay was found to be 100% (Figure 1). In a study in which PCR assay was done for detection of *Chlamydia trachomatis,* the specificity of PCR assay was documented as 99% (Ostergaard, Birkelund, & Christiansen, 1993).

The detection limit for this assay on reference samples was 0.01 ng. This showed that the methodology was highly sensitive and dependable (Figure 2). Some researchers showed the detection limit of 0.25 ng in their experiments through PCR (Di Pinto, Forte, Conversano, & Tantillo, 2005) (Matsunaga et al., 1999). A minimum detection limit of 0.1% was observed for different samples as is found in literatures (Dalmasso et al., 2004) (Soares, Amaral, Mafra, & Oliveira, 2010) (Soares, Amaral, Oliveira, & Mafra, 2013). Detection limit of 1% was also mentioned in the literature (Cheng, He, Huang, Huang, & Zhou, 2014). In another study, minimum detection limit of 2% was observed in their experiment (Dalmasso, Civera, La Neve, & Bottero, 2011). This was thus seen that as compared to previous studies documented in the literature, the sensitivity of our assay was much better because of the minimum detection limit of 0.01 ng. This methodology could likely be adopted for the detection and characterization of several other bacteria along with *E. coli* in raw milk.

In the present study, 43 samples (43%) out of 100 raw cow milk samples were found to give positive results for *E. coli* through PCR assay (Table 2 & 3). A study conducted in Bangladesh had documented 75% of raw cow milk samples positive for *E. coli* (Islam, Kabir, & Seel, 2016). Another study conducted in Pakistan revealed that the percentage of raw milk samples contaminated by *E. coli* was found out to be 65%, 60%, 50% and 45% in samples obtained from milk vending shops, milk vendors on donkey, milk vendors on bicycles and dairy farms respectively (Soomro et al., 2002). Zafalon et al. (2008) showed that the prevalence of *E. coli* was 57.3%. In another study, the contamination of milk samples by *E. coli* was found out to

be 26%, 20% and 6.6% collected from milk vendors, dairy farms and houses respectively (Zafalon, LONGONI, Benvenutto, Castelani, & Broccolo, 2008) (Kumar & Prasad, 2010) (Hossain et al., 2012). Still in another study, the percentages of *E. coli* in milk samples were found to be 59%, 62% & 68% in milk samples collected from dairy farms, dairy shops and street vendors respectively (Amin et al., 2017).

Because of the danger related to human health with bacterial contamination of milk, there is requirement for adoption of fast, sensitive, specific and inexpensive identification techniques for the examination of milk samples. The present study was also focused on these points to make this method specific, sensitive and inexpensive for detection and characterization of bacteria in milk. This study makes use of simple and traditional methods with high degree of sensitivity and specificity. The sample processing procedures meet many criteria already established. It is not dependent on any extra processing measures such as specific expertise, distinctive equipment or chemicals. The PCR assay was not expensive in order to perform the analysis on large scale. The procedure effectively removed PCR inhibitors, avoiding severe settings which would hamper the recovery of microorganisms from concentrated samples.

On the basis of observations done during the collection of samples, it could be concluded that erroneous hygienic practices and bad management before and during milking might contribute to contamination of milk by *E. coli*, and the traditional farms show more susceptibility regarding its infection. Tendency of contamination at a noticeably high percentage shows the risky situation for dairy farming and public health equally. The presence of *E. coli* in milk samples in the present study is a matter of great importance and consideration. On the basis of results obtained, the current study suggests that we should have to adopt proper and important developmental technologies in the field of dairy industry in last few decades. These areas include production of cattle, herd rearing, food processing and hygiene, proper storage facilities and refrigeration. Processes like pasteurization and ultra-high- temperature treatments should be adopted so as to decrease the danger of transmission of infection through milk to the consumers and to avoid the outbreaks of GIT diseases. Precise food safety standards, regulations of law and their strict enforcement, regular scrutiny and quality testing are some other measures which can help in improvement of milk quality thus decreasing the incidence of outbreaks of infection.

In order to check the effectiveness of antibiotics against *E. coli* present in milk samples, antimicrobial susceptibility test was performed through well diffusion method. Results of antimicrobial susceptibility test revealed that most of the isolates of *E. coli* have shown sensitivity towards azithromycin, streptomycin, gentamicin, norfloxacin and ciprofloxacin. However, resistance of *E. coli* was seen for amoxycillin and erythromycin, which was consistent with previous researches conducted by (Memon et al., 2013) and (Bedada & Hiko, 2011). *E. coli* showing resistance to amoxicillin and erythromycin indicate that they possess resistance property to these antibiotics which might be attributed to the improper application of these antibiotics.

## Conclusion

The results of current research showed that raw milk obtained from cattle presented high contamination by *E. coli*. The present study was performed with an intention to isolate and characterizes *E. coli* present in cow's raw milk samples obtained from several areas of Bahawalpur district. From 100 samples, 43 were detected to have *E. coli*. *E. coli* isolates were amplified by 16S rRNA gene-based PCR. Thus, the results of the present study indicate that strict precautionary and preventive measures should be taken while handling with milk. To avoid contamination by pathogenic strains of *E. coli*, regular washing and sterilization of dairy equipment and utensils, hands of the workers involved in collection and distribution of milk and udders of the animal are necessary measures to be taken. Also, these researches should also be carried out in various areas of Pakistan which are affected with repeated epidemics of gastroenteritis. Resistance pattern shown for broad spectrum antibiotic (e.g., amoxycillin) points to a dangerous situation which should be considered carefully and suggests that indiscriminate use of antibiotics for precautionary or therapeutic purposes should be avoided as it could be the cause of increasing antimicrobial resistance.

## References


Amin, W., Ahmed, E., Embarak, M., Abo-Shama, U., Thabit, A., & Ismail, S. (2017). Molecular Detection of Enterotoxigenic E. coli in raw Milk and Milk Products. *Int. J. Curr. Microbiol. App. Sci, 6*(11), 856-864.

Bedada, B., & Hiko, A. (2011). Mastitis and antimicrobial susceptibility test at Asella, Oromia Regional state, Ethiopia. *J. Microbiol. Antimicrob, 3*(9), 228-232.

Cheng, X., He, W., Huang, F., Huang, M., & Zhou, G. (2014). Multiplex real-time PCR for the identification and quantification of DNA from duck, pig and chicken in Chinese blood curds. *Food Research International, 60*, 30-37.

Christensen, D. G., Orr, J. S., Rao, C. V., & Wolfe, A. J. J. A. E. M. (2017). Increasing growth yield and decreasing acetylation in Escherichia coli by optimizing the carbon-to-magnesium ratio in peptide-based media. *83*(6), e03034-03016.

Dalmasso, A., Civera, T., La Neve, F., & Bottero, M. T. (2011). Simultaneous detection of cow and buffalo milk in mozzarella cheese by real-time PCR assay. *Food Chemistry, 124*(1), 362-366.

Dalmasso, A., Fontanella, E., Piatti, P., Civera, T., Rosati, S., & Bottero, M. T. (2004). A multiplex PCR assay for the identification of animal species in feedstuffs. *Molecular and cellular probes, 18*(2), 81-87.

Dell'Orco, F., Gusmara, C., Loiacono, M., Gugliotta, T., Albonico, F., Mortarino, M., & Zecconi, A. J. R. i. v. s. (2019). Evaluation of virulence factors profiles and antimicrobials resistance of Escherichia coli isolated from bulk tank milk and raw milk filters. *123*, 77-83.

Di Pinto, A., Forte, V., Conversano, M., & Tantillo, G. (2005). Duplex polymerase chain reaction for detection of pork meat in horse meat fresh sausages from Italian retail sources. *Food control, 16*(5), 391-394.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M.,... Relman, D. A. (2005). Diversity of the human intestinal microbial flora. *Science, 308*(5728), 1635-1638.

Gan, S. D., & Patel, K. R. (2013). Enzyme immunoassay and enzyme-linked immunosorbent assay. *J Invest Dermatol, 133*(9), e12.

Guetouache, M., Bettache, G., & Samir, M. (2014). Composition and nutritional value of raw milk. *Issues Biol. Sci. Pharm. Res, 2*(10), 115-122.

Heo, J., & Hua, S. Z. J. S. (2009). An overview of recent strategies in pathogen sensing. *9*(6), 4483-4502.

Hossain, S. Z., Ozimok, C., Sicard, C., Aguirre, S. D., Ali, M. M., Li, Y., & Brennan, J. D. (2012). Multiplexed paper test strip for quantitative bacterial detection. *Analytical and bioanalytical chemistry, 403*(6), 1567-1576.

Islam, M., Kabir, S., & Seel, S. (2016). MOLECULAR DETECTION AND CHARACTERIZATION OF ESCHERICHIA COLI ISOLATED FROM RAW MILK SOLD IN DIFFERENT MARKETS OF BANGLADESH. *Bangladesh Journal of Veterinary Medicine, 14*(2), 271-275.

Kretzer, J. W., Lehmann, R., Schmelcher, M., Banz, M., Kim, K.-P., Korn, C., & Loessner, M. J. (2007). Use of high-affinity cell wall-binding domains of bacteriophage endolysins for immobilization and separation of bacterial cells. *Applied and environmental microbiology, 73*(6), 1992-2000.

Kumar, R., & Prasad, A. (2010). Detection of E. coli and Staphylococcus in milk and milk products in and around Pantnagar. *Veterinary World, 3*(11), 495.

Law, J. W.-F., Ab Mutalib, N.-S., Chan, K.-G., & Lee, L.-H. J. F. i. m. (2015). Rapid methods for the detection of foodborne bacterial pathogens: principles, applications, advantages and limitations. *5*, 770.

Lazcka, O., Del Campo, F. J., & Munoz, F. X. (2007). Pathogen detection: a perspective of traditional methods and biosensors. *Biosensors and Bioelectronics, 22*(7), 1205-1217.

Link, A. J., Jeong, K. J., & Georgiou, G. (2007). Beyond toothpicks: new methods for isolating mutant bacteria. *Nature Reviews Microbiology, 5*(9), 680.

Matsunaga, T., Chikuni, K., Tanabe, R., Muroya, S., Shibata, K., Yamada, J., & Shinmura, Y. (1999). A quick and simple method for the identification of meat species and meat products by PCR assay. *Meat Science, 51*(2), 143-148.

Memon, J., Yang, Y., Kashif, J., Yaqoob, M., Buriro, R., Soomro, J.,... Hongjie, F. (2013). Genotypes, Virulence Factors and Antimicrobial Resistance Genes of Staphylococcus aureus Isolated in Bovine Subclinical Mastitis from Eastern China. *Pakistan Veterinary Journal, 33*(4).

Mortazavi, S. A., & Gharehyakheh, S. (2014). Comparison of microbial contamination of milk from traditional and industrial farms. *European Journal of Experimental Biology, 4*(1), 195-197.

Mungai, E. A., Behravesh, C. B., & Gould, L. H. J. E. i. d. (2015). Increased outbreaks associated with nonpasteurized milk, United States, 2007–2012. *21*(1), 119.

Oliver, S. P., Jayarao, B. M., & Almeida, R. A. (2005). Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. *Foodbourne Pathogens & Disease, 2*(2), 115-129.

Ostergaard, L., Birkelund, S., & Christiansen, G. (1993). Use of polymerase chain reaction for detection of Chlamydia trachomatis. *Journal of Clinical Microbiology, 31*(11), 3081.

Paul, M., Van Hekken, D. L., & Brewster, J. D. (2013). Detection and quantitation of Escherichia coli O157 in raw milk by direct qPCR. *International Dairy Journal, 32*(2), 53-60.

Pingoud, A., & Jeltsch, A. (2001). Structure and function of type II restriction endonucleases. *Nucleic acids research, 29*(18), 3705-3727.

Pukančíková, L., Lipničanová, S., Kačániová, M., Chmelová, D., & Ondrejovič, M. (2016). Natural Microflora of Raw Cow Milk and their Enzymatic Spoilage Potential. *Nova Biotechnologica et Chimica, 15*(2), 142-155.

Razzaq, A., Shamsi, S., Nawaz, A., Ali, A., Malik, K. J. P., & Biology, A. (2016). Occurrence of Shiga toxin producing E. coli from raw milk. *5*(2), 270.

Soares, S., Amaral, J. S., Mafra, I., & Oliveira, M. B. P. (2010). Quantitative detection of poultry meat adulteration with pork by a duplex PCR assay. *Meat Science, 85*(3), 531-536.

Soares, S., Amaral, J. S., Oliveira, M. B. P., & Mafra, I. (2013). A SYBR Green real-time PCR assay to detect and quantify pork meat in processed poultry meat products. *Meat Science, 94*(1), 115-120.

Soomro, A., Arain, M., Khaskheli, M., & Bhutto, B. (2002). Isolation of Escherichia coli from raw milk and milk products in relation to public health sold under market conditions at Tandojam. *Pakistan Journal of Nutrition, 1*(3), 151-152.

Srinivasan, R., Karaoz, U., Volegova, M., MacKichan, J., Kato-Maeda, M., Miller, S.,... Lynch, S. V. J. P. o. (2015). Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. *10*(2), e0117617.

Tadesse, H. A., Gidey, N. B., Workelule, K., Hailu, H., Gidey, S., Bsrat, A., & Taddele, H. J. V. m. i. (2018). Antimicrobial Resistance Profile of E. coli Isolated from Raw Cow Milk and Fresh Fruit Juice in Mekelle, Tigray, Ethiopia. *2018*.

Tahira, B., Ullah, K., Samad, A., Ali, S., Nabi, S., Naeem, M.,... RESEARCH. (2017). ISOLATION AND MOLECULAR CHARACTERIZATION OF SHIGA TOXIN PRODUCING E. COLI O157: H7 IN RAW MILK USING mPCR. *8*(7), 3107-3112.

Tamerat, N., Muktar, Y., & Shiferaw, D. (2016). Application of molecular diagnostic techniques for the detection of E. coli O157: H7: a review. *J Vet Sci Technol, 7*(362), 1-9.

Tenaillon, O., Skurnik, D., Picard, B., & Denamur, E. (2010). The population genetics of commensal Escherichia coli. *Nature Reviews Microbiology, 8*(3), 207.

Tunegová, M., Toman, R., & Tančin, V. (2016). HEAVY METALS–ENVIRONMENTAL CONTAMINANTS AND THEIR OCCURRENCE IN DIFFERENT TYPES OF MILK. *Slovak Journal of*, 122.

Wang, C., Xing, K., Zhang, G., Yuan, M., Xu, S., Liu, D.,... Lai, W.-H. J. F. c. (2019). Novel ELISA based on fluorescent quenching of DNA-stabilized silver nanoclusters for detecting E. coli O157: H7. *281*, 91-96.

Weintraub, A. (2007). Enteroaggregative Escherichia coli: epidemiology, virulence and detection. *Journal of medical microbiology, 56*(1), 4-8.

Yaici, L., Haenni, M., Saras, E., Boudehouche, W., Touati, A., & Madec, J.-Y. J. J. o. A. C. (2016). bla NDM-5-carrying IncX3 plasmid in Escherichia coli ST1284 isolated from raw milk collected in a dairy farm in Algeria. *71*(9), 2671-2672.

Zafalon, L. F., LONGONI, H., Benvenutto, F., Castelani, L., & Broccolo, C. R. (2008). Aspectos epidemiológicos da mastite bovina causada por Staphylococcus aureus. *Embrapa Pecuária Sudeste-Artigo em periódico indexado (ALICE)*.