MICROBIAL HAZARDS OF RAW MILK MARKETED IN KHULNA CITY

A THESIS
BY
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Examination Roll No.: MS 070728
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Biotechnology and Genetic Engineering Discipline
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Khulna-9208, Bangladesh
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DECLARATION

This thesis reports the original research work of the author, except as otherwise stated. The material presented has not been submitted either in whole or in part for a degree from this or any other university. The findings of the research has not been/will not be published anywhere without the concurrence of the concerned supervisor/co-supervisor.

11.10.09

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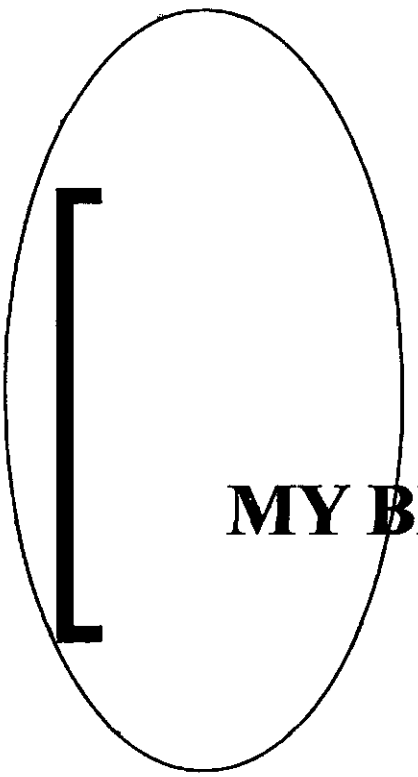
ABSTRACT

The aim of this study was to evaluate the main microbiological parameters of raw milk (total bacterial count, coliform bacteria of feacal and non-feacal origin, *Salmonella* sp.). Raw milk was assessed from eight (08) selected areas in Khulna city. In all of the samples, total bacterial count was beyond acceptable range. Fifty five percent (22 of 40) of the samples were found to be contaminated with feacal coliform (*E. coli*) while non-feacal coliform bacteria (*Enterobacter*) were detected in 45% (18 of 40) of raw milk samples. *Salmonella sp.* was found in only one milk sample (0.25%, 1of 40). Results of this study suggest that raw milk marketed in Khulna City without pasteurization might be hazardous for the consumers.



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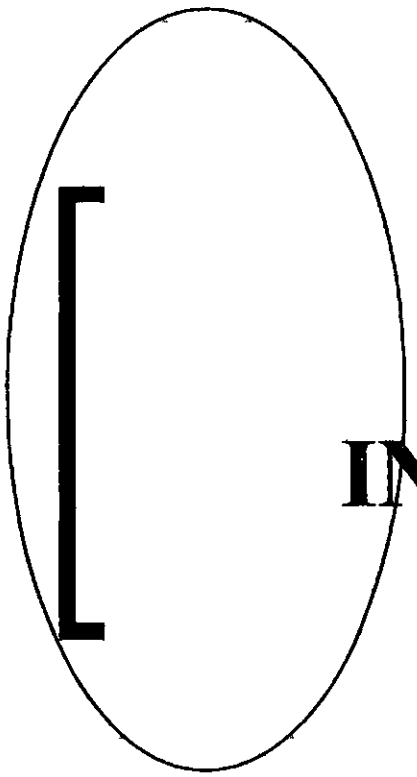


Chapter One

INTRODUCTION

INTRODUCTION

Milk is an ideal food. It contains all human body requirements except vitamin C. For this reason it is known as a complete food and being consumed by all categories of people in its natural state (Islam, 1999). Food habit of common people is changing. They prefer more healthy delicious foods form fresh raw milk. The composition and amount of microflora in the raw material has a decisive effect on the quality and safety of dairy products. Consequently, the total bacterial count is one of the accepted criteria for milk intended for processing by dairy plants.

Dairy products include market milk and cream, butter, frozen desserts, cheese, fermented and condensed milk and dried milk products. Raw milk contains relatively few bacteria when it leaves the udder of a healthy cow. Micrococci and streptococci have been recovered from aseptically drawn milk. During the normal milking operation, however, milk is subject to contamination from the animal, especially the exterior of the udder and adjacent areas. Bacteria found in manure, soil, and water may enter from this source. Such contamination is reduced by clipping the cow, especially the flanks and udder, grooming the cow, and washing udder with water or a germicidal solution before milking. Contamination of the cow with soil, water, and manure is reduced by paving and draining barnyards.

Probably the most significant sources of contamination are dairy utensils and milk-contact surfaces, including the milk pail or milking machines, strainers, milk cans or pipelines. If dairy utensils or the milk contact surfaces are inadequately cleaned, sanitized, and dried, bacteria may grow in large numbers in the dilute milk like residues and cause contamination of next batch of milk. Undesirable bacteria from these sources include lactic streptococci, coliform bacteria, psychrotropic gram-negative rods, and thermodurics, those which survive pasteurization, e.g., micrococci, enterococci, bacilli, and brevibacteria. In general, these bacteria grow well in milk and hence endanger its keeping quality.

Other possible sources of contamination are the hands and arms of the milker of dairy workers and flies. Normally these sources would contribute very few bacteria, but they might be sources of pathogens or contaminate for spoilage. The quality of farm water supply used in the milking parlor for cleaning, rinsing, etc., will have some effect on the

quality of the milk. The numbers of bacteria per milliliter of milk added from the various sources depends on the care taken to avoid contamination. For example, the exterior of the cow contributes comparatively few organisms if precautions are taken and a milking machine is used, but under very poor conditions thousands per milliliter could enter the milk. Hazard Analysis and Critical Control Point (HACCP) involves a system approach to identification of hazard, assessment of chances of occurrence of hazards during each phase, raw material procurement, manufacturing, distribution, usage of food products, and in defining the measures for hazard control.

Fresh milk and its products have made a major contribution to infants and adults diet in all countries all over the world. Mould growth on dairy products is quite common. Up to the recent decades this was considered to be a rather technological than a hygienic problem. In Brazil, raw milk used by powder manufacturers is required to match at least the same characteristics of Grade C Milk, for which no threshold levels have been established on microbiological parameters such as total bacterial count and pathogenic bacteria. Therefore, some dairy industries have set their own standards on the quality of raw milk purchased for processing.

Coliform is considered as normal flora of intestinal tract of human and animals. They have been used as indicator organisms for bacteriological quality of milk and its products. Coliform count is always being taken as a definite index of fecal contamination of milk and its products, afford an indication of possible presence of enteric pathogens which may constitute health hazards to the consumers. The quality of raw milk marketed by marginal farmer is controversial for the hygienic loopholes already mentioned. The consumers do not know the quality of this raw milk. Moreover the prices of this raw milk vary so much that consumers often get confused.

Hence, there is a great controversy concerning with the actual quality raw milk found in the local markets of Khulna City Corporation. Till to date no research records are available so far in our country to compare the quality of existing market raw milk. Therefore, in order to assess the quality of local market raw milk, the present research was undertaken with the following objectives:

I. To determine of the total aerobic plate count (APC).
II. To determine whether the coliform organism present (if any) is of faecal origin.
III. To determine of the *Salmonella* spp. present (if any) in raw milk.

Chapter Two
LITERATURE REVIEW

LITERATURE REVIEW

On December 18, 2000, the California Department of Public Health (CDPH) approved a motion by Mayor Antonovich, instructing the Director of Health Services and County Counsel to report back to the Board within 30 days with changes to the Los Angeles County Ordinance which will align the County Code with Me State Code permitting the sale of two additional categories of raw milk. Grade A and Guaranteed, The Board also approved an amendment by Supervisor Yaroslavsky, instructing the Director of Health Services to report back in 30 days with findings and recommendations on the grading of raw milk, including the public health implications associated with the different levels of inspection and grading. Finally, County Counsel was asked to report back on any liability issues concerning the proposal to permit additional categories of raw milk.

Agaoglu *et al.* (2000) compared the membrane filters and multiple tube fermentation by the colilert and enterolert method for detection of water coliform bacteria, *E.coli* and enterococci used in drinking raw milk.

About 10% of processed milk samples were reported to be contaminated by coliform bacteria (Ledford *et al.* 1983). However the soul samples showed 60% positive result if storage for 10 days at 4^0C.

The high number of micro-organisms were reported on the inner surfaces of washed milking machines before milking revealed ineffective cleaning (washing) by about 60% of dairy milkers (Torkar and Teger, 2004). There were no seasonal differences in the number of micro-organisms, except that the number of coliform was higher in spring. The average of total number of micro-organisms was $4.9 \cdot 10^5$ cfu/ml in raw milk, kept for about 18–24 hours at room temperature.

(Costello et *al.* 2001) was showed an association between the SPC and the coliform counts. SPC numbers were classified into five categories; SPC class 1 (log CFU/ml < 2), class 2 (log CFU/ml ≥ 2 and < 3), class 3 (log CFU/ml ≥ 3 and < 4), class 4 (log CFU/ml ≥ 4 and < 5), and class 5 (log CFU/ml ≥ 5 and < 6). The average coliform counts were 0.0, 0.69, 1.17, 1.88, and 2.73 logs CFU/ml in SPC class 1, 2, 3, 4, and 5, respectively. There was strong agreement between SPC classes and average coliform numbers. These data suggest that it is possible to estimate coliform counts based on SPC of raw milk.

Chatterjee et *al.* (2006) reported that standard plate count (SPC) method, the microbial colonies were found to be high in raw milk samples and in pasteurized milk samples the colonies were low. Bacterial colony was found to be opaque and metallic sheen in colour prepared from raw milk samples and by biochemical characterization it was identified as *Escherichia coli.*

Ekici *et al.* (2004) reported the importance of milk testing for public health in two regions in Rio de Janerio: a focus on fecal coliform, nitrates and aluminum. *Salmonella* spp. could not be isolated in any of the samples.

Coliform isolates from the bovine faeces were identified as *Escherichia coli.* The coliforms present in the milk were *Hafnia alvei, Serratia liquefaciens, Yersinia enterocolitica* and *Enterobacter amnigenus.* No *E. coli, Enterococcus* or *Aerococcus* from the bovine faeces were found in the milk (Vancanneyt *et al.* 2007).

Samples of raw milk purchased from farmers by ten dairy plants. The milk was analyzed at the "Lab-Mlek" Sp. z o.o., Milk Analyses Laboratory in Olsztyn. The total bacterial count was determined instrumentally, with a Bactoscan apparatus, manufactured by Foss-Electric A/S. No significant improvement in the microbiological quality of the raw milk from the region of Warmia and Mazury was observed (Szteyn *et al.* 2005).

Curiale *et al.,* (1997) inoculated a dry-film coliform count plate that with 5 ml sample was compared with the Violet Red Bile Agar plate method in a collaborative study by 18 laboratories. Significantly ($P < 0.05$) higher numbers of coliform were recovered by the dryfilm method from 2% milk samples at the 3 inoculum levels, the chocolate milk at the low and high-inoculum levels, and the cream at the high-inoculum level.

Baylis *et al.,* (2000) compared the ability of four rapid methods and a standard cultural method to detect low levels of heat-injured cells of *Salmonella tvphimurium* in ice cream and skimmed milk powder. The detection of *Salmonella* in samples contaminated with low levels (< 10 cfu 25 g-1) was significantly greater with the novel broth method than with the other methods ($P < l= 0.01$).

Bindschedler, De Man and Curiat (1981) evaluated five culture media for the detection of coliform, *E.coli,* and *Enterobacter* in several varieties of dairy products (fresh and dehydrated) and dehydrated cocoa products.

Twedt *et al.,* (1994) conducted a collaborative study on the recovery of viable *Listeria monocytogenes* from milk and dairy products (Camembert cheese, Limburger cheese, skim milk powder, and ice cream).

Vasavada (1988) focused attention on milk, cheese, and ice cream contaminated with pathogenic bacteria, *viz., Listeria monocvtogenes, Yersinia enterocolitica, (Campylobacter jejuni,* and enteropathogenic *Escherichia coli.* This review presents information on characteristics of these pathogens and illnesses caused by them.

Chapter Three

MATERIALS & METHODS

MATERIALS AND METHODS

The research work was conducted in laboratory of the Discipline of Biotechnology and Genetic Engineering of Khulna University.

Sampling site

Raw milk samples were collected from 40 different shops at Khulna City. The study area included Gallamari, Nirala, Moylapota, Sonadanga, Shibbarimor, Rupsha, Khalispur, Doulatpur, New market and Khulna University campus.

Sample collection

For the collection of samples some clean, dry and sterile screw cap tubes were taken to the sampling site. Sufficient amount of raw milk was collected from each site, kept in screw cap tubes and labeled. Measures were taken to avoid contamination as far as possible. Samples were processed immediately for microbiological analysis.

Preservation of sample

Samples were transported to the laboratory as soon as possible after sampling. Samples were preserved at 4^0 C for immediate use. However, for long term usage samples were stored at -20^0 C.

Preparation of samples and Microbiology tests

The milk samples were mixed well before diluting. Milk samples were ten times diluted mixing 1 ml raw milk with 10 ml of phosphate buffered saline (PBS). The diluted samples were mixed again by using vortex machine. These diluted samples were used for ten fold serial dilutions in one drum vial. A measured volume (0.1 ml) of the sample was transferred onto each sterile Petri plates, carefully spread and stored at overnight in the incubator. Eosin Methylene Blue (EMB) agar was used as media because it contains methylene blue which inhibits gram positive bacteria (Atlas and Bertha, 1997). Gram-negative lactose fermenters (coliform) that grow on this medium will produce "nucleated colonies". Colonies of *Escherichia coli* and *Enterobacter aerogenes* can be differentiated on the basis of size and the presence of a greenish metallic sheen (Atlas, Parks and Brown, 1995).

The method of aerobic plate count proposed by Andrews (1992) was followed to determine the total colony count of the samples. A series of dilutions of the samples (10^2, 10^4 and 10^6) was made and measured volume (0.1ml) was transferred onto Petri dishes and carefully spread. After incubation at 37^0 C for 24 h, the colonies were counted. Samples of measured volume were transferred onto EMB plate and carefully spread by glass rod. After incubation overnight characteristics metallic sheen (MS) and non-metallic sheen were observed and colonies were stored in one drum vials which were used for further biochemical test.

Measured volume (0.1 ml) was transferred onto the Xylose Lysine Deoxycholate (XLD) agar and carefully spread. The plates were then incubated at 37^0 C for 24 hours and observed for characteristics colonies of *Salmonella* sp. with or without black centre on XLD.

Biochemical test for identification of bacteria

Indole test

Typical colonies were inoculated into individual tube of tryptone water and incubated at 37°C for 48 hours. KOVAC's reagent was mixed drop wise into the culture to observe the characteristics indole ring. Organisms that posses the enzyme tryptophanase can break down the amino acid tryptophan to indole. When indole reacts with para-dimethyl-aminoberzaldehye (Kovac's reagent) a pink colour is produced.

Methyl Red (MR) Test

Using sterile technique, each experimental organism was inoculated into tubes containing 5ml of 1% peptone water. Then the inoculated tubes were incubated up to 48 hours at 37°C. One third of each culture was transferred into an empty tubes and set for the Voges-Proskauer test. Five drops of the methyl red indicator were added to the remaining aliquot of each culture and observed for the color of the culture turning red as an indicative of a positive test.

Voges - Proskauer Test

To the aliquots of each broth culture separated during the methyl red test, 10 drops of Barritt's reagent-A was added and culture was shaken. Immediately 10 drops of Barritt's reagent-B was added and the culture was re-shaken by every 3 to 4 times. The culture was observed up to 15 minutes for the formation of pink complex as an indicative of positive VP test.

Citrate Utilisation Test

Using sterile technique, the suspected isolate were inoculated into its appropriately labelled Simmon Citrate slant by means of loop inoculation and one tube should be served as a control. Then the inoculated tubes were incubated for 24 to 48 hours at 37°C and observed for the presence of growth and turning the color from deep green to prusian blue.

Biochemical test for the identification of *Salmonella*

Urease Test

Using sterile technique, each experimental isolate was inoculated into it appropriately labelled urea agar tube by means of loop inoculation. Then the inoculated tubes were incubated at 37 °C for 24 hours. Deep pink colour indicates splitting of urea resulting production of ammonia. Ammonia is alkaline and turns the indicator a bright pink colour, which constitute the positive result.

Triple Sugar Iron Agar

Principle

KIA contains three sugars: glucose (0.1%), lactose (1.0%), and sucrose (1.0%). It also contains phenol red to indicate a change in pH and ferrous sulfate to demonstrate H_2S production. This is a complex medium that contains a large amount of lactose and a very small amount of glucose; a pH indicator (yellow in acid and red in base); and iron, which is precipitated as a black sulfide if H2S is produced by an organism. Theses ingredient allow determining four biochemical properties of the organisms. Salmonella culture produce an alkaline slant (red) and acid butt (yellow) with or without production of H_2S but no gas.

Procedure

1. All tests in this media relied on anaerobic conditions. To provide this the organism must be introduced into the media, not on the surface. Using a sterile inoculating needle, touch the organism to be tested and stab the SI media penetrating to the bottom of the tube. Then removing the needle streak the slant.
2. Tubes were incubated for at least 18 hours at 35-37°C.
3. After incubation following observation were made according to the type of sugar utilized and nature of end product (acid or alkali). 3.6.2.3 H_2S Production:

The TSI agar medium also contains sodium thiosulfate, a substrate for hydrogen sulphide (H_2S) production, and ferrous sulphate for detection of this colourless end product. Following incubation, only cultures of organisms capable of producing H2S will show an extensive blackening in the butt because of the precipitation of the insoluble ferrous sulphide.

Gas formation

If an organism forms gas from glucose or lactose, the agar in the butt shows bubbles or cracks. If only glucose is fermented, the amount of gas formed may be too small to detect as gas in the agar.

Motility

Inoculate a tube of motility agar medium by stabbing to a depth of l cm from the bottom or up to upper third or less of the medium. After incubation at 37°C for 24 to 48 hours, examine the growth pattern of the organisms. A motile organism migrates from the stab line and diffuses into the medium, causing turbidity; or it may exhibit fuzzy streaks of growth. Growth of non-motile organisms is concentrated along the stab line, with the surrounding medium remaining clear.

Glycerol Fermentation Test

Glycerol- Peptone water broth was prepared adding measured amount of peptone, sodium chloride, glycerol, and bromothymol blues in distilled water and dispersed in 3 to 4 ml volume in test tube. A negative reaction can help to exclude the possibility of selecting *Hafnia* and *Citobacter*.

100 ml of raw milk sample was added to 150 ml buffered peptone water and incubated at 37^0 C for 24 hours (pre-enrichment)

↓

Enrichment was done in Selenite Broth (SB) or Tetrathionate Broth (TB) in Mc carty's bottle by incubated at 37^0 C for 24 hours

↓

Colonies were streaked onto the surface of XLD plate and incubated at 37^0 C for 24 hours

↓

Typical colonies from XLD plates were picked (i.e., red smooth with or without black dark centre)

Fig 1: Flow chart for the isolation of *salmonella sp.*

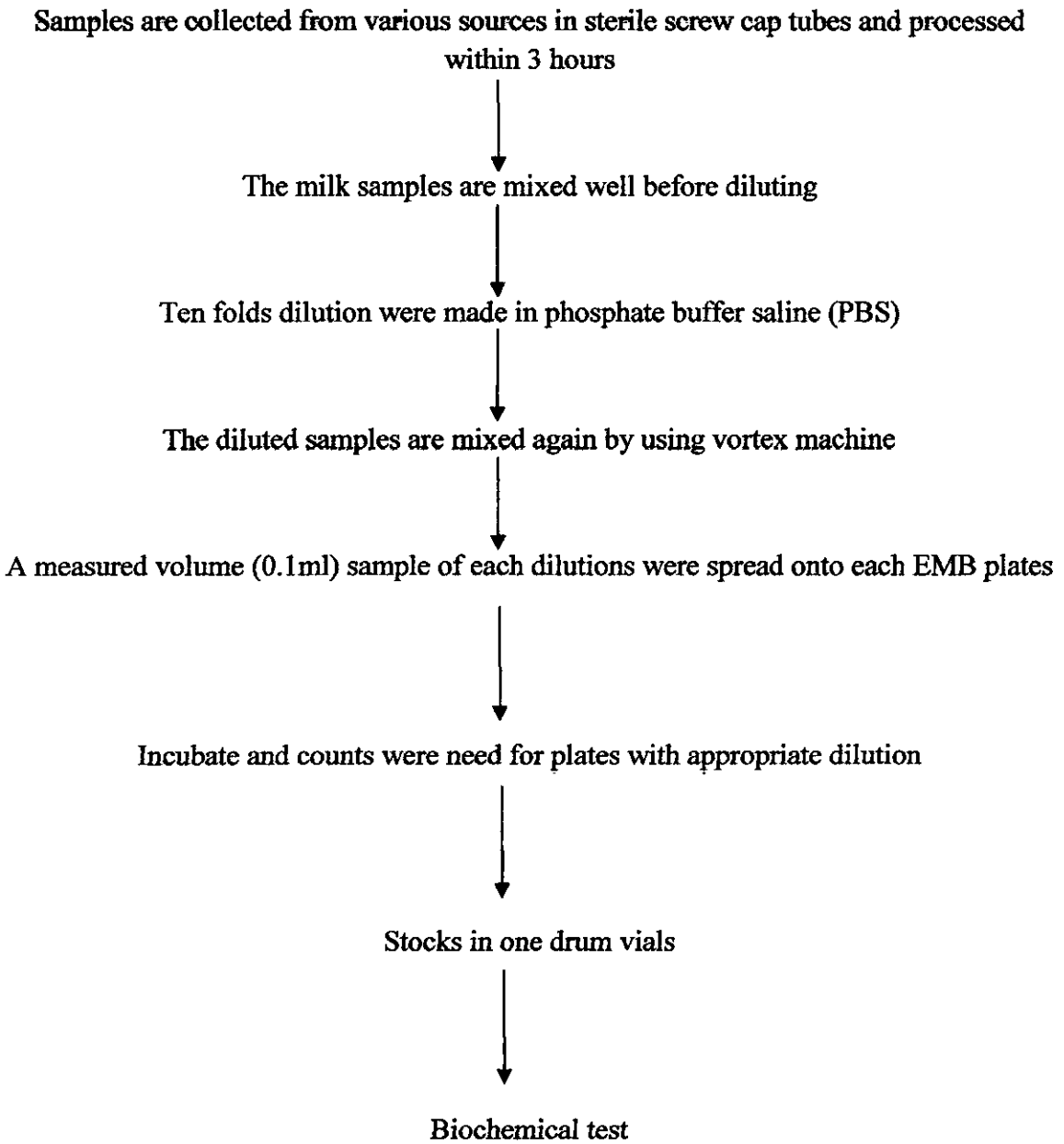
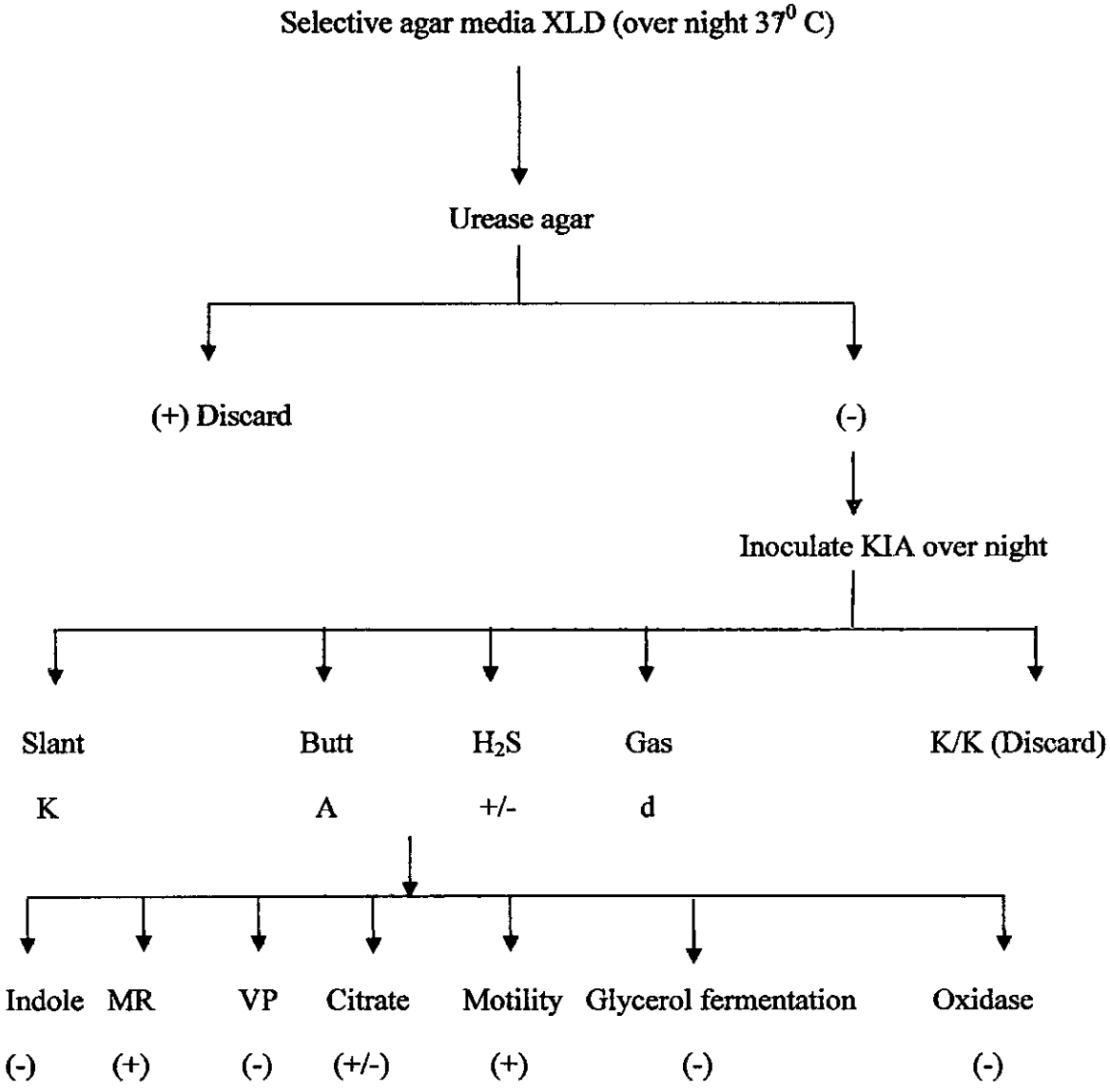


Fig 2: Flow diagram for estimation of faecal coliform.



Symbols:

K = alkaline (red) reaction, A = acid (yellow) reaction, + = positive reaction,
- = negative reaction, d = different biochemical types.

Fig 3: Flow chart for the identification of *Salmonella sp.*

Chapter Four
RESULTS & DISCUSSION

RESULTS AND DISCUSSIONS

Measurement of total aerobic count onto Plate Count Agar

The study was undertaken to assess the potable quality of raw milk in Khulna City Corporation. Forty raw milk samples were collected from various shops at Khulna City and were used for checking the microbiological quality. Total aerobic count is determined by standard plate count method. Standard Plate Count (SPC) determines the number of visible colony forming units (numbers of individual or tightly associated clumps of bacteria) per 1 ml of milk. If SPC exceeds 5000 cfu/ml which is highly contaminated. SPC is usually less than 5000 cfu/ml, the milk sample is good. A SPC of 10000 should be achievable by most farms (Murphy, 1997). The Extension Milk Quality Leadership Council has recommended that quality milk should have a SPC of 5000 and less. In this experiment, 0.1ml volume samples from three different dilutions (10^2, 10^4 and 10^6) were used. In most of the cases 10^4 was found to be countable. All of the samples, total bacteria count was exceeding to be acceptable range.

Bacterial growth on the EMB plate

Coliform count provides an indication of unhygienic production practices and mastitis infection. A count of less than 100 cfu/ml was considered acceptable. Counts of 10 cfu/ml or less were achievable and desirable. To confirm the presence and identification of feacal coliform in the raw milk, the diluted samples were inoculated in the EMB agar plates and incubated at 37^0 C for 24 hours. After incubation, the morphology of the colony characteristics was recorded. Results were shown in table 1. Results indicated that all of the samples onto the EMB agar plate produced black centered colonies with or without metallic sheen. The characteristics growth of all samples confirmed the presence of coliform of both feacal (*E.coli)* and non-feacal (*Enterobacter*) origin. Then both MS and NMS colonies were picked and stored at 4^0 C for biochemical characterization.

In 40 samples, R-1, R-2, R-3, R-4, R-5, R-10, R-11, R-12, R-13, R-14, R-15, R-17, R-24, R-27, R-28, R-29, R-30, R-31, R-32, R-34, R-36, R-40 milk samples onto the EMB plate produced black centered colonies with metallic sheen and R-6, R-7, R-8, R-9, R-16, R-18, R-19, R-20, R-21, R-22, R-23, R-25, R-26, R-33, R-35, R-37, R-38, R-39 milk samples onto the EMB plate produced only black centered colonies without metallic sheen. "-" sign indicates no growth of colonies on EMB plates.

Biochemical test for identification of bacteria

To differentiate feacal coliform (*E. coli*) form coliform of non-feacal (*Enterobacter*) origin isolates were subjected to indole, methyl red, Voges-Proskauer and citrate utilization test. Feacal coliform (*E. coli*) shows indole and methyl red positive while *Enterobacter* shows positive reaction in Voges-Proskauer and citrate utilization test.

In 40 samples, R-1, R-2, R-3, R-4, R-5, R-10, R-11, R-12, R-13, R-14, R-15, R-17, R-24, R-27, R-28, R-29, R-30, R-31, R-32, R-34, R-36, R-40 milk samples onto the EMB plate produced black centered colonies with metallic sheen and showed indole and methyl red positive in biochemical test. On the other hand, R-6, R-7, R-8, R-9, R-16, R-18, R-19, R-20, R-21, R-22, R-23, R-25, R-26, R-33, R-35, R-37, R-38, R-39 milk samples onto the EMB plate produced only black centered colonies without metallic sheen and showed positive reaction in Voges-Proskauer and citrate utilization test. So samples, R-1, R-2, R-3, R-4, R-5, R-10, R-11, R-12, R-13, R-14, R-15, R-17, R-24, R-27, R-28, R-29, R-30, R-31, R-32, R-34, R-36, and R-40 were feacal (*E. coli*) origin and samples R-6, R-7, R-8, R-9, R-16, R-18, R-19, R-20, R-21, R-22, R-23, R-25, R-26, R-33, R-35, R-37, R-38, R-39 were non-feacal (*Enterobacter*) origin.

Observation of *Salmonella* sp. growth on the XLD plate

100 ml of raw milk samples were transferred to 150 ml of buffered peptone water and homogenized for one minute. After incubated at 37^0 C for 24 hours, 1 ml mixture was added to each of 10 ml of tetrathionate broth (TB). The broths were incubated at 37^0 C for 24 hours. After incubation, a loopful from each of selective enrichment broths were transferred to the surface of the Xylose Lysine Deoxycholate (XLD) agar medium and incubated. Five samples (R-16, R-26, R-30, R-35 and R-38) were shown characteristics red smooth colonies which were stored for biochemical analysis. In urease test, five samples were shown negative reaction. In Triple Sugar Iron, three samples were shown K/A and two samples were shown A/A reaction. Three samples were H_2S positive and could not produce gas. Two samples were H_2S negative and could produce gas. Only R-38 sample was shown methyl red positive, indole, VP and citrate negative. So, R-38 sample was *Salmonella* sp. positive.

Table1. Enumeration of microorganisms in different milk samples by standard plate count methods

Sample ID	Total aerobic count (cfu/ml)	Number of coliform (MS) count on EMB (cfu/ml)	Number of coliform (NMS) count on EMB (cfu/ml)
R-1	$5x10^7$	$1.2x10^4$	-
R-2	$29x10^6$	$2x10^4$	-
R-3	$27x10^6$	$1.9x10^4$	-
R-4	$2x10^7$	$1.7x10^4$	-
R-5	$44x10^6$	$1.8x10^4$	-
R-6	$19x10^6$	-	$2.2x10^3$
R-7	$64x10^6$	-	$8.8x10^3$
R-8	$2x10^7$	-	$2.2x10^3$
R-9	$19x10^6$	-	$3.2x10^3$
R-10	$34x10^6$	$1x10^4$	-
R-11	$27x10^6$	$1.6x10^4$	-
R-12	$21x10^6$	$1x10^4$	-
R-13	$28x10^6$	$1.2x10^4$	-
R-14	$15x10^6$	$1.8x10^4$	-
R-15	$19x10^6$	$1x10^4$	-
R-16	$35x10^6$	-	$1x10^4$
R-17	$18x10^6$	$1.9x10^4$	-
R-18	$4x10^7$	-	$1.5x10^4$
R-19	$6x10^7$	-	$2.8x10^4$
R-20	$67x10^6$	-	$2.5x10^4$
R-21	$29x10^6$	-	$1.2x10^4$
R-22	$39x10^6$	-	$2x10^4$
R-23	$42x10^6$	-	$1.9x10^4$
R-24	$33x10^6$	$1.7x10^4$	-
R-25	$31x10^6$	-	$1.9x10^4$
R-26	$28x10^6$	-	$1.2x10^4$
R-27	$17x10^6$	$1.3x10^4$	-
R-28	$26x10^6$	$1.9x10^4$	-
R-29	$36x10^6$	$2x10^4$	-
R-30	$25x10^6$	$1x10^4$	-
R-31	$3x10^7$	$1x10^4$	-
R-32	$44x10^6$	$2x10^4$	-
R-33	$66x10^6$	-	$2.2x10^4$
R-34	$72x10^6$	$3.1x10^4$	-
R-35	$45x10^6$	-	$2x10^4$
R-36	$5x10^7$	$2.1x10^4$	-
R-37	$53x10^6$	-	$2.3x10^4$
R-38	$42x10^6$	-	$2.8x10^4$
R-39	$52x10^6$	-	$1.9x10^4$
R-40	$51x10^6$	$2.3x10^4$	-

R=Raw milk, MS=Metallic sheen, NMS=Non metallic sheen, - = Negative

Table 2: Biochemical test for identification of coliform (both feacal and non-feacal)

Sample ID	Total number of sample	Colony characteristics	Biochemical test (IMViC)				
			Indole	Methyl Red	Voges-Proskauer	Citrate	Remark
R-1, R-2, R-3, R-4, R-5, R-10, R-11, R-12, R-13, R-14, R-15, R-17, R-24, R-27, R-28, R-29, R-30	17	Metallic sheen	+	+	-	-	*E. coli*
R-6, R-7, R-8, R-9, R-16, R-18, R-19, R-20, R-21	9	Non-metallic sheen	-	-	+	+	Enterobacter
R-22, R-23, R-25, R-26, R-31	5	Non-metallic sheen	-	+	-	-	*E. coli*
R-32, R-33, R-34, R-35, R-36, R-37, R-38, R-39, R-40	9	Metallic sheen	-	-	+	-	Enterobacter
Grand total	40						

R=Raw milk, + =Positive test, - = Negative test

Table 3. Identification of Salmonella sp. from raw milk samples and biochemical test.

Plating on XLD	Urease	Triple Sugar Iron				Indole	MR	VP	Citrate	Oxidase	Glycerol fermentation	Remark
		Slant	Butt	H_2S	Gas							
Red smooth colonies	-	A	A	+	-	NA	-	-	-	+	-	-Ve
Red smooth colonies	-	K	A	-	+	NA	-	-	-	+	+	-Ve
Red smooth colonies	-	A	A	+	-	NA	-	-	-	-	+	-Ve
Red smooth colonies	-	K	A	-	+	NA	-	-	+	-	-	-Ve
Red smooth colonies	-	K	A	+	-	-	+	-	-	-	-	+Ve

R=Raw milk, NA=Not applicable, + =Positive test, - = Negative test, K= alkaline (red) reaction, A = acid (yellow) reaction.

Fig 8. Growth of feacal coliform bacteria on EMB plate showing pure colonies with metallic sheen at incubated at 37^0 C.

Fig 9. Growth of non- feacal coliform bacteria on EMB plate showing pure colonies at incubated at 37^0 C.

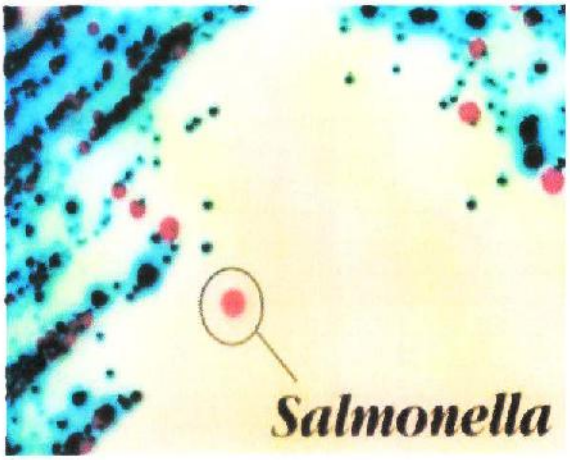


Fig10. Isolation plate of *Salmonella sp.* after 24 hours incubation of milk sample on XLD agar medium.

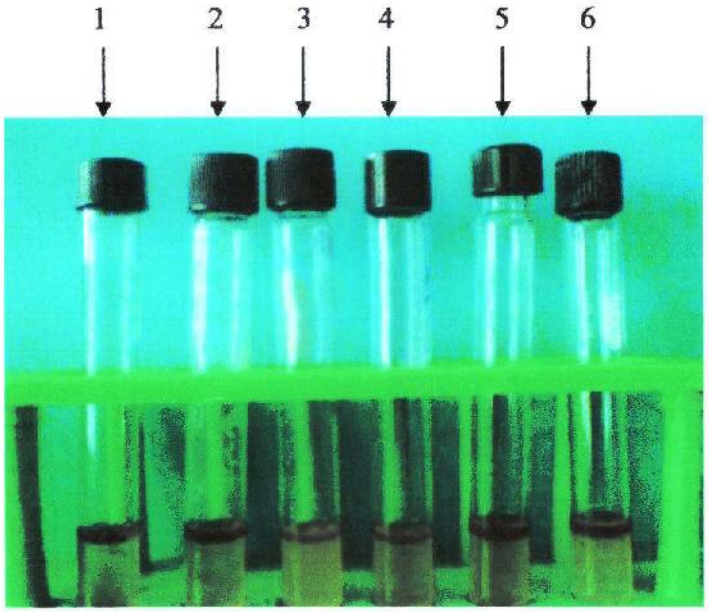


Fig11. Indole test to determine indole production from tryptophan as shown by feacal coliform (*E. coli*). Tube 1, 2 &3= show indole positive test, tube 4=shows negative test, tube 5 =positive control and tube 6 =negative control.

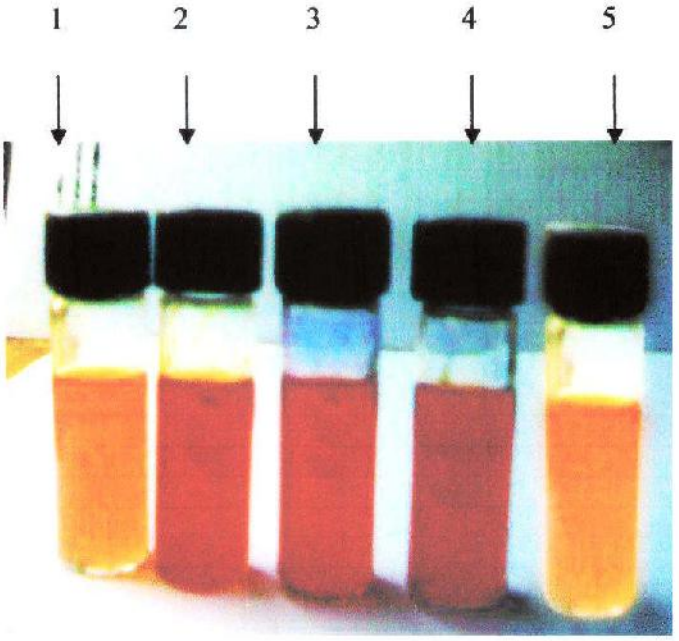


Fig12. Methyl red test to determine acidic end product from glucose. Tube 1=shows negative test, tube 2&3=cherry red colour represents positive test, tube 4=positive control and tube 5=negative control.

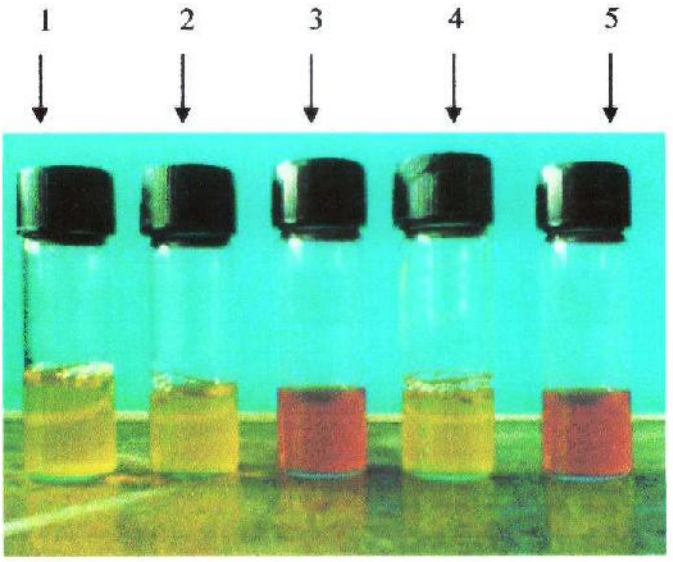


Fig13. Voges –proskauer test to detect the presence of acytylmethyl carbinol as produce by *Enterobacter*, coliform bacteria of non-feacal origin. Tube 1&2=represents negative test, tube 3= represents positive test,, tube 4=negative control and tube 5=positive control.

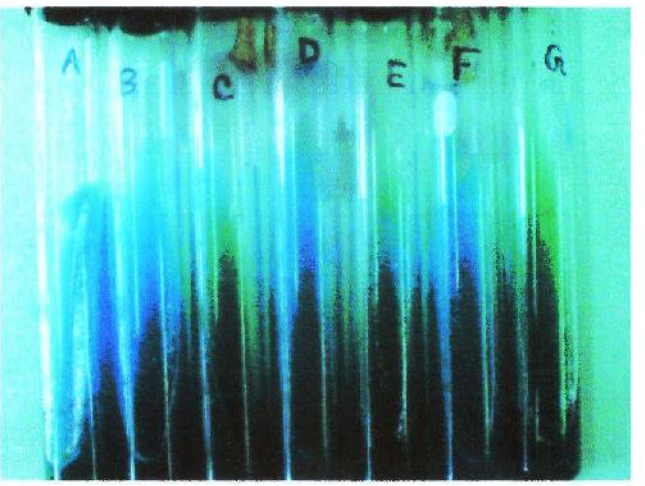


Fig14. Citrate test to determine the utilization of citrate as shown by *Enterobacter*. A, B &D=indicates positive test, C&E= indicates negative test, F=positive control and G=negative control

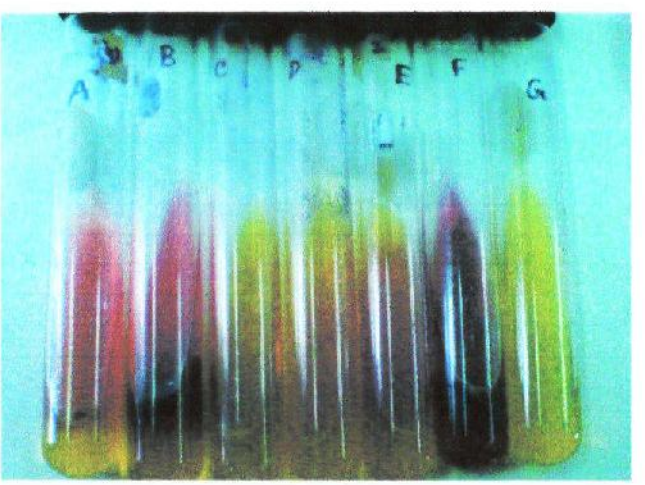
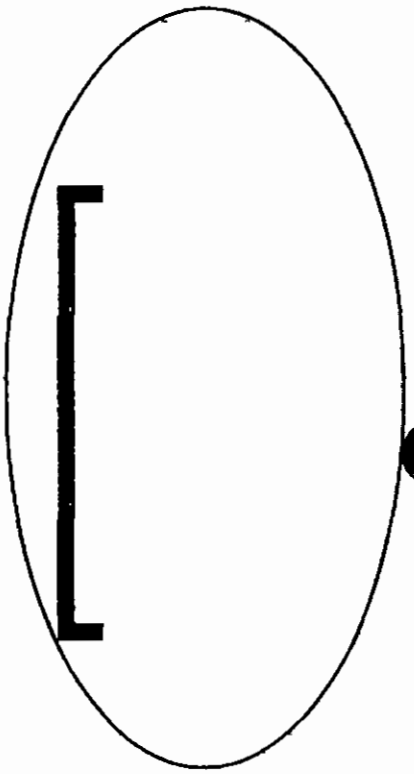


Fig15. Triple Sugar Iron Agar test for screening Salmonella isolates: F=positive control and G=negative control. B= represents K/A reaction (alkaline in slant and acid in butt) which produce H_2S. Tube A, C, D&E=represents H_2S production without gas and A/A reaction (acid in slant and acid in butt).

Fig16. Urease test determines ammonia production. A-C=negative result, D= positive control and E= negative control.



Chapter Five
CONCLUSION

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Microbial quality control of raw milk is very important before the product being marketed. Considering high count and prevalence of total coliform and faecal coliform in most of the raw samples under investigation, it is anticipated that the milk samples are exposed to feacal contamination due to faulty distribution system. Exposure to feacal contamination increases the probability of different infectious disease that is disseminated by orofeacal-root evidenced by the detection of *Salmonella* in one milk sample. HACCP overcomes shortcomings of reliance only on microbial testing. So, HACCP is to be promoted and incorporated into Food Safety Legislation in out Country. Following recommendation can be made to prevent the repeated demonstration of coliform organism:

- Clean and sanitize after each uses and before using.
- Udder sanitation practices; teats need to be cleaned and dried.
- Pure water supply should be needed.
- Teat cup liners should be cleaned free of cracks.
- Dirty animals should be cleaned.
- Hands must be cleaned properly before milking.

Chapter Six
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REFERENCES

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APPENDICES

APPENDICES

EMB Agar:

Source- HiMedia Laboratories Limited, Mumbai, India

Typical formula (g/L)

Peptic digest of animal tissue	10.0
Di-potassium phosphate	2.0
Lactose	5.0
Sucrose	5.0
Eosin-Y	0.40
Methylene blue	0.065
Agar	13.50

pH 7±0.2 at temp. 25^0 C

Used for gram negative bacteria.

Preparation:

36.0 gm agar powder was suspended in 1000 ml distilled water and then heated to dissolve completely. After dissolving, the medium was sterilized by autoclaving at 15 lb pressure (121^0 C) for 15 minutes. Then it was cooled to 50^0 C and shaked the medium in the order to oxidized the methylene blue.

Simmons Citrate Agar:

23 gm citrate agar was suspended in 1000 ml. of distilled water and boiled to dissolve completely. Then the medium was distributed as 7-8 ml. aliquots to each of the screw cap tubes and sterilized by autoclaving at 15 lb. pressure (121^0 C) for 15 minutes and cooled to room temperature and allowed to solidify as slopes with 1 inch. butt leaning against a sterile glass pipette.

Source-Oxoid Ltd. Basingstoke, Hampshire, England

Typical formula (g/L)

Magnesium sulfate	0.2
Ammonium dihydrogen phosphate	1.0
Di-potassium phosphate	1.0
Sodium chloride	5.0
Sodium citrate	2.0
Bromothymol blue	0.08
Agar	15

pH 7±0.2 at temp. 10-25^{0}C

Buffered Peptone Water (BPW)

20 gm dehydrated buffered peptone water base was suspended in 1000 ml of distilled water and boiled to dissolve completely. Then the medium was dispensed as desired and sterilized by autoclaving at 15 lb pressure at 121^0 C for 15 minutes.

Selenite Broth (SB)

23 gram of powder (SB) was suspended in 1000 ml distilled water in a conical flask and warmed to dissolve completely. Then the medium was distributed into sterile tubes and sterilized in a boiling water bath or free flowing stream for 10 minutes.

Tetrathionate Broth (TB)

46 gm. of powder was suspended in 1000 ml distilled water in a conical flask completely and heated to boiling and cool to 45^0 C. Then 20 ml of iodine solution were added into the medium and mixed well and distributed into the tube 10 ml amount of sterile tubes. Iodine solution was prepared by dissolving 6 gm of iodine crystals and 5 gm potassium iodine in 20 ml of water.

Xylose Lysine Deoxycholate (XLD):

56.5 gm of XLD agar was suspended in 1000 ml. of distilled water, heated with frequent agitation until the medium was dissolved and then transferred to a water bath at 50°C. After cooling, the medium was poured into sterile Petri dishes

Kliger's Iron Agar (KIA):

55.6 gm of KIA powder was suspended in 1000 ml. of distilled water in a conical flask and boiled to dissolve completely. Then the medium was distributed as 7 ml. into required number of screw capped test tubes. The tubes were sterilized by autoclaving at 15 lb. pressure (121°C) for 15 minutes. Then the medium was allowed to solidify as slopes with 1 inch. butt leaning against a sterile glass pipette.

Composition;

Beef extract	3g
Yeast extracts	3g
Peptone extract	15g
Proteose peptone	5g
Lactose	10g
Glucose	1g
$FeSO_4$	0.2g
Sodium thiosulphate	0.3g
Agar	15g
Phenol red	0.024g
p^H	7.4

Urea Agar:

2.4 gm dehydrated urea agar base was suspended in 95 ml. distilled water in a conical flask and boiled to dissolve completely. Then the medium was sterilized by autoclaving at 115 °C for 20

minutes. The sterilised medium was cooled to 50 °C and aseptically introduced into 5 ml. of filter sterile 40% urea solution. Then the medium was mixed well. 2 ml. of this was distributed into sterile one drum vial.

Composition:

Urea	20g
Yeast extracts	0.1g
KH_2PO_4	9.0g
K_2HPO_4	9.5g
Phenol red	0.01g
Distilled water	1litre
p^H	6.8

MacConkey Agar (MCA)

40.0 gm of MacConkey agar powder was suspended in 1000 ml. of distilled water and heated if necessary to dissolve the medium completely. Then autoclaved at 121^0 C temperature for 15 minutes at 15lb pressure. The medium was distributed into the sterile plates and allowed to solidify.

Indole

Source-Oxoid Ltd. Basingstoke, Hampshire, England
Typical formula (g/L)

Total nitrogen	14.0
Amino nitrogen	2.6
Sodium chloride	1.6

Source of sodium chloride-Fluka Switzerland

MR-VP broth

Source-Oxoid Ltd. Basingstoke, Hampshire, England

Typical formula (g/L)

Peptone	5.0
Glucose	5.0
Phosphate buffer	5.0

P^H 7±0.2 at temp. 10-25^0 C

Measured amount (17 gm) of dehydrated MR -VP broth base was dissolved in 1000 ml of distilled water. The suspension was aliquate in 9 ml at each screw cap tubes, then autoclaved 151b pressure at 121^0 C for 15minutes.

Plate Count Agar

Suspended 28.0g in 1litre distilled water. Heat to boil to dissolve the medium completely and autoclaved 151b pressure at 121^0 C for 15minutes.

Composition:

Ingredient	**Amount**
Bacto peptone	5.0 gm
Beef extract	3.0 gm
Agar	15.O gm
Distilled water	1000 ml

P^H adjusted to 6.8 ± 0.2 at 25°c.

KOVAC'S reagent, for detection of indole

P -dimethylaminobenzaldehyde	5g
Iso -Amyl alcohol	75ml
Conc. HCl	25ml

Dissolve the aldehyde in the alcohol by gently warming in a water bath (about 50 -55^0 C. Cool and add the acid. Protect from light and store at 4^0 C.

Note: The reagent should be light yellow to light brown in colour; some samples of amyl alcohol are unsatisfactory, and give a dark colour with the aldehyde.

Methyl Red Solution, detection for acid

Methyl red	0.1 gm
Ethyl alcohol	300.0 ml
Distilled water	200.0 ml

Note: Dissolve the methyl red in the 95% ethyl alcohol. Dilute to 500 ml with distilled water.

Barrit's reagent, for detection of acetylmethyl -carbinol

Solution A:

Alpha -naphthol	5.0 gm
Ethanol, absolute	95.0 ml

Note: Dissolve the alpha -naphthol in the ethanol with constant stirring.

Solution B:

Potassium hydroxide	40.0 gm
Creatine	0.3 gm
Distilled water	100.0 ml