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Research article

MOLECULAR AND IMMUNOLOGICAL STUDIES OF PATHOGENIC *ESCHERICHIA COLI* IN MEAT SAMPLES COLLECTED FROM DIFFERENT LOCALITIES OF LAHORE

Kausar Malik* and Hafsa Memona

Department of the Zoology, Lahore College for Women University Lahore, Jail Road, Lahore, Pakistan.

Escherichia coli (E. coli) **is the predominant nonpathogenic facultative flora of the human intestine. A minority of *E. coli* strains are capable of causing human illness by producing several different toxins. *E. coli* O157:H7, an emerging cause of food-borne diseases with the occurrence of an estimated 20,000 illnesses and 250 deaths each year in the USA, has now been reported from several developing countries including Pakistan. Meat contamination occurs during slaughtering, processing and storage due to ineffective sanitary and handling practices. This research work was based on Microbial, Immunological screening and Molecular study of pathogenic bacteria *E. coli* in meat samples (Beef, Mutton, Quail and Fish). Three hundred Meat samples were collected from different localities of Lahore. Tryptic soy broth (TSB) and MacConkey agar were used for growth and identification of *E. coli* while Enriched media (mEC) supplemented with Novobiocin, broth was prepared for isolation of *E. coli* O157:H7. Out of 300 samples, 225 samples were identified as contaminated with *E. coli* by culture method. Polyclonal antibody production against pathogenic *E. coli* O157:H7 was performed by immunizing rabbits with pure grown culture of pathogenic *E. coli* O157:H7. Immunological studies {Dot Blot assay and Enzyme Linked Immunosorbent Assay (ELISA)} were performed for all meat samples by using produced polyclonal antibody and verified the results by using commercial monoclonal antibody (anti *E. coli* O157:H7) which confirmed the presence of pathogenic *E. coli* in collected contaminated meat samples. Molecular studies include DNA and RNA extraction by using commercial kits. To study genomic DNA band patterns and RNA analysis of pathogenic *E. coli* O157:H7, agarose gel electrophoresis was performed. Crude proteins were extracted from pure culture of *E. coli* O157:H7 grown on selective media and their band patterns were observed by resolving on SDS-Polyacrylamide gel electrophoresis.**

Key words: *Escherichia Coli*, Pathogenic, Molecular, Immunological, Electrophoresis, Monoclonal, Antibody, Polyclonal.

Introduction

Escherichia coli (*E. coli*) being a microbial, commensal organism, abundant in the lower digestive tract of both humans and animals, was firstly described in 1885 by German paediatrician Theodor Von Escherich (1857-1911). It is important in suppressing the growth of harmful bacterial species and vitamins synthesis (Chen and Frankel, 2005). *E. coli* belongs to the family *Enterobacteriaceae* which also includes many other pathogens such as *Salmonella*, *Shigella*, and *Yersinia* (Ewing, 1986). *E. coli* is commonly non virulent but some strains have adopted pathogenic or toxigenic virulence factors that make them virulent for humans and animals. *E. coli* are a Gram negative bacteria that can be distinguished serologically on the basis of their 'O' (lipopolysaccharides) and 'H' (flagellar) antigens

**Correspondence author Email: kausarbasit7576@yahoo.com*

and sometimes by their 'K' (capsular) antigens (Rogers, 2004). *E. coli* infections are wide-ranging according to serotypes. Some serotypes cause only diarrhea and Hemolytic Uremic Syndrome (HUS) in severe condition. Other infections are Urinary Tract Infection (UTI), neonatal meningitis, respiratory illness and intestinal diseases (gastroenteritis) (Griffin and Tauxe, 1991).

One of the most common causes of infantile diarrhea is enterohemorrhagic *E. coli* (EHEC). EHEC is a main food-borne and water-borne pathogen that causes diarrhea, Hemorrhagic Colitis (HC), and HUS in animals and humans. EHEC produce shiga or verotoxin, intimin for attaching and effacing (A/E) lesion, watery and very bloody stool while live in colon. Low dose is too infective to cause disease in humans and animals. It includes O157:H7, O26 and O111 (Clarke et al., 2003).

The most notable EHEC strain is O157:H7 discovered in 1982. The Center for Disease Control and Prevention (CDC) reported 75,000 human infections per year of *E. coli* O157:H7. Around 5% of the patients develop more serious health problems, such as hemolytic anemia, kidney failure, and thrombocytopenia. The route of infection of *E. coli* O157:H7 is usually fecal-oral transmission (Weimer, 1999).

E. coli O157:H7 is non-invasive, elaborates no colonization factors (CFA/I nor CFA/II), does not produce heat stable or heat labile toxins and is non-hemolytic. It can't ferment sorbitol whereas 93% of all *E. coli* ferment sorbitol. *E. coli* O157:H7 does not grow at 45ºC in the presence of bile salts (Doyle and Schoeni, 1984). Infections of pathogenic *E. coli* 0157:H7 may be restricted to colonization of a mucosal surface or can distribute throughout the body (Chen and Frankel, 2005). Colonization of the human gastrointestinal tract by *E. coli* O157:H7 is essential step to maintain members of the normal microflora in the intestine but also the critical early phase in all diarrhoeal infections (Torres et al., 2005). The attaching and effacing (A/E) virulence mechanism of EHEC is encoded by the chromosomal locus of enterocyte effacement (LEE). After initial adherence it translocate their Tir receptors and other proteins to the eukaryotic host cell by means of an LEE encoded type III secretion apparatus (Batchelor et al., 2000).

Eating undercooked contaminated meat also cause infection because the bacteria were not killed in the heating process (Balci *et al*., 2006). Undercooked or raw hamburger (ground beef) had been documented firstly for *E. coli* O157:H7 outbreak in 1982. In production of ground beef, meat from multiple cattle is often ground together, so contamination from a single animal occurs (Szalanski et al., 2004). The U.S. Department of Food Safety and Inspection Service (FSIS) declared that the presence of <1 cfu/25 g of ground beef is dangerous to human health (Weimer, 1999). Objectives of the research work were; Culturing, Detection and isolation of pathogenic *E. coli* O157:H7 present in collected meat samples, Easy and economical anti-*E.coli* polyclonal antibody production for immunological screening of pathogenic *E. coli*. Its analysis at Molecular level was also performed by using agarose gel electrophoresis and SDS-PAGE.

Materials and Methods

Culturing, Isolation and identification of Pathogenic *E. coli* O157:H7:

Total 300 raw meat samples of four different types (Beef, Mutton, Fish and Quail) were collected from retailers and local meat stores of different localities of Lahore to test the presence of pathogenic bacteria *E. coli*. Tryptic Soy Broth (TSB) media and broth was used for culturing of wide variety of microbes present in meat samples. Meat samples were streaked on TSB agar media and incubated for 18 hours at 37˚C. Colonies appeared on the TSB media were inoculated into the TSB broth and incubated for 18 hours at 37C. Colonies grown on TSB media plate were streaked on Sorbitol MacConkey Agar media (SMAC) in order to isolate pathogenic *E. coli* O157:H7 strains. mEC with Novobiocin is used for the detection of pathogenic *E. coli* O157:H7 in meat samples. This media is specific for the growth of pathogenic *E. coli* O157:H7. mEC broth was inoculated with grown culture in TSB broth and incubated at 37˚C and 150rpm for 18 hours.

Polyclonal Antibody Production in Rabbits:

Polyclonal antibody was produced in albino rabbits by injecting them heat killed grown culture of *E. coli* O157:H7 on selective media in conjugation with Freund's Complete Adjuvant. Two boosting injections of heat killed pure grown culture of *E. coli* O157:H7 in conjugation with Freund's Incomplete Adjuvant were given after 2 weeks, respectively. Production bleed was collected and antiserum was separated. Titer determination of antiserum was done by Dot Blot Immunoassay.

Immunoasays of *E. coli* O157:H7 in grown Meat Samples:

Dot Blot Immunoassay was done for all 300 samples in order to determine cross reactivity and concentration of pathogenic *E. coli* O157:H7. Nitrocellulose membrane was blotted with produced primary antibody followed by

blocking with skimmed milk. Grown cultures of meat samples were blotted on membrane and incubated. Secondary antibody (Horseradish Peroxidase conjugated anti-rabbit antibody) was blotted on membrane and color developed by treating with DAB tablets using as chromogenic agent. Three times washing was done with PBST after above described each step. Every incubation was done at 37°C for 2 hours every time. Results were verified by using commercial monoclonal Primary antibody.

ELISA protocol was performed for confirmation of *E. coli* O157:H7 presence in our samples. Sterilized Microtiter plate was coated with produced primary antibody and incubated for 2 hours at 37°C. Blocking with skimmed milk followed by incubation with pure grown culture of meat samples. Washing was done with PBST and plate was incubated with secondary antibody (Horseradish Peroxidase conjugated anti-rabbit antibody). After washing plate was incubated with DAB tablet as horeserasish peroxidase substrate, for 30 minutes. Finally washed the plate and configure its absorbance at 480nm wavelength on ELISA reader.

Molecular Study of Pathogenic *E. coli* O157:H7:

DNA and RNA were extracted from *E. coli* O157:H7 grown on selective media (mEC media with Novobiocin) using Puregene Genomic DNA purification kit (Lie Technologies, USA) and Purescript RNA purification kit (Lie Technologies, USA) according to the procedure given in the kit protocol. Extracted plasmid DNA was digested with restriction enzyme (HindIII). Genomic DNA, RNA and digested DNA were electrophoresed on agarose gel to analyze band patterns. Total protein was extracted from bacterial cells grown on selective media (EC media modified with Novobiocin) by buffer lysis method. 500µl grown culture was centrifuge to get pellet. This pellet was treated with cell lysis solution and proteinase inhibitor. Total protein was extracted from supernatants and. secondary structure was linearized by keeping on boiling water bath for 5 minutes. Extracted protein was electrophoresed on SDS-PAGE.

Results

Presence of Pathogenic *E. coli* in meat samples:

Meat samples were grown on TSB media and broth considered as contaminated samples. Pink colonies grown on Sorbitol MacConkey agar identify the *E. coli* O157:H7 serotype. mEC with novobiocin broth was got turbid which confirmed presence of pathogenic *E. coli* O157:H7. Out of total 300 collected samples, 225 samples were seen contaminated with pathogenic *E. coli* O157:H7 serotype (Table 1).

Immunoassays of *E. coli* O157:H7:

Dot Blot assay for *E. coli* O157:H7 show presence of pathogenic *E. coli* O157:H7 in meat samples. Dot Blot results were categorized as: +, ++, +++ and ++++. This categorization is according to the ascending concentration of pathogenic *E. coli* O157:H7 in meat samples, respectively. ELISA reading at 480nm showing optical density (OD) and comparison of OD with cut off value (COV) categorized samples in Positive (++), Negative (-) and Borderline sample (+). Elisa results are shown in Table 1.

Molecular Analysis of Pathogenic *E. coli*:

Extracted DNA of Pathogenic *E. coli* was approximately 23Kb in size because DNA band was lying parallel to the upper band of HindIII Marker (Figure 1). Digested DNA was appeared as smear and no defined size was observed (Figure 2). RNA band was observed but it did not match with any size of ladder so it was of high molecular weight and lying at start of the gel (Figure 3). All protein bands are lying between 6.5 – 30 KDa bands of marker. Major proteins found have size about 23 KDa in almost all samples and other most common proteins bands lies in the 10 and 17 KDa size. These crude proteins are analyzed as a mixture of many proteins (Figure 4).

Discussion

Although meat is widely used daily and a basic source of animal protein in diet of the people of developing countries but little research has been conducted in Pakistan on meat and its contamination issues. In Pakistan the conditions of slaughterhouses in large cities are terrible as compared to villages which are almost without slaughter houses facilities. Common observation of carcasses is on the floor of shops or in an open environment. Blood, dirt, dung and soil left for several hours before being transported (Jafri, 2003). Diarrhea is the most widespread disease in children in Pakistan. Statistical analysis showed that the number of diarrhea patients is much higher than number of patients infected by other infectious diseases as seen in a Civil Hospital Lahore where the reported average number of diarrhea patients are 35/day. It is expected that similar situations exist in other parts of Pakistan as well. It is very relevant to assess the frequency of different diarrhea causing pathogens and characterize them.

Zhao et al. (2001) observed that *E. coli* 0157 strains were isolated from samples of hamburger with

Food category	Microbial screening				Immunological Screening		Contaminated samples
	Cooked		Uncooked		Cut Off Value	Average OD at 480nm	
	Collected samples	Contaminated samples	Collected samples	Contaminated samples			
Beef	40	31	40	36	0.2398	0.7313	67/80
Mutton	40	33	40	40	0.185	0.7144	73/80
Fish	35	22	35	28	0.2079	0.3191	50/70
Quail	35	14	35	21	0.1929	0.2259	35/70

Table 1. Results of Microbial Screening of different Meat Samples.

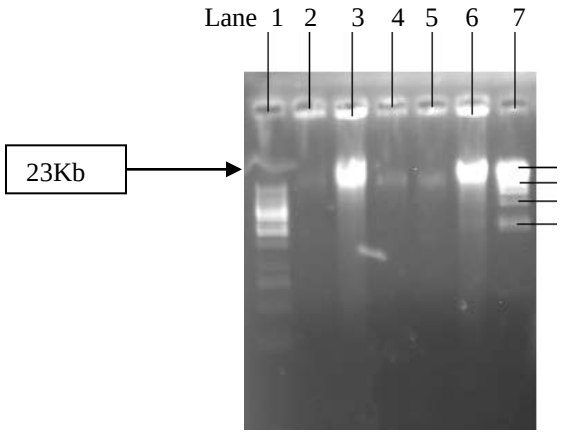

Figure 1. DNA band pattern on Agarose gel. (lane 1=1Kb DNA ladder, lane 2-6= DNA samples, lane 7= HindIII Marker).

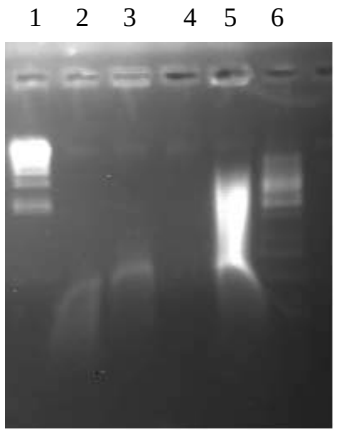

Figure 2. Digested DNA pattern on Agarose gel. (lane 1= 100bp DNA ladder, lane 2-5= digested DNA, lane 6= HindIII Marker).

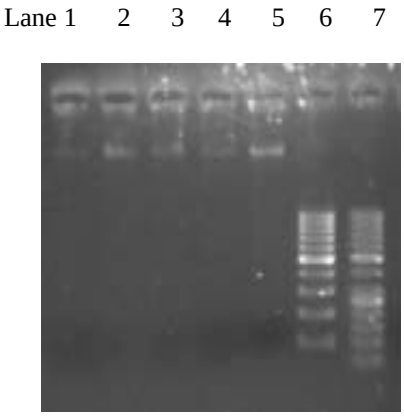

Figure 3. RNA samples electrophoresed on gel. (lane 1-5= RNA samples, lane 6,7= 100bp,250bp DNA ladder,

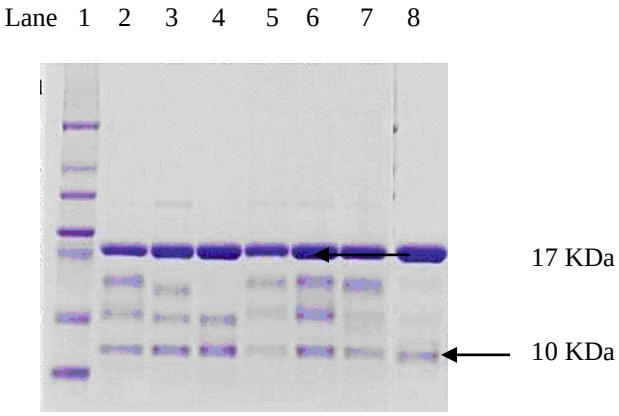

Figure 4. Crude protein band pattern on SDS-PAGE. (lane 1= protein marker, lane 2-8= protein sample)

vegetable. Samples which were positive for *E. coli* on the whole, have 30.2% *E. coli* 0157 positive. All the *E. coli* O157 strains recovered from meat samples obtained from small retailers, translates that supermarkets generally have a faster and greater turnover of sales that prevents the accumulation of unsold stock. However, the meat products are wrapped and sealed to prevent its contact with the surrounding environment. Differences between different stores have also been observed that might be due to variations in storage conditions, handling practices and product availability.

When Mutton samples were tested for the presence of pathogenic *E. coli*, we found that 73 samples, including 33 cooked and 40 uncooked, were positive for pathogenic *E. coli* presence. 67 positive samples, including 31 cooked and 36 uncooked samples, were found in total beef samples. This data revealed that cattle are the main reservoir of *E. coli*. *E. coli* is recognized as predominant alimentary flora of bovine species. Different fish samples (Mackeral, Bekti, Lady Fish, River sole, Round Sole, White Pomfret and Chakori) were analyzed and 50 samples were found positive for *E. coli* (including 22 cooked and 28 uncooked samples). Cooked samples showed 62% *E. coli* prevalence, conversely uncooked samples that showed 80% *E. coli* prevalence. Fish is still not considered as carrier of Pathogenic *E. coli* but its presence in samples can be due to many reasons such as cross contamination, unhygienic handling and processing with other food items. Cooked samples showed low percentage prevalence as compared to uncooked samples. Cooking can destroy the bacterium colonies and appropriate cooking will make our food eatable. Samadpour et al. (1994) detected *E. coli* O157:H7 in retail pork (18%), lamb (48%), chicken (12%), turkey (7%), fish (10%), and shellfish (5%) most of which have not yet been identified as biological carrier in human infections.

The minimum level of sensitivity of the ELISA assay was 0.5 ng of VT1 per ml of milk and 1 ng of VT1 per g of ground beef. The ELISA developed by Weeratna and Doyle, (1991) had a sensitivity of 0.5 to 1 ng/g of food and takes approximately 5 h to complete, when enzyme immunoassay plates pre-coated with antibody are used. While, current study provided an ELISA method which is relatively rapid and capable of detecting *E. coli* O157:H7 in foods or food extracts with good sensitivity while, previously described ELISAs developed to detect VT1 in pure cultures grown in culture media, are quite time-consuming. *E. coli* prevalence on raw meats and many other foods is controlled by several interconnected factors such as temperature of storage, atmosphere composition and competitive microflora (ICMSF, 1998).

Conclusion

E. coli O157:H7 are a significant cause of hemorrhagic gastrointestinal disease and the hemolytic uremic syndrome. The prevalence of *E. coli* is very high in Pakistan and is increasing day by day. The established method in this study proposed two step methods of detection of Pathogenic *E. coli*: Culture method and Immunological tests. This method meets the current demands for rapid detection of pathogenic *E. coli* O157:H7 at commercial level. This is simple and rapid method and low number of pathogenic *E. coli* can be detected reproducibly. This method is advantageous on others because it focused on analysis time and sensitivity and helpful to detect presence of pathogenic *E. coli* O157:H7 in cultures grown for just few hours. It proposed effective sampling plan for determination of raw meat concerning contamination of pathogenic *E. coli* O157:H7. This primary information will form the basis for the future development of a decent combative programmed research at gross route level. This research work expose the major causes of the contamination pressure in the primary processing plants and will helpful to prevent further spread of the pathogenic *E. coli* O157:H7 into other foods and in the environment

Recommendations

E. coli 0157:H7 are frequently associated with diarrheal diseases in humans and animals. This strain causes diarrhea, HUS and thrombotic thrombocytopenic purpura in humans by producing different toxins. Infection from *E. coli* 0157:H7 usually occurs as a result of the ingestion of contaminated food and water. The CDC predictably estimates 3000 cases of HUS, and at least 60 deaths annually are because of *E. coli* O157:H7 in the United States. Therefore some recommendations regarding to this pathogenic *E. coli* 0157:H7 are given as:

- Detection of pathogenic *E. coli* 0157:H7 was hampered by the lack of specific culturing techniques that allows direct discrimination of pathogenic *E. coli* from nonpathogenic strains so there should be advancement in culturing method to isolate pathogenic *E. coli* swiftly.
- Develop a sensitive method to detect enterotoxins in food and explore unnoticed conditions for production of these toxins in food.

- Pathogenic *E. coli* 0157:H7 is hazardous in very low amount (10 cells) but its critical detection

  can be done only at 10000 cells so advance procedures should be developed to rapid detection of minute quantities of *E. coli* 0157:H7.
- Continued molecular subtyping of *E. coli* O157 strains from both humans and the environment should be done in detecting causes of illness and identification of multi-state, geographically dispersed diseases due to contaminated commercial products.
- Conventional microbial detection techniques pertaining to analysis time and specificity should be introduced which will detect pathogenic organisms in no time.
- An Advisory Board should be established by the Secretary of Health Unit which will include consumers as well as industrialists to reach a decontamination criteria and appropriate actions to reduce the public health risk.
- Use properly cooked meat products and make awareness in public about ingestion of contaminated or undercooked foodborn illnesses.

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