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Morphological, biochemical and polymerase chain reaction based studies for isolation and identification of *Salmonella* in milk samples of Kathua city

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Abstract: The study was conducted on milk samples of Kathua city. The purpose of study was to get hand on training on various techniques of microbiology and molecular biology by using the morphological, biochemical characteristics and polymerase chain reaction identifying the *Salmonella* which is disease causing pathogen. *Salmonella typhimurium* and *Salmonella entireties* are accounted nearly 75% of all *Salmonella* isolated, because of the increased incidence of salmonellosis in human, much of which in associated with poultry, eggs, and milk. Control of salmonella infections in animals prevents the food borne salmonellosis**.**

Key words: Disease; pathogen; salmonellosis; *Salmonella entireties*; *Salmonella typhimurium*;

1. Introduction

Food borne diseases are recognized as one of the most serious public health concerns today. With travel and trade increasing over the years, the risk of dissemination of pathogens grows continuously. Microbiological quality control programmes are increasingly applied throughout food production in order to minimize the risk of infection for customer. Thus, the availability of reliable and rapid test systems to detect the presence or absence, even the degree of contamination of pathogens, become important for the agricultural and food industry. Such systems would also find a place with the framework of legislative control measures.

Traditional and standardized analysis of food for the presence of pathogen (bacteria) relies on the enrichment and isolation of presumptive colonies on the solid media, using approved diagnostic artificial media. This is usually followed by biochemical identification. Traditional methods of detection require several days to weeks to obtain the result.

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Furthermore, the traditional methods are based on the phenotypic properties, yet the phenotypic properties by which the bacteria are identified may not always expressed, and when expressed, they may difficult to classify.

For about 100years, microbial detection, identification and characterization have relied on the ability to cultivate, purify and characterize the organisms by morphological, biochemical and immunological characteristics; a particular pattern of which may define a specific group or an individual species.

The macroscopic or macromolecular features are collectively known as phenotypes. Although traditional methods of microbial identification are reliable, there are many short coming associated. These methods are usually time consuming, some phenotypes displayed by a single organism may vary, as a function of the particular growth conditions used in the and therefore appear to be identical.

Advances in molecular biology technology, particularly the *Polymerase Chain Reaction* have allowed for more reliable microbial identification.

The PCR and other recently developed amplification techniques have simplified and accelerated the in vitro process of nucleic acid amplification. The amplified products known as amplieons may be characterized by various methods, including agarose gel electrophoresis, nucleic acid probe hybridization, analysis of fragments after restriction endonuclease digestion or direct sequence analysis. Rapid techniques of nucleic acid amplification and characterization have

significantly broadened the microbiologist`s diagnostic arsenal.

The objective of study was to detection of *salmonella* and find out the incidence of this pathogen in milk samples. Furthermore, the aim was to get hand on training on various techniques of microbiology and molecular biology.

2. Material and Methods

2.1 Growth media

The composition per liter of the growth used is as follows. 18g of agar per liter added for preparation of solid media expect where mention otherwise.

2.2 Nitrate Test

Nitrate broth, bacto peptone 10g. beef extract 5g, sodium chloride 5g (pH 7.2). or bacto peptone 5g, beef extract 3g potassium nitrate 5g (pH 7.2), sol. A (sulphanic acid 8g, 5 N acetic acid 1 liter) and sol. B (alfa-Naphthyllaine 5g, 5 N Acetic acid 1 liter). 3ml of nitrate broth was inoculated with the sample and incubated at 37 ºC for 24h, five drops of each solution A (sulphanilie) and solution B (alfa-naphthylamine) were added. The development of red color indicated a positive test. The samples that did not generate a red color were subjected to further confirmation for a negative test.

2.3 Oxidase Test

Oxidase reagent (tetramethyl-p-phenylenediamine 0.5g ,distilled water 50ml). The bacterial culture was streak inoculated into Tripton Soya Agar and incubated at 37^{o}C for 24h. A few drops of oxidase reagent were added, the development of blue color within 10-15 sec, indicated an oxidase positive test.

2.4 Citrate Utilization

Simmons citrate medium (pH 6.9), ammonium dihydrgen phosphate 1g, dipotassium phosphate 1g, sodium chloride 5g, sodium citrate 2g, magnesium sulphate, 0.2g brom thymol blue 0.08g, potassium iodide 2g and distilled water 300ml.

The bacterial culture was streak-inoculated into the Simmons Citrate Agar slant and incubated for 48 hr at 37 $^{\circ}$C. the growth of culture and development of blue color indicate the citrate positive test.

2.5 Indole production and H_2S Production

Kovac`s reagent, P-Dimethlaminobenzaldehyde 5g amyl alcohol 75ml, conc. Hydrochloric acid 25ml (P-dimethlaminobenzaldehyde was dissolved in amyl alcohol followed by the addition of conc. Hydrochloric acid). SIM medium pH 7.3, bacto peptone 30g, beef extract 3g, ferrous ammonium sulphate 0.2g, sodium thiosulphate 0.035g, and agar 3g.

For the detection of indole production, the culture was stab inoculated into the sim agar deep tubes and incubated for 48 hat 37^{o} C. the presence of indol was detected by the addition of 10 drops of Kovac`s reagent which produces a cherry red colour layer in positive culture. The presence of black coloration was taken as a confirmation for the production of H_2S.

2.6 Triple Sugar Iron Agar test and H_2S Production

Triple sugar iron agar (TSI, pH 7.4) , beef extract 3g, bacto yeast extract 3g, bacto paptone15g, protesone peptone 5g, lactose 10g sachharose10g, dextrose 1g, ferrous sulphate 0.2g sodium chloride 5g, sodium thio-sulphate 0.3g, phenol red 0.002g.

The culture was inoculated into the slant of triple sugar iron agar slant by means of staband streak inoculation and inoculated at 37^{0}C for 24hr. The slants were examined for the acid and gas production and results recorded as per protocols given Cappiccino and Sherman (1996)

2.7 Methyl red and voges-proskauer test

Barrit`s reagent sol.A, alfa-naphthol 5g dissolved in95ml absolute alcohol with constant shaking. Sol. B, potassium iodide 40g, creatine 0.3g, distilled water was added to make the fine volume of 100ml. methyl red solution, methyl red 5g, ethyl alcohol 300ml, distilled water 200ml. The culture was inoculated into 10ml of MR-VP broth and incubated for 24h at 37 $^{\circ}$C. Five drops of methyl red indicator were added to 1 ml aliquot of this culture. The production of red color was taken as positive methyl red test.

The other aliquot of the MR- VP broth culture, 10 drops of Barrit`s reagent A were add mixed gently, immediately, add 10 drops of Barrit`s reagent B were added and mixed again. The development of pink color within 5 min. was taken as a Voges-Proskauer positive test.

2.8 PCR Condition

Templates were usually prepared from pure culture of bacterial species. For *Salmonella*, the PCR protocol used was initial denaturaion at 94 $^{\circ}$C for 3 min followed by 35 cycles of 94 $^{\circ}$C for 15 sec at 60 $^{\circ}$C of or 30 sec 72 $^{\circ}$C for 30 sec and final

extension at 72 °C for 5 min. The PCR were run on 15% agarose gets and visualized on a UV illuminator. PCR protocol for the detection of the *Salmonella* by primers QVR 184-QVR 185. The primers used for species yielded a specific PCR product of 752bp. A reaction mixture with all PCR components without DNA was used as a negative control. A 1kb DNA ladder was used as a DNA size maker or examined the DNA fragments by using the agrose gel electrophoresis.

3. Results and Discussion

Salmonella strains were isolated from milk samples using conventional microbiological methods. The complete isolation of *Salmonella* and its identification by biotyping takes 2-3 days. The samples were isolated by pre-enrichment in senite F broth followed by plating on deoxycholate citrate agar and picking up the black colonies for further identification by morphological and biochemical testing 20 milk samples. 15 samples were founded positive. Table 1 shows the biochemical characteristics of the *Salmonella* strains isolated from milk. The identified of culture was also subjected to PCR for molecular diagnosis and both the methods gave the same results. A PCR reaction was set up for the detection of *Salmonella* in enriched milk samples and identification of pure cultures isolated from milk, DNA from other micro organisms in the milk did not interfere with the PCR reaction indicating that the PCR is very specific and suitable for the detection of *Salmonella* spp. The method has been already developed and validated by IIIM Jammu. The primers based in this study are based on flgB gene which is involved in the basal body of flagellum of salmonella. About 50 genes are involved in the structure and function of *Salmonella* flagellum. The protocol used here are highly sensitive, specific, rapid and cost effective. The results are clear and easy to interpret.

Table 1. Biochemical test at *salmonella* strains isolated from raw milk samples

Biochemical tests	Cultures samples			
	1	**2**	**3**	**4**
Nitrate test	+ve	+ve	+ve	-ve
Oxidase test	-ve	-ve	-ve	-ve
Citrate test	+ve	+ve	+ve	-ve
Indole test	-ve	-ve	-ve	-ve
Methyl red test	+ve	+ve	+ve	+ve
Voges proskauer test	-ve	-ve	-ve	-ve

This study indicates that milk is an important carrier of *Salmonella* spp. and could be major health hazard if not processed properly before human consumption. This also indicated the unhygienic condition prevalent in the dairy farms and ignorance of the dairy farm workers.

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