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Research article

MICROBIAL AND IMMUNOLOGICAL STUDIES OF PATHOGENIC *E. coli* IN RAW MILK SAMPLES COLLECTED FROM DIFFERENT LOCALITIES OF LAHORE.

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Contamination of raw milk is very common in Pakistan due to unhygienic conditions. Present work was based on the study of bacterial contamination (human pathogenic *E. coli)* of raw milk samples. Samples of raw milk were collected from different localities of Lahore and were tested for the presence of human pathogenic *E. coli*. Microbial screening of all the samples was done by using different media i-e, TSB, MacConkey and Novobiocin. Gram staining of these bacteria confirmed the presence of Gram negative pathogenic *E. coli*. All of the 100 samples had shown positive results. In this study influence of NaCl and pH adjusted with lactic acid and HCl on survival of *E. coli* in TSB agar was also determined. The organism grew in TSB containing ≤6.5% NaCl or at a pH of 5, adjusted with HCl and when TSB was acidified with lactic acid, it grew at pH of 4.5. Polyclonal antibody production against pathogenic *E. coli* was performed by immunizing rabbits with pure culture of pathogenic *E. coli* grown on selective media. Immunological studies (Dot Blot assay) were performed for 50 milk samples by using produced polyclonal antibody which confirmed the presence of pathogenic *E. coli* in collected milk samples. The present study will be helpful for detection of pathogenic *E. coli* and creation of knowledge about these pathogenic bacteria and diseases they caused among people.

Key words: *Escherichia Coli,* Pathogenic, Contamination**,** Molecular, Immunological, Milk, Lahore.

Introduction

A large population of Pakistan uses raw milk commonly sold in local market unpacked and unprocessed. Vast numbers of pathogenic bacteria are present in raw milk that confers spoilage and consequently causes serious health hazards. Even milk drawn in very asept ic conditions always contains micro-organisms. Different microbes associated with raw milk are *Pseudomonas, Acitobacter, Moraxella, Flavobacterium, Lactobacillus* and *Coliforms* but the theme of present studies was the screening, detection and microbial studies of pathogenic *E.coli*. By virtue of this work, awareness among the people can be raised about the serious health hazards it can impose and importance of timely detection of pathogenic *E.coli*. This work can be further elaborated to sort out the preventive measures against *E.coli.*

Raw milk is defined as original milk from cows and buffaloes, which has not been processed or heat treated (Speer, 2003). Milk is a very nutritious food (Saha et al., 2003). It adds high quality of protein (Casein), Carbohydrates (Lactose), fats, essential minerals and vitamins to our daily diet. This nutrition also provides an excellent bacteriological medium. Our environment contains a large number of micro-organisms. Some of these micro-organisms can directly enter the teat canal through teat opening and finally into milk. From the time when milk leaves the udder until it is poured into the container everything with which it come in contact is a potential source of contamination. This includes air, milking equipments and the workers. Poor sanitary

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conditions will result in heavily contaminated milk that spoils rapidly. Whereas milking performed under good hygienic conditions and strict sanitary practices, result in milk with low bacterial content and good keeping quality (Pelczar et al., 1985) .Fresh milk from healthy animal contains bacteria most of which are beneficial or harmful. Some conditions like health of an animal or handler, dirt, manure, air, cuts, wounds and contaminants from polluted water can make milk very dangerous if consumed (Malaria and Gashe, 1990).

Many farms rely on untreated water supplies from bore holes, lakes, river, wells, springs; some of these may be contaminated at source with micro-organisms of fecal origin e .g. *Coliforms*, fecal *Streptococci* etc. If this untreated water entered into the milk or is used for rinsing equipment and containers, any of the microbes present in water may contaminate the milk (Keene, 1997). Milk and its products have potential to transmit pathogens to humans. The nutritional components in milk and milk products make it a very important part of human diet and it also supports the growth of micro-organisms. Drinking of raw milk can cause food borne illness. In 1900 it was discovered that contaminated raw milk can transmit tuberculosis, Q-fever, diphtheria and scarlet fever to human (Jayarao and Henning, 2001).

E. coli was named by a German pediatrician and bacteriologist named Theodor Von Escherich (1857-1977). He was the first person who isolates this bacteria belonging to the family Enterobacteriaceae. This bacterium is a common inhabitant of human intestine as well as other warm blooded animals. They reached the victim through food or water. It adheres to the mucus of large intestine in bowl (Vogt and Dippold, 2005). *E.coli* is a facultative anaerobic, Gram-negative bacterium and is non-sporulating. Cells are typically rod shaped with the diameter of 0.5µm and are 2µm long. The cell volume is 0.6-0.7 μm^3 (Madigan and Martinko, 2006).

Optimal growth of *E.coli* occurs at 37º C but some strains in laboratory can also multiply at temperatuture of about 44º C (Cody and Giynm, 1999; Vogt and Dippold, 2005) . *E. coli* can respond to environmental signals such as temperature, chemicals, pH, osmolarity, etc., in a number of very remarkable ways (Reid et al., 2001). Many strains of *E. coli* like, O157:H7, O121 and O104:H21 produce toxins, besides these other strains of *E.coli* include, O118:H16, O171:H25, O177:H11. Some strains of *E.coli* like, ER1793, ER1821, ER2267, ER2738, ER2925, JM101, JM109, NM522, ER2507, TB1, CAG597, CAG626, CAG629, PR1031, ER2508KS1000, UT5600 and CJ236 are used in cloning. Most of the strains are used for protien expression like, POP2136 with pFOS1, ER2420 with pBeloBAC11, ER2420 with pACYC184. The most common non-O157:H7 serotypes associated with human disease include O 26:H11, O 103:H2, O 111NM and O113:H21. Other remaining strains are O2:H5; H6, O48:H21, O112:H2, O145:H25;H-, O50:H7, O146:H8, O55:H6; H7; H10;H-, O46:H31, etc. (Vogt and Dippold, 2005).

E. coli 0157:H7 secretes a very powerful toxin called a verotoxin (Vtx). The ability of these bacteria to secrete this toxin was identifiet in 1982. It binds to the receptors on human kidney, brain and gut cells bringing them to death (Well et al., 1983). Shiga like toxin are iron regulated toxins. These are functionally identical to toxins produced by virulent *Shigella* species strain of *E.coli* that express shiga-like toxins, gained this ability due to infection with a prophage containing the structural coding for the toxin, and non-producing strains may be come infected and produce shiga-like toxins after incubation with shiga toxin positive strains (Karch et al., 2005). Serotyping is important in distinguishing the small number of strains that cause diseases in human. *E. coli* can cause infection in the urinary tract and brain stem (meningitis) as well as intestinal diseases referred to as gastroenteritis. There are five classes of *E. coli* that produce disease. They are classified by the method of pathogenesis: 1) toxins (enterotoxigenic), 2) invasive (enteroinvasive), 3) hemorrhagic (enterohemorrhagic), 4) pathogenic (enteropathogenic), and 5) aggregative (clumping or enteroaggregative) (Sonnenwieth, 1973; Toddar, 1997).

Many infections are caused by virulent as well as harmful strains of *E.coli.* These include gastroenteritis, urinary tract infection and neonatal meningitis. In rare cases virulent strains are responsible for hemolytic uremic syndrome, peritonitis, mastitis, septicemia and Gram-negative pneumonia. Although virulent strains within healthy adult humans cause only bloody diarrhea but transfer of genetic material lead to the production of new strains i.e. *E.coli* 0157:H7 responsible for serious illness or death in elder stages (Fotadar et al., 2005). 90% of urinary tract infections (UTI) are caused by Uropathogenic *E.coli.* The bacterium usually colonizes the feaces or perineal region and goes up the urinary tract to the bladder (Todar, 2007; Nicolle, 2008). Hemolytic uremic syndrome (HUS) is a leading cause of kidney failure in children, which often requires dialysis and may eventually leads to death. Other symptoms of illness due to Pathogenic *E. coli* include a central nervous system involvement (Nataro and Kaper, 1998). *E.coli* 0157:H7 is inhibited by NaCl at the concentration greater then 8.5%. An experiment had revealed that cells grew vigorously in NaCl of concentrations up to 2.5%,

whereas at 4.5% the rate of multiplication was increased three folds. The growth starts to retard at 6.5% and is completely inhibited at ≥ 8.5%. Moreover *E.coli* grows well at pH 5.0 when acidified with HCl and at pH 4.6 in presence of lactic acid. *E.coli* 0157:H7 also grow well at alkaline pH as high as 9.0 (Glass et al., 1992).

Antibodies (Abs) are made by immunizing animals (mouse, rabbit, goat, horse, etc.) with purified antigen (Ag). The animal responds by producing Abs that particularly recognize and bind to the specific Ag. Polyclonal antibodies (PAbs) can be produced in several animal species, predominantly in rabbit, horse, goat, and chicken. Polyclonal antibodies are a group of immunoglobulin molecules which are secreted from different B-cell lines against a specific antigen. This antibody consists of structurally different epitopes that recognize different antigen. Antigens are poor immunogen itself. Most complex protein antigens induce different B-cell clone to produce polyclonal response. Non-protein antigens are poor immunogen so adjuvants are used as immunostimulant (Jennings, 1995). PAbs have higher affinity, wide reactivity and lower specificity as compared to MAbs (Hayat, 2002). Selection of antigen quantity for immunization differs with the characteristics of antigen and adjuvant selected. Selection of appropriate adjuvant is affected by the purpose of antibody production (for research or vaccine development). Most common adjuvants for antibody production are Freund's, The Ribi adjuvant system and Titermax (Hanly et al., 1995).

Dot Blot is a type of immunoassay to measure antigen antibody interaction. It is a western blotting related technique for detecting proteins/antigens in samples that are marked through circular templates directly onto the membrane. This is different technique in which protein samples are not separated electrophoretically but are marked through circular templates directly onto the membrane or paper substrate and hybridized with an antibody probe. Antigens may be applied directly to nitrocellulose membrane as a separate spot (dot) to give a simple and reliable assay (Nakamura, 1985). The nitrocellulose membrane used for blotting were 125 µm (110-140 µm) thick microporous with 3, 5 or 8 µm small pore size diameter, packed with a 100 µm thick optically clear polyester film impermeable to water. The dry membrane absorbs aqueous solutions (buffers) easily and quickly becomes homogeneously wet (Lye and Birge, 1981).

Material and methods:

Ten zones of District Lahore (Sultan Pura, Ghari Shau, Town Ship, Model Town, Wapda Town, Mughal Pura, Shad Bagh, Gulberg, Samnabad & Defence) were selected for the present study. Ten raw milk samples were then collected from each zone, randomly from 10 different shops. The samples were placed in ice buckets after collection to keep temperature below 10ºC during transportation.

Preparation of media:

7.5 g of tryptic soy broth and 0.75 g of yeast extract were measured and dissolved in 250ml of distilled water with the help of magnetic stirrer. pH was adjusted up to 7-7.2 by adding NaOH [diluted solution] drop wise in the media. Then 3.75g agar was added in it. The media was autoclaved for 2-3 hours. Liquid media was prepared in the same way as above only it was lacking agar. Another media named Sorbitol-MacConkey Agar which is a differential media for the identification of *E.coli* O157:H7 was also used. 25.8g of the powder Sorbitol-MacConkey Agar was added in 500ml of distilled water in a beaker, stirred and then autoclaved.

Microbial screening of samples:

After autoclaving, solid media (TSB Agar media and Sorbitol-MacConkey Agar media) were poured to the sterile Petri plates and then were incubated at 37º C. Added 5 ml of the liquid media into the test tubes and were placed in shaker for 24 hrs. After incubation plates were streaked with samples. The inoculating loop was sterilized, poured in milk sample and were streaked on the plates with in laminar flow. Plates were then incubated at 37°C for 16-18 hrs. Sample cultures which were grown in solid media were introduced in the test tube with help of inoculating loop. The test tubes were left in shaker for overnight to obtain the bacterial growth. Glycerol stock were formed by taking 700µl of culture sample from test tubes containing colonies grown in liquid broth and poured in eppendrof with micropipette containing about 300µl of glycerol in it.

Detection of pathogenic *E. coli:*

About 5g of novobiocin containing media was dissolved in 150ml of distilled water in a beaker, stirred and autoclaved for 2-3 hours and then cooled at room temperature. 1ml of autoclaved media was poured in each eppendrof and was incubated for 16-18 hrs. 10µl of previously grown TSB media was added in each eppendrof having 1ml of novobiocin containing media. It was placed in the shaker at 37°C and at 150rpm for overnight. Poured plates containing solid agar media were streaked by the bacterial culture grown in

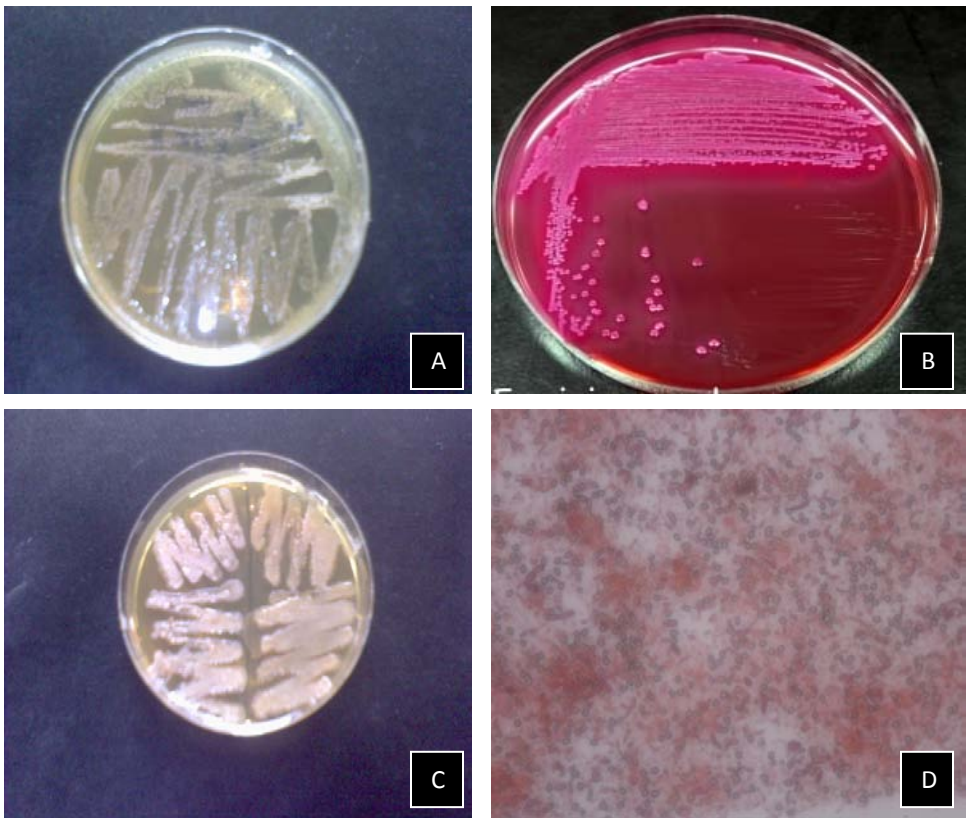

Fig: 1 (A) Bacterial growth on TSB-Agar petri plate, in raw milk Sample; (B) Sorbitol-MacConkey Agar Plate media showing growth *E. coli* colonies, in milk sample; (C) Petri plate showing growth of pathogenic *E. coli in* raw milk samples on Novobiocin containing media; (D) Slides showing Gram staining Pathogenic *E.coli* from of growth of pathogenic *E.coli* under microscope.

novobiocin containing media. Bacterial colonies grown on that media were further used for gram straining. Glycerol stokes were made by same method as above and were stored at -20ºC.

Effect of NaCl and pH on growth of E. *coli* TSB agar media:

250ml of TSB agar media was prepared and divided into 7 portions, where each portion contains 35.7ml of media. 7 different concentrations of NaCl were prepared (0.5, 1.5, 2.5, 3.5, 4.5, 6.5, and 8.5%). Then 1ml of each concentration was added to 35.7ml of TSB media. It was then autoclaved. Media was poured in the Petri plates, incubated at 37º C overnight. Next day *E. coli* grown in the novobiocin containing media were streaked on each petriplate with different NaCl concentrations. Petri plates were again incubated. TSB agar media was adjusted to different pH values, using 85% lactic acid (pH 3.4, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0), and 6N HCl (pH 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0). Media was autoclaved, poured, incubated, and streaked with the *E.coli.*

Production of polyclonal antibody:

250µl produced pure grown culture of pathogenic *E. coli* was first treated with heat shock by keeping it in boiling water bath for 5 min. These heat killed culture were mixed with an equal volume of Freund's Complete Adjuvant (FCA) (Sigma F-5881) and injected to the experimental rabbit subcutaneously as priming injection. Just before first injection, 5ml blood sample was taken from control and experimental rabbits, as preimmune serum. Second boost injection was produced by mixing 250µl heat killed grown colonies of *E. coli* with 250µl FIA (Sigma F -5506) and was given to experimental rabbit two weeks later. Third boost injection was prepared with FIA using the same method and was injected to experimental rabbits two weeks later. No Injection was given to control rabbits during this period. 1 week after the third injection, rabbit was dissected and heart was punctured to collect maximum blood. About

1ml serum was recovered from each 5ml blood sample. Separated Antiserum was stored at -20˚C for further testing. Produced antibody authentication and titer determination was carried by performing Dot Blot protocol. 6 dilutions of antiserum were prepared as (1:1000, 1:2000, 1:4000, 1:8000, 1:12000 and 1:16000 µl). Anti-rabbit IgG (A-6154) used as secondary antibody whose substrate is Horseradish peroxidase. DAB tablets were used for reduction of secondary antibody substrate. Intensity of color development and cross reactivity with antibody will confirm its authentication and defined its titer. Titer is the dilution that gives maximal absorbance in this assay.

Dot Blot Assay

Dot Blot protocol was performed for confirmatory presence of pathogenic *E. coli* in our samples. Samples were tested with self produced polyclonal primary antibody in rabbits.

Results:

After incubation of 16-18 hrs growth of microbes was observed on all plates containing solid media (TSB Agar media plates streaked with milk samples, Sorbitol-MacConkey plates and TSB Agar media plates streaked with grown cultures of pathogenic *E. coli* in novobiocin containing media). All of the 100 samples had shown positively for presence of pathogenic *E. coli*. These are shown in following figure 1. For confirmation of pathogenic *E. coli* Gram staining procedure was performed. Slides were observed under microscope. All of the 100 samples after staining had shown presence of Gram –ve, pink colored, rod shaped pathogenic *E. coli.*

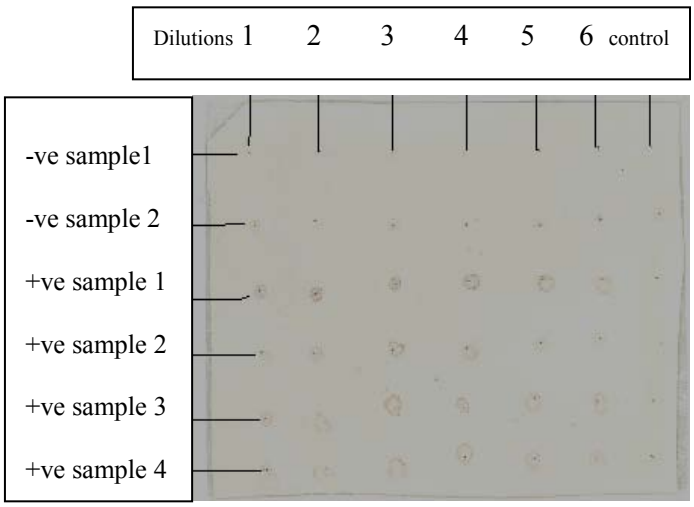

Fig. 2. Dot Blots showing results of reactivity of different samples with different dilutions of polyclonal antibody.

Effect of NaCl:

The petriplates had shown that *E.coli* was inhibited by NaCl at concentration of≥8.5%. It was growing quickly in NaCl concentrations up to 2.5%, whereas at 4.5% NaCl the growth was decreased. At 6.5% the cells were growing to a maximum population before death had occurred. And finally at 8.5% and 10.5% concentrations of NaCl the growth was totally inactivated. So, the higher concentrations of salt had the ability of retarding growth of pathogenic *E. coli*.

Effect of pH:

It had shown that *E. coli* grew well in media acidified with HCl at levels as low as pH 5.0. At 4.5 the growth has decreased, whereas at 4.0 and 3.5 the population was inactivated after few days. On the other hand lactic acid had greater inhibitory effects on *E. coli* at an equivalent pH than did HCl. Media acidified with lactic acid at pH 4.5 had shown that the growth was inactivated in very few days, whereas at pH 4.0 and 3.5 the growth was completely inhibited after 24 hrs.

Immunoassay:

Polyclonal antibody was produced and its authentication was determined with titer determination. Dot blot assay of only 50 milk samples was performed with freshly prepared antibody. All of the 50 samples were positive for pathogenic *E. coli* (Figure 2-3).

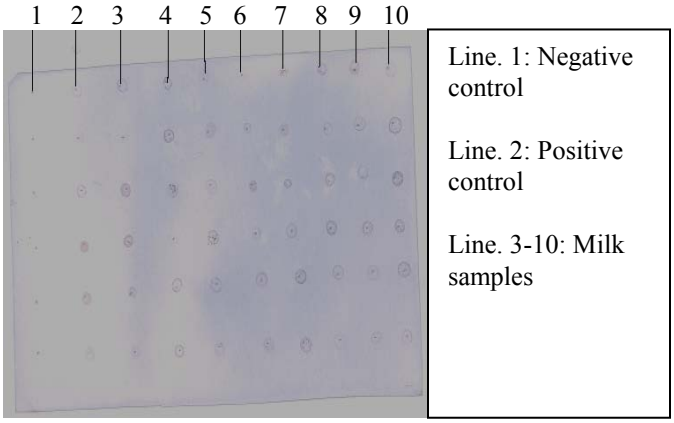

Fig. 3. Dot Blot results of 50 Milk samples showing presence of pathogenic *E. coli* in all samples

Discussion:

The main objective of present study was to examine human pathogenic *E.coli* in raw milk samples collected from different localities of Lahore. The main aim was the detection of pathogenic *E.coli* and to raise public awareness about this pathogenic bacterium and the diseases it would cause. In present research, it was noted

that all the 100 samples of raw milk showed the positive result for bacterial growth. Solid, agar-based media was used to identify colonial characteristics (shape, size, elevation, margin type), but that also served to select for particular bacterial groups and differentiate between two or more different species. The colony formed was of circular, rounded and lobed edges. The size was punctiform with off-white color and cloudy appearance. The texture was shiny and opaque. Older colonies often have a darker center. These results were clear on Petri plates. After that the grown colony was inoculated in liquid broth, by placing it in a shaker. Turbidity confirmed the bacterial growth. For the growth of pathogenic *E.coli*, Novobiocin and Sorbitol-MacConkey agar media ware used. The plates were streaked with TSB liquid broth containing grown cultures of *E.coli*. On the next day, the colonies appeared on the plates were mucoid colonies, which have entire margins and were slightly rose. *E.coli* has 94% ability to ferment sorbitol and makes the colony on Sorbitol- MacConkey appear pink to red. Pathogenic *E.coli* on the contrary cannot ferment sorbitol resulting of a colorless colony. While on mTSB with Novobiocin containing media the results were the same, with off-white colonies. Therefore the purpose of study was to establish a screening procedure for the detection of pathogenic *E.coli*, from the raw milk samples to investigate its prevalence from different localities of Lahore. Gram staining has confirmed the presence of pathogenic *E.coli*.

In this study effect of NaCl and pH (adjusted with lactic acid and HCl) on the growth of pathogenic *E.coli* in media was also noted. Results revealed that *E.coli* 0157:H7 is inhibited by NaCl in TSB at concentration of ≤ 8.5%. It had showed vigorous growth at concentrations up to 2.5%, whereas at 4.5% NaCl, the doubling time was about three fold longer. *E.coli* grew in 6.5% NaCl, although slowly. It was completely inhibited in 8.5 and 10.5% NaCl. Effect of pH on growth of *E.coli* 0157:H7 was also noted. It grew well in TSB acidified with HCl at levels as low as pH 5.0. At pH 4.5, the generation time of *E.coli* was increased, whereas at pH 4.5 and 3.5 the population was inactivated. Lactic acid has greater inhibitory effects on *E.coli* 0157:H7 at an equivalent pH than HCl. At all pH values be low 6.5, the organism grew constantly more slowly in TSB acidified with lactic acid than in TSB acidified with HCl.

Antibody production was done by immunizing rabbit with pure grown cultures of pathogenic *E. coli* in combination with FCA and FIA as boosting injection. Production bleed was collected and serum was tested for immunoassay to confirm its substantiation and titer determination. It was analyzed in Dot blot assay all serum dilutions (1:1000, 1: 2000, 1: 4000, 1: 8000, 1:12000 and 1: 16000) reacted with positive samples except sample 2 which was not reacted with 1:16000 so exact titer of our antibody is 1: 12000. This dilution is effective for all types of positive samples and was used for immunological studies of Milk samples. Polyclonal antisera comprising several different Abs of the target protein but irrelevant Abs can also be present in high concentration (up to 10 mg/ml) if not affinity purified. The advantage of PAbs over MAbs in that they are more liable to identify multiple isoforms (epitopes) of the target protein.

Dot Blot analysis of 50 milk samples was done with polyclonal antibody produced in rabbits and it was analyzed that positive samples were detected most clearly on nitrocellulose membrane. Positive samples are categorized in four categories (+, ++, +++, ++++) according to intensity of *E. coli* present in samples. + Samples have least number of *E. coli* while ++, +++ and ++++ shows the increase in number of *E. coli* in different milk samples. Alkaline phosphatase conjugated anti-rabbit secondary antibody reacted with proteins present in positive samples and develops an obvious distinctive color on membrane.

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