International Journal of Cell & Molecular Biology (IJCMB)
Accepted on 9 December, 2010, Vol. 2 (1), pp 446-451, 1st January, 2011
Available online at http://www.ijcmb.yolasite.com/archive.php
ISSN (2220-7589) 2010

Research article

IMMUNOASSAY BASED SCREENING OF PATHOGENIC *E. coli* IN FOOD SAMPLES COLLECTED FROM DIFFERENT LOCALITIES OF LAHORE AND GUJRANWALA

Kausar Malik* and Saffora Riaz

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

Some bacterial pathogens cause food borne or water borne bacterial disease only when they are in large number while ten pathogenic *E. coli* are enough to cause human infections like diarrhea and other complicated diseases. Food poisoning caused by *Escherichia coli* is commonly caused by eating squalid vegetables or unwashed fruits. However most infectious agents do not multiply on foods, but use them as vector to gain entrance to human body. The aim of this study was based on the isolation and immunological detection of pathogenic bacteria present in hawkers foods i.e. dahi bhala and fruit chat. Dahi bhala and fruit chat samples were collected from different localities of Lahore and Gujranwala. Microbial screening of this pathogenic bacterium was performed, Nutrient agar and MacConkey agar was used for growth of bacteria Tryptic soy broth (TSB) and Tryptic soy broth modified with novobiocin was prepared for identification of pathogenic *E. coli*. *E. coli* was isolated by streak plate and pour plate method by using selective media. To perform immunoassays, antibodies against *E. coli* were produced by immunization of rabbit. Immunization was carried out by injecting mixture of *E. coli* grown culture and Freund's complete adjuvant in first immunization. *E. coli* grown culture and Freund's incomplete in subsequent immunization. Immuno dot blot assay was performed to detect the concentration or intensity of *E. coli* isolates from the samples. ELISA was also performed to check the degree of pathogenic *E. coli* contamination in food samples (dahi bhala and fruit chat). For molecular level studies *E. coli* DNA, RNA and crude proteins were isolated. For the size determination of DNA and RNA agarose gel electrophoresis was performed. Proteins were extracted from the *E. coli* cells by using cell lysis solution and proteinase inhibitor that inhibit the degradation of protein. Then SDS PAGE (Sodium Dodesyl Sulphate Poly Acryl amide Gel Electrophoresis) was performed to examine crude proteins in pathogenic *E. coli*.

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

Introduction

Many food borne and waterborne diseases have a bacterial cause. Bacterial gastrointestinal diseases may rise from intoxication or infections. The food borne and water borne bacterial diseases are characterized as either intoxication or infections. (Dupton, 2007). According to world health organization (WHO) waterborne diseases account for an estimated 1.7 million deaths worldwide each year. Most of these deaths are from diarrheal diseases especially in children in developing nations (Adak et al., 2002).With warm moist conditions and plenty of nutrients lightly contaminated by the next day (Delaquis et al., 2007). *Escherichia coli* (*E. coli*) articulated and named after its discoverer is a Gram negative bacterium that is usually originate in the lower intestine of warm blooded organisms. Most *E. coli* strains are non-toxic but some such as serotype 0157:H7

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

can initiate severe food poisoning responsible for products recalls. The non-dangerous strains are part of the normal flora of the gut and can assistance bacteria within the intestine (Bachouri et al., 2002).

Different immunoglobulin classes were found in most vertebrates, signifying the value of having antibody with diverse effector functions. These are clssically generated by immunization of an appropriate mammal such as a mouse, rabbit or goat(Altschuh et al., 1992). Adjuvant is used to enhance an immune reaction to antigens. Adjuvants are commonly used with soluble protein antigens to augment antibody titers and stimulate a prolonged response with accompanying memory (Karlsson et al., 1991). Enzyme immunoassay are based on two important biological phenomenon (i)extraordinary discriminating power of antibodies, based on the ability of immune system of vertebrates to produce a virtually unlimited variety of proteins each with an affinity for specific foreign compound and (ii)the extremely high catalytic power and specificity of enzymes which may quite often be detectable with great ease. (Tijseen, 1999).

Enzyme linked immunosorbent assay also called ELISA or EIA is a biochemical technique used mostly in immunology to perceive the existence of an antibody or an antigen in a sample. Formally an ELISA involves at least one antibody with specificity for an exacting antigen (Crowther, 1995). Gel electrophoresis provides a versatile, temperate, high resolution technique for fractionation and physical-chemical characterization of molecules on the basis of size, conformation, and net charge (Charmabach and Rodbard, 1982).

Materials and Methods

A survey of *Escherichia coli* prevalence in a variety of retail foods was undertaken in two cities of Pakistan (Lahore and Gujranwala) with samples haphazardly collected from a number of outlets in each area. A total of 300 food samples (150 samples of Dahi bhalla and 150 samples of Fruit chat) were procured. Samples were shipped to the laboratory on the day of purchase/collection in eppendorfs tubes placed in ice packs.

Microbial Screening of Samples:

Preparation of enriched or selective Medias and broth: Prepared and autoclaved the Broth (TSB) and media. After autoclaving pour the agar media to sterile petriplates and broth into eppendorfs tube. After incubation of 24hrs at 30 °C streaked the sample on Petri plates containing agar media and also inoculate the culture into broth.

Gram Staining:

Microbes were stained by using Gram Christian Staining method. Slides were observed under the microscope.

Antibody production: Male albino rabbits at 1-2 years of age were obtained. Two basic types of adjuvants freund's incomplete adjuvant and freund's complete adjuvants and grown culture of *E. coli* were used to inject to rabbit for antibody production. Each injection was given after two weeks of interval.

Immuno-dot-blot:

Make about 1000-16000 ml dilutions and dispense in 6 ml aliquots that will be frozen until needed. There are recommended concentrations; Original stock1 µl, 1:1000, 1:2000,1:4000,1:8000,1,12000 or 1:16000. Labeled the nitrocellulose membrane and pipette out 1µl of primary antibody ont om embrane. Then after incubation washed the membrane in 1x PBS three times and pipette out 1 µl cultured samples. After the incubation and washing of culture sample, Incubate the membrane with 1 µl secondary antibody o each spot at 37 °C for 1½ hour . Again wash the membrane with 1x PBS three times. Dip the membrane in DAB (3, 3 -di-aminobenzidine) tablets. Rinsed the membrane in distilled water and air dry the membrane. Based on subjective evaluation of the intensity of the detected strains, tested bacterial isolates were determined to have a negative (–), or various degrees of a positive (+, ++, +++ or + +++) reaction with polyclonal *anti E .coli* serum.

ELISA:

Cultured samples were tested by ELISA for antibodies recognizing different bacterial strains. The plates were incubated for 20 min at room temperature and the reactions were stopped by the addition of 2N H_2SO_4 50 µl/well. The absorbance was measured at 490 nm by an ELISA plate reader (BioRad).

DNA and RNA extraction:

DNA was extracted from the *E. coli* culture by using Puregene Genomic DNA Purification kit (cat# d-5000) Gentra Systems, USA. Total RNA was isolated from bacterial cells grown on selective media (EC media modified with Novobiocin) using Purescript RNA purification kit. Agarose gel electrophoresis was done for visualizing DNA.

Protein extraction:

Centrifuge the media containing colonies to pellet down. Discard the supernatant and added 400 µl cell lysis solution (Tris [hydroxymethyl] aminomethane, ethylenediamine tetraacetic acid and sodium dodecyl

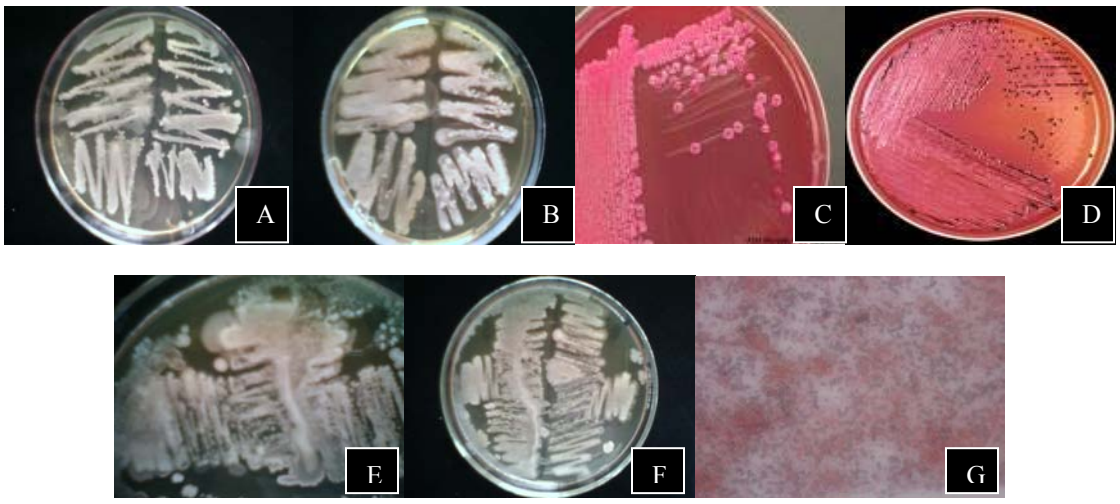

Fig. 1: Petri plates showing results of Streaking of TSB Media of (A) Dahi bhala, (B) Fruit chat; Petri plates showing grown bacterial colonies on Sorbitol-MacConkey Agar (C) Dahi bhala sample and (D) Fruit chat sample. Petri plates showing grown bacterial colonies from (E) Dahi bhala and (F) Fruit chat on mTSB Media with Novobiocin, (G) Rod shaped Bacteria of pathogenic *E. coli* under microscope.

sulphate) and proteinase inhibitor (to inhibit degradation of the protein. Centrifuge it and took supernatant and then added the 100 µl loading dye to each eppendorfs tube and boil it in bubble rack for 15min. Placed the eppendorfs at -20°C for 5min. Observed the proteins by SDS-PAGE.

Results:

Microbial analysis of samples:

Two categories of media enable macroscopic study of bacteria: enriched and selective e.g. Tryptic soy broth (TSB) is commonly used enriched media. Selective media are formulated with ingredients Novobiocin that inhibit the growth of some bacteria, such as an antibiotic, but enhance growth of the target organism as sorbitol MacConkey agar (Figure 1). Sorbitol-MacConkey agar usually comes with lactose in it so that one can see that *E. coli* ferments lactose to make *E. coli* differs from most other strains of *E. coli* in being unable to ferment sorbitol. Thus *E. coli* would make red colonies as shown in the figures 1 (C) and (D), while other Gram (-) bacteria would not be red and actually be a very light pink. Novobiocin is an antibiotic; it is also known as, albamycin or cathomycin, an aminocoumarin. Identification of bacterial species and testing for antibiotic sensitivity require pure and isolated, bacterial colonies. Results of grown colonies of *E.coli* are shown in figure 1 (E) and (F).The most important differential strain used in bacteriology is the Gram Stain, named after Dr. Christian Gram. Differential staining requires the use of at least three chemical reagents, Primary stain, Decolorizing agent the Counter stain (Figure 1 (G).

Polyclonal Antibody Production and Immuno dot blot assay:

Polyclonal antibody production was carried out in albino rabbits by immunizing them fresh and pure *E. coli* O157:H7cultures. This serum was tested by immuno dot blot assay for its authentication and titer determination (Figure 2). Immuno dot blot assay was used for detection of *E. coli* in food samples. Rabbit polyclonal antiserum raised against *E. coli* strain was used for immuno dot blot assay. Immuno dot blot assay detect the presence of pathogenic *E. coli*. Figure 10 showed the results of Immuno dot blot assay of food samples (dahi bhala and fruit chat) using monoclonal (commercial) and polyclonal antibodies (Figure 3).

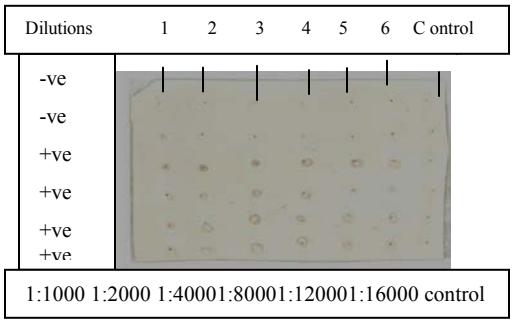

Fig 2: Titer analysis results of (Anti *E. coli* antibody) generated by rabbit immunization.

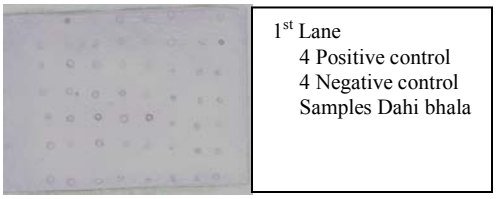

Fig 3: Immuno dot blot assay of food samples (dahi bhala a nd f ruit c hat) using monoclonal (commercial) and polyclonal antibodies.

ELISA Analysis:

ELISA was used to check c ontamination r ate of f ood samples (Dahi bhala and Fruit chat). ELISA was carried out, primary antibody that was produced in rabbit coated in t he 96 w ell plates (100 µl/well), and wells were blocked by s kimmed m ilk for 1 h at r oom temperature. They were incubated with *E. coli* grown culture for 2 h at 37 °C, and the ne gative cont rol w as t he ser um obtained from r abbit w hich w ere not i mmunized. T he absorbances of samples were measured at 480 nm by an ELISA pl ate reader (BioRad). ELISA results are shown in figure 4.

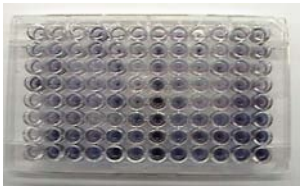

Fig 4: Microtitter plate s howing E LISA r esults using monoclonal and polyclonal antibodies.

Contamination rate of dahi bhala samples was 98% and fruit chat s amples w as 97.3%. D ahi bha la s amples 147 and 146 fruit chat samples showed the positive result for bacterial growth as has been mentioned in Table 1.

Agrose gel electrophoresis for total genomic DNA:

The e xtracted D NA w as e lectrophoresed on 1.2% Agarose g el. Results of D NA g el electrophoresis are shown. DNA bands lie above to the upper most band of HindIII marker s o t heir s ize i s a pproximately 23k b (Figure 5).

RNA Gel Electrophoresis:

RNA samples were loaded in first 5 w ells, 100bp D NA Ladder i n 6th well a nd 250bp D NA L adder i n 7th well, An RNA ba nd does not m atch w ith a ny l adder s ize because it is lying above of t he l adder si zes. So RNA size is greater than 100bp ladder.

SDS-PAGE for Total Protein electrophoresis:

Results of protein electrophoresis are shown in figure 6. Protein Marker l oaded i n f irst w ell w hile r emaining lanes ar e prot ein samples. All prot ein bands ar e l ying between 6.5 – 30 KDa bands of marker. M ajor pr otein size lying at 23 KDa in almost all samples and second most common pr otein ba nds l ies i n t he 10 a nd 17 K Da range. T hese pr oteins ar e cr ude ext racted and have a mixture of proteins (Figure 6).

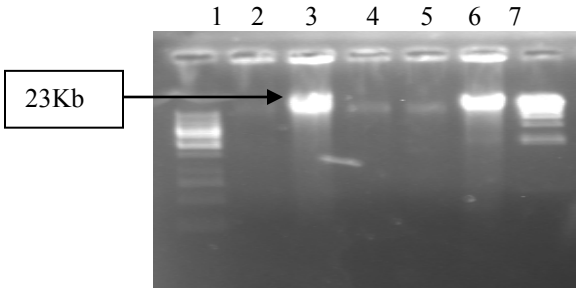

Fig 5: Agarose gel showing patterns of deoxyribonucleic acid (DNA) of pathogenic *E. coli*. *E. coli* genomic DNA are in 2nd, 3rd, 4th, 5th and 6th well, 1 Kb D NA la dder in 1st well a nd H indIII marker in 7th. DNA bands lie p arallel to th e upper most b and o f H indIII m arker s o th eir s ize is approximately 23kb.

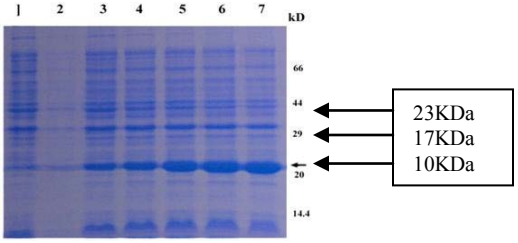

Fig 6: Characterization of crude proteins. 10% SDS–PAGE g el s tained with c oomassie b rilliant blue showing crude proteins extracts.

Discussion

E. coli are ubi quitous or ganisms w ithin the human intestine; w hen these org anisms acqui re virulence factors and become pathogenic, they are able to produce a v ariety of di arrheal di sease syndromes. Since shiga toxin-producing *E. coli* (STEC), especially of ser otype O157:H7, were recognized as important food pathogens, several selective m edia have be en described for the detection of t hese org anisms. Strains of *E. coli* O157, unlike t he m ajority of *E. coli* strains, do not f erment sorbitol within 24 h and s how ne gative g rowth. P oor growth of these strains at t emperatures above 42°C has been observed. Differential characteristics are applied in several media for the isolation of *E. coli* O157. The most widely accepted method of screening for *E. coli* O157

Table 1: Results of microbial screening and immunoassays of pathogenic *E. coli* in food samples.

Samples	**Locality**	**Growth of *E. coli* on different medias**			**Gram staining**	**Immuno Dot blot assay**		**ELISA**			
Dahibhala(D.B.) FruitChat (F.C)	Gujranwala (Grw) Lahore (Lhr)	Tryptic soy broth (TSB)	Sorbitol-MacConkey agar	mTSB with Novobiocin	Bacilli (+or -)	Intensity of the *E.coli* isolates		Sam p-les	Cut off value	Result	
D.B. 75	Grw	+	+	+	-	18	+	16	.24	15	-
						21	++	59	.17	8	+
						7	+++			42	++
						29	++++				
F.C 75	Grw	+	+	+	-	34	+	75	.20	19	-
						15	++			16	+
						3	+++			40	++
						20	++++				
D.B. 75	Lhr	+	+	+	-	17	+	33	.17	11	-
						16	++	42	.20	9	+
						9	+++			55	++
						33	++++				
F.C 75	Lhr	+	+	+	-	29	+	75	.20	12	-
						22	++			5	+
						8	+++			58	++
						16	++++				

involves observation of Sorbitol MacConkey (SMAC) agar for colourless (sorbitol negative) colonies.However, the possible presence of other sorbitol non-fermenters *E. coli* often makes it necessary to test many colonies for confirmation.

The low infective dose of VTEC requires the use of enrichment media when screening foods for the presence of these organisms. Selective enrichment media used include modified tryptic soy broth with novobiocine(mTSB+n). Contamination rate of dahi bhala samples was 98% and fruit chat samples was 97.3%. Dahi bhala samples 147 and 146 fruit chat samples showed the positive result for bacterial growth as has been mentioned in table 1. Therefore the purpose of study was to establish a screening procedure for the detection of pathogenic *E. coli*, also known as pathogenic bacteria from food samples (dahi bhala and fruit chat) its % prevalence from different localities of Gujranwala and Lahore.

Gram staining has confirmed the presence of pathogenic *E. coli*. After all procedure the smear was of pink color. By observing them under the microscope it was noted that all bacteria were rod shaped in single and clusters arrangement. A major purpose of this study was to produce the primary antibody by using the grown *E. coli* culture on selective media. Since pathogenic *E. Coli* O157:H7 infection causes systemic immune responses, we intend to induce humoral reactivity by the adjuvant used Complete Freund's adjuvant(CFA) in first vaccination and Incomplete Freund's adjuvant (IFA) in boosting through the intra peritoneal pathway. The important index for humoral immunity is expanding of serum immunoglobulin. *E. coli* culture grown on TSB+n was subjected to rabbit to determine sera parameters which were compared with several control groups. The new approach had several advantages. First, our results demonstrated that the Antibody produced by the new approach had much better specificity than that produced by the process of the single sample immunization.

Second, whole cell of *E. coli* could be used smoothly to immunize animals in the approach, which was timesaving. Third the established approach was convenient, since these antibody could be used in ELISA analysis and dot blot analysis without further purification.

The specificity of immuno dot blot assay for detection of *E. coli* is relatively high and these tests may be potentially useful for identification of false positive reactions generated by other detection methods. In this study, an indirect ELISA was also carried out for the detection of *E. coli* in food samples (dahi bhala and fruit chat). The ELISA approach made use of a polyclonal antibody against *E. coli.* An advantage of using this technology for *E. coli* detection is that it allows the continuous production of polyclonal antibodies of consistent specificity. Lysis and DNA extraction is performed afterwards by heat treatment. DNA was extracted by using Puregene Genomic DNA Purification kit (cat# d-5000) Gentra Systems, USA.Total RNA was isolated from bacterial cells grown on selective media (EC media modified with Novobiocin) using Purescript RNA purification kit.Agarose gel electrophoresis was done for visualizing DNA, digested DNA and RNA. Protein was extracted from the *E. coli* colonies by using cell lysis solution and RNAase to degrade the RNA and Proteinase inhibitor that inhibit the protein degradation and visualized by SDS-PAGE.Despite the critical points observed in the street food market, it is opportune to emphasize its importance as food source and income for a growing population. Therefore, it deserves the attention of local authorities throughplanning, investments, mass media campaigns, regulations and education.

Conclusion:

Our perspective on *E. coli* has undergone a significant transformation in recent decades and certainly will continue to evolve. Once dismissed as a harmless inhabitant of the intestinal tract, *E. coli* is now seen as a pathogenic species with outstanding adaptability to cause disease in humans and animals. Pathogen-specific virulence factors have been revealed that adversely affect a wider range of eukaryotic cell processes. The antibody determines to a large extent the specificity and sensitivity of the resulting immunochemical technique. These features may be modulated by judicious design of the immunogen and by rational immunoassay development.

References:

Adak, G. K., Long, S. M. and Brien, S. J. (2002). Trends in indigenous food borne disease and deaths. An international journal of Gastroenterology and Hepatology. 51:832-841.

Altschuh, D., Dubs, M. C., Weiss, E., Zeder, L. G. and Regenmortel, M. (1992). Determination of kinetic constants for the interaction between a monoclonal antibody and peptides using surface plasmon resonance. Biochemistry. 31:6298–6304.

Bachouri, M., Quinto, J. and Moram, T. 2002. Survival of *Escherichia coli* O157:H7 during storage of yogurt at different temperatures. Journal of food science. 11:212-215.

Chrambach, A. and Rodbard, D. (1982). Polyacrylamide gel electrophoresis. Science. 30(172):440-451.

Crowther, J. R. (1995). ELISA, Theory and practice In: *Methods in Molecular Biology.* Humana Press, New Jersey. Pp.211-215.

Delaquis, P., Bach, S. and Dinu, LD. (2007). Behavior of *Escherichia coli* O157:H7 in leafy vegetables. Journal of Food Protection. 70:1966–1974.

Dupont, H. L. (2007). Food safety: the growing threat of food borne bacterial enteropathogens of animal origin. Clinical Infectious Diseases. 45:1353-1361.

Karlsson, R., Michaelsson, A. and Mattson, L. 1991. Kinetic analysis of monoclonal antibody antigen interactions with a new biosensor based analytical system. *Journal of Immunological Methods*. 145:229-240.

Tijssen, P. (1999). Laboratory Techniques in Biochemistry and Molecular Biology In: Practice and Theory of Enzyme Immunoassay. Elsevier Science Publishers, Netherlands. Pp. 321-376.