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Isolation and Identification of *Salmonella sp.* from different food

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Abstract

The consumption of meat as well as dry fish and many different types of processed foods are dramatically increasing in recent years. Although the productions are increasing, the quality is not maintained up to standard. The microbial safety status of raw meat, dry fish and pudding was investigated in this study to determine the incidence of *Salmonella sp.* contamination. Isolation and identification of *Salmonella sp.* by conventional tests (microscopy and culture in differential media combined with several biochemical tests) was performed. Presence of *Salmonella sp.* in the foods indicates fecal contamination.

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Introduction

The causative agent of Salmonellosis is *Salmonella sp*. (Cross *et al.,* 1989; Tauxe *et al.,* 1991; Smith *et al.,* 1994; Baumler *et al.,* 2000), a gram-negative, non-spore forming rod and facultative anaerobe (Grimont *et al.,* 2000). Most species are motile (except *S. gallinarum* and *S. pullorum*) by peritrichous flagella. Although optimum temperature for *Salmonella sp*. is 32-37°C, it is also capable of growing at 6-46°C. Generally, detection methods are based on physiological and biochemical markers of the organism (Williams, 1981). Cultural methods are based on nutrient acquisition, biochemical characteristics, and metabolic products unique to *Salmonella sp*. (Ricke *et al.,* 1998).

The natural habitat of *Salmonella sp*. is the intestinal tract of human and other animals (domestic and wild birds, reptiles, amphibians, and insects). They are regular pathogen of human and other animals. They possess a complex antigenic structure and produce variety of toxins and other virulence factors. These bacteria can enter via oral route usually with contaminated food or drink and colonize in the intestine within 8-48 hours to 7-20 days. The mean infective dose for *Salmonella sp*. to produce clinical or sub-clinical infection in humans is 10^6-10^8 CFU/ml, although as few as 10^3 CFU/ml can produce disease to infants and immune compromised individuals. Host factors (gastric acidity, normal intestinal flora, and local intestinal immunity, age of person) contribute to resistance to *Salmonella sp*. infection. People previously exposed to the organism have a high resistance to the organism. According to World Health Organization (WHO) about 80% of all diseases and sickness in the developing world are caused by inadequate sanitation, contaminated water or unavailability of safe water (CDC training, 2012). In Asia and Pacific region, fecal pollution is one of the most serious problems affecting both surface water and ground water bodies and leading to a tenacious persistence of waterborne diseases. When *Salmonella sp*. is released from human or animal host to the environment, it encounters nutritional and physicochemical shocks causing limited time of survival. Although it is an enteric organism, recent research indicates its survival in the environment. *S. typhi* and the paratyphoid bacteria are normally caused septicemic and produce typhoid or typhoid-like fever in humans. Foods other than eggs have also caused outbreaks of *S. enteritidis* disease. *S. typhi* and *S. paratyphi* A, B, and C produce typhoid and typhoid-like fever in humans. In Bangladesh, meat, dry fish, and processed foods are all very popular. Meat, major protein source, may contaminate by cross contamination with carcass, contaminated water used in cleaning, processing and transportation, handlers, container (Aseel *et al.,* 2013). Presence of Salmonella sp. in dry fish and processed food indicates cross contamination and unhygienic food handling. Therefore, it is important to check microbiological quality and take necessary measures to the quality as per international standard and remove any existing flaws.

Materials and methods

Sample Collection

Dry fish (Chittagong), meat (Dhaka) and processed foods (Dhaka) were collected in a clean box and transported to laboratory promptly.

Pre-enrichment and Enrichment

25g of each sample was placed into 175 ml lactose broth and 10g of sample was dissolved in 90ml of Ringer's solution without homogenization to prevent injury to bacterial cells (Birol Özkalp *et al.,*).

Lactose broth (LB) was used for the pre-enrichment and homogenization of food samples were performed as this could damage bacterial cell and incubated at 37°C for 24 hour. Selenite broth (SB) (AOAC international, 2000; June *et al.,* 1995; Hammack *et al.,* 1999) was used for the selective enrichment media for *Salmonella sp*. for 24 hour at 37°C.

Total Microbial Count

Pour Count Agar (PCA) plate was used for the counting of heterotrophic bacteria after incubation at 37°C for 24 hours. Potato Dextrose Agar (PDA) was used for the total fungal count after incubation at

room temperature for at 72 hours without any movement. As most fungal species are spore former and movement will cause the spore to spread and that will give false count.

Colony Morphology

Bismuth Sulfite Agar (BSA) was used as selective media for the growth of *Salmonella sp*. A flow diagram of the total work is given in Fig. 1.

Morphological characteristics (size, shape, surface texture, edge, elevation, color, opacity etc.) developed after 24 hour of incubation, were carefully studied and recorded.

Microscopic Study

Microscopic characteristics (size, shape, arrangement, color, Gram reaction) were observed according to the methods described in Manual of Methods for General Bacteriology by American Society of Microbiology (ASM, 1981). Gram staining was performed to observe size, shape, arrangement, and Gram reaction.

Biochemical Tests

Several biochemical tests (CDC training, 2012) were performed to detect the physiological activities of the selected isolated strain.

Oxidase Test

A positive reaction forms a deep–purple hue within 5-10 seconds. A delay reaction forms color within 10-60 seconds. A negative reaction do not form color or form color after 60 seconds.

Catalase Test

Few drops of hydrogen peroxide was placed onto a clean glass slide and then a small amount of 24 hour old culture was picked and mixed with hydrogen peroxide and immediate bubble formation indicates positive reaction.

Motility Test

The SIM (sulfide, indole, motility) medium containing McCartney bottle was inoculated with the test organism by a single stab with a straight wire and incubated for 24 hour at 37°C. A positive result of motility was indicated by the spread of the stab line. As only the motile bacteria can spread through the semi solid SIM media with their flagella and pilli. And non-motile bacteria do not spread as they lack organelle required for motility.

Indole Production Test

This test was performed to determine the production of indole from the amino acid tryptone. The production of indole is indicated by the formation of cherry red color layer at the top after the addition of Kovac's reagent. And the negative reaction is indicated by dark brown color.

Hydrogen Sulfide Production Test

This test was performed on KIA (Kliggler's Iron Agar). The butt was inoculated with the 24 hour old bacterial culture and the slant was inoculated by streaking. It was incubated at 37°C for 24 hour. A positive reaction for the fermentation of glucose and lactose was indicated by the yellow coloration of both the butt and slant. Presence of cracks indicates the production of acids. Blackening of the medium indicates hydrogen sulfide production. Pink-red slant but yellow but indicates glucose fermentation but not lactose.

MR-Test (Methyl red)

Each broth media tube was inoculated with organisms from sample and incubated at 37°C for 24 hour.

Voges-Proskauer Test (VP)

This test is used to determine the capability of some organisms to produce non-acidic or neutral compound like acetylmethylcarbonyl from organic acids derived from glucose metabolism. Most *Salmonella sp*. is VP-negative (CDC and prevention training, 2012). Each tube was inoculated by loop using sterile technique and incubated over night at 37°C. Barritt's reagent was added and waited for 15 minutes.

Citrate Utilization

In the absence of fermentable sugar some microbes

are able to use citrate as their energy source if they possess citrase enzyme. Citrate agar were stabbed and streaked with a needle by maintaining sterile technique and incubated at 37°C for 24 hours.

Carbohydrate Fermentation

Each tube was inoculated with a loop and no shaking was done as it may force bubble of air in the inverted glass vial which may give false positive result and incubated at 37°C for 24 hour.

Results

Sample Collection

Dry fish, meat and processed food were collected from different places (Dhaka, Chittagong).

Pre-enrichment and Enrichment

Lactose broth (LB) was used for the pre-enrichment and no homogenization of food samples were performed as this could damage bacterial cell and turbidity after 24 hour incubation at 37°C (Fig. 2a).

Table 1. Colony characteristics on BSA.

Colony characteristics	BSA agar
Size	Moderate to small
Shape	Round
Elevation	Convex
Margin	Entire
Surface	Smooth
Color	Black colony with silver metallic sheen at the edge
Opacity	Opaque
Texture	Gummy

Table 2. Microscopic observation.

Parameters	Observation
Size	Small
Shape	Rod
Arrangement	Single
Gram reaction	Negative (Pink)

Salmonella sp. growth in selenite broth is indicated by changing of broth color after 24 hour incubation (Fig. 2b).

Total Microbial count

After overnight incubation the cultural characteristics of the organisms on PCA agar (24 hour) and PDA (72 hour) agar was observed. And the bacterial and fungal count was performed (Fig. 3a & 3b). From bar diagram it is clear that total bacterial count was highest in pudding and least in dry fish (Fig. 4).

Table 3. Biochemical test results.

Test / Sample	Dry fish	Meat	Pudding
Oxidase	-	-	-
Catalase	+	+	+
Motility	+	+	+
Indole production	-	+	-
KIA	A/A	A/A	A/K
Gas production	+	+	+
H_2S production	-	-	-
Glucose fermentation	+	+	+
Lactose fermentation	-	-	-
Sucrose fermentation	-	-	-
Starch hydrolysis	+	+	+
Gelatin hydrolysis	+	+	+
Citrate utilization	+	+	+
MR test	+	+	+
VP test	-	-	-

However highest fungal spore was present in raw meat and least in pudding. Moderate amount of microbes were present in dry fish (Fig. 5).

Growth on BSA agar Plate

Colony characteristics (Table. 1) of the isolates were observed and Shiny black color colony on BSA plate after incubation at 37°C for 48 hour confirms that the isolates are potential *Salmonella sp.* (Fig. 6b).

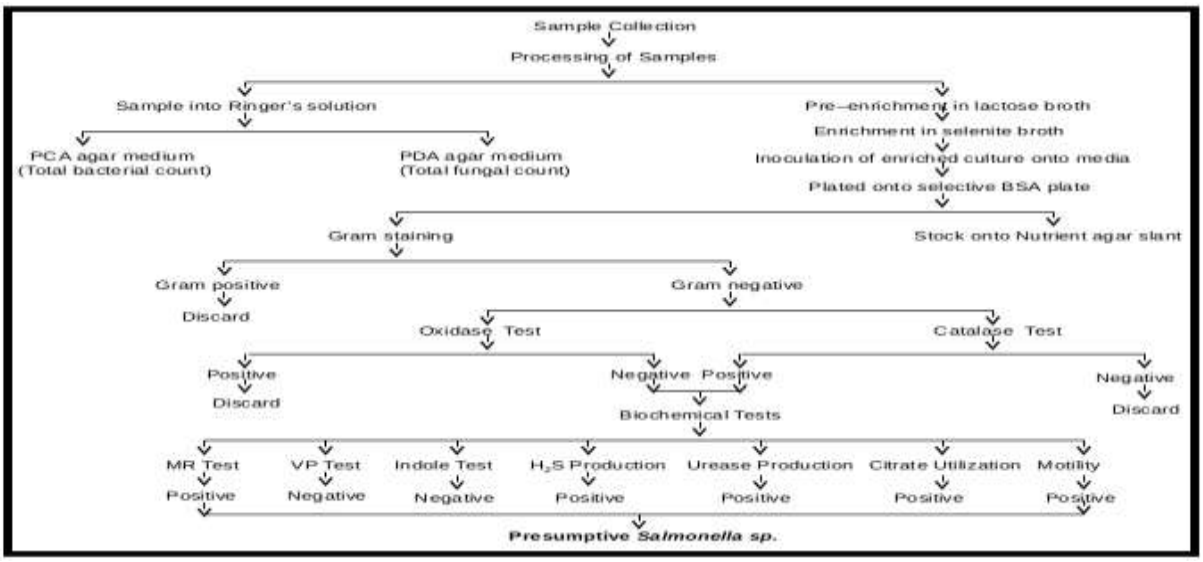

Fig. 1. Flow Diagram of conventional culture and biochemical tests to identify *Salmonella sp.* from different sample.

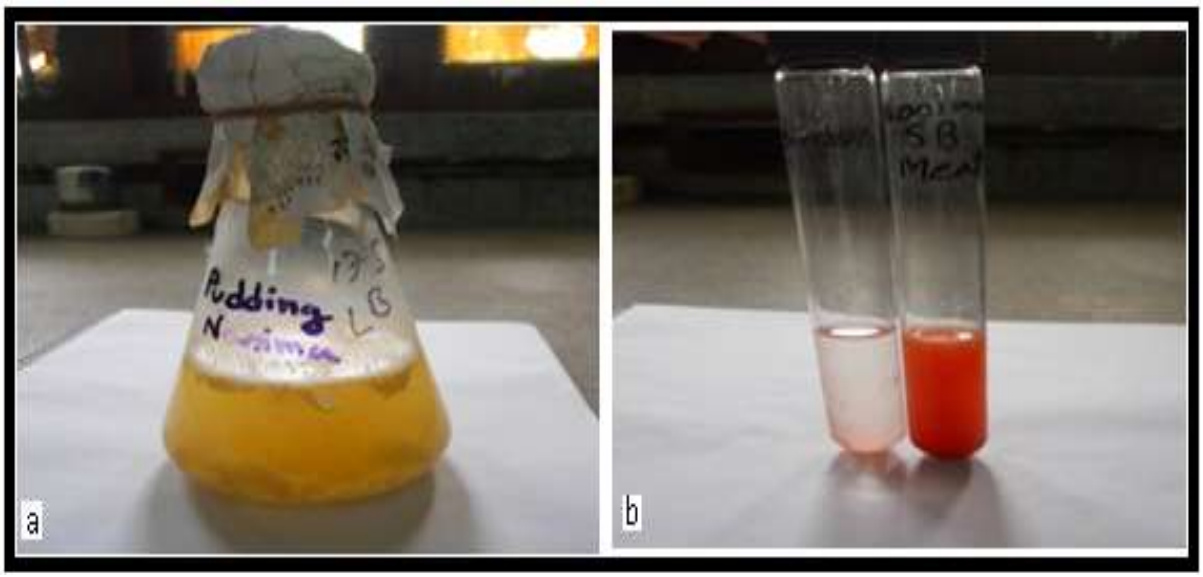

Fig. 2. (a) Pre-enrichment and (b) Enrichment of bacteria.

Microscopic Observation

Gram reaction and microscopic observation (Table. 2) revealed that the isolates are small gram negative rod shaped bacteria that are singularly arranged (Fig.6a).

Biochemical Tests

Biochemical tests results presented in Table. 3 revealed that the isolates are motile facultative anaerobes possessing catalase but not oxidase enzyme. They are Methyl Red positive but VP negative. They can utilize citrate as carbon source. They showed variation in Kliglers Iron Agar test result that is normally used for differentiating certain members of the Enterobacteriaceae by demonstrating

hydrogen sulfide production. The isolates did not produce H_2S but produced gas. There was also variation in indole production tests (Fig. 7).

Carbohydrate Fermentation

This bacterium is capable of breaking monosaccharide such as glucose and produces acidic by products that turn the red media yellow by decreasing the pH of the media but they are unable to ferment disaccharide such as sucrose and lactose and hence the media color remains unchanged (Fig. 8).

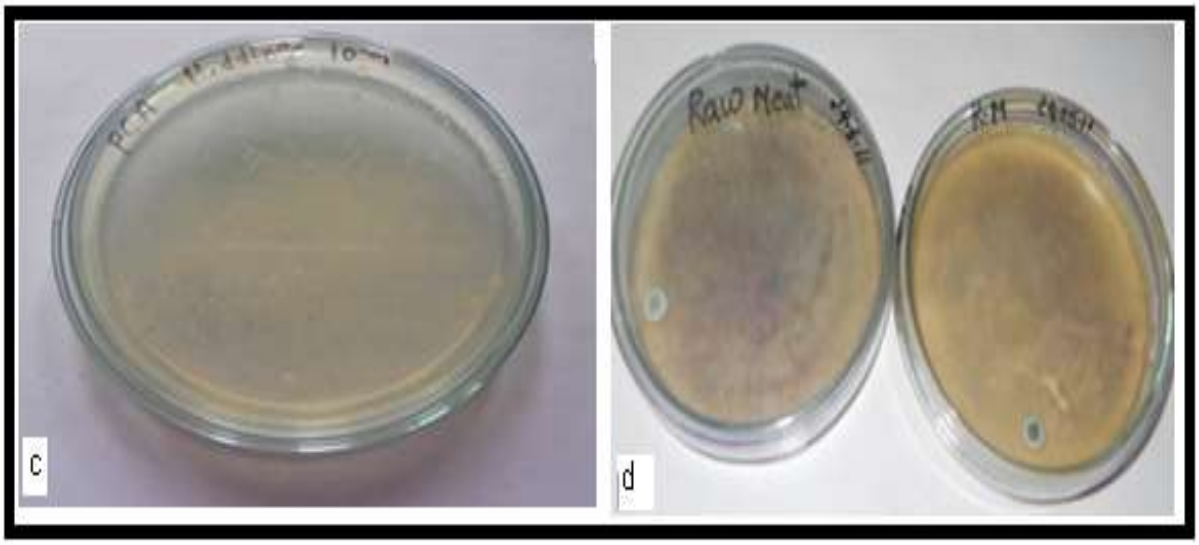

Fig. 3. (a) Total bacterial count (b) Total fungal count.

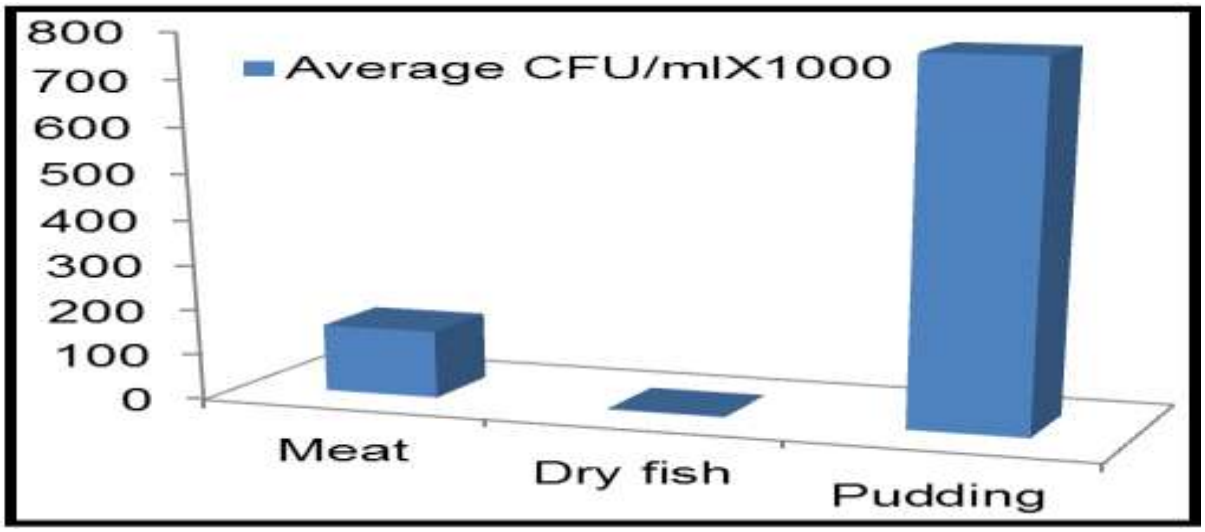

Fig. 4. Bar diagram showing total bacterial count of different samples.

Discussion

Most *Salmonella sp*. is pathogen to human and other animals. They are not normal inhabitants of environment as they are normally reside in human and animal feces so their presence in food indicates fecal contamination. Different types of methods for sample preparation, isolation and detection of *Salmonella* in foods and food ingredients are used. Molecular methods, immunological, and bacteriophage detection systems are used in many diagnostic food microbiology labs. Novel technologies such as the application of biosensors, microarrays, and nanotechnology might be used in near future. Present study was designed to investigate the occurrence, distribution, and persistence of *Salmonella sp*. in different food samples by using cost

effective microscopic, cultural and biochemical tests results. Although total bacterial count was much higher in processed food (pudding) than raw meat and dry fish, fungal count was much lower. Raw meat contained highest number fungal spore compared to dry fish and pudding. On the other hand dry fish contained lower number of microbes indicating lower survivality of bacteria due to lower water activity (a_w), moderate number of fungal spore was present indicates their lower withstanding capacity at lower a_w.

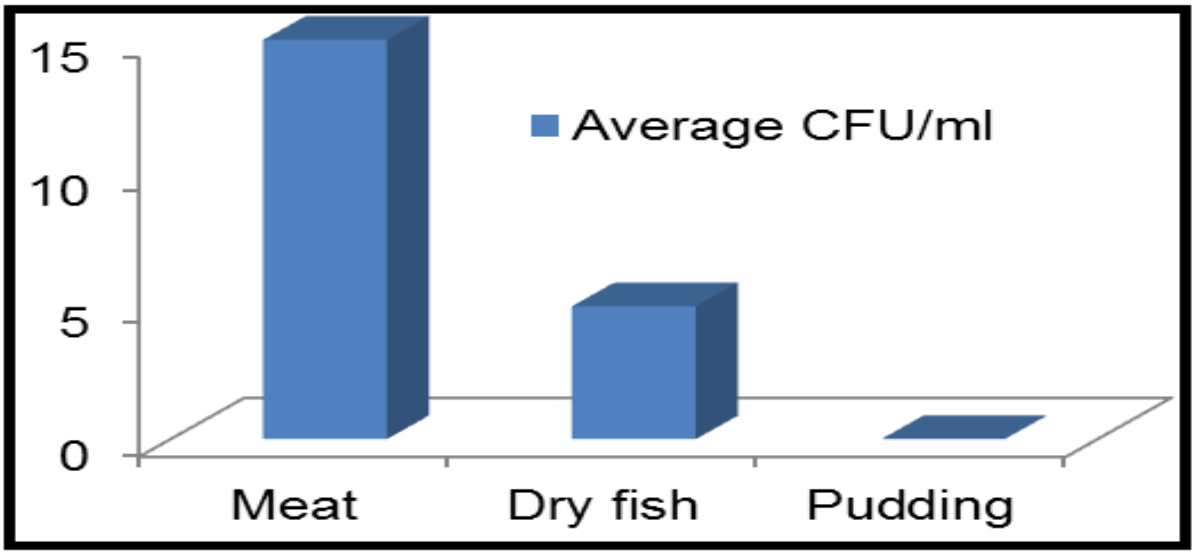

Fig. 5. Bar diagram showing total fungal count of different samples.

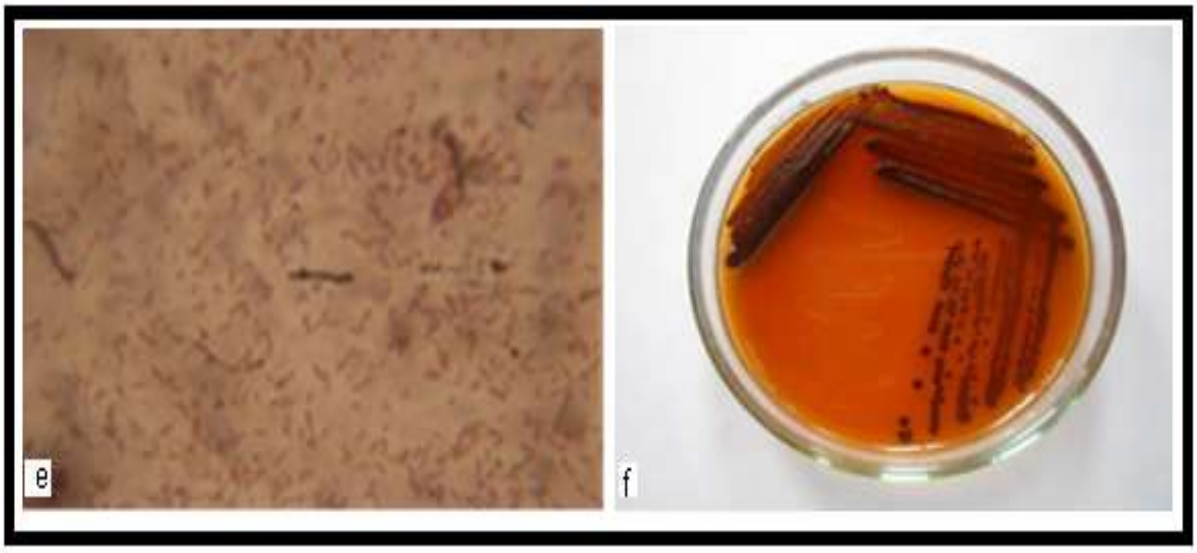

Fig. 6. (a) Microscopic view of the organism (b) Colony characteristics on Bismuth Sulfite Agar.

The isolates producing black shiny colony on selective media (BSA) were selected as potential *Salmonella sp*. showed characteristics, indicating that the isolates were *Salmonella sp*.

Microscopic characteristics and gram reaction results as well as biochemical tests and glucose fermentation confirmed the isolated isolates are *Salmonella sp* as these species can only ferment monosaccharide but unable to ferment disaccharides.

Most of the strains showed similar, but some showed different biochemical characteristics indicating strain variation.

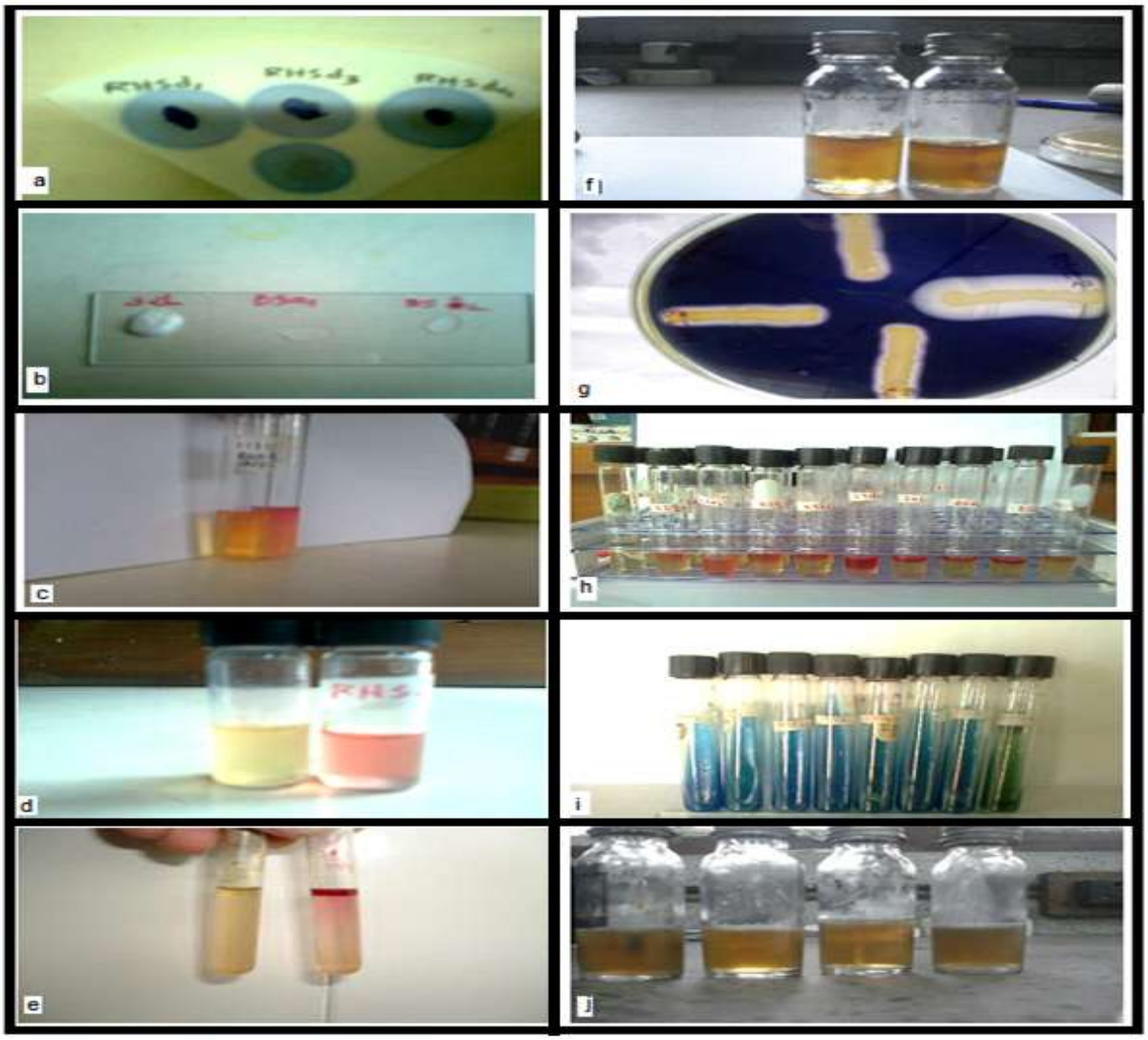

Fig. 7. Biochemical tests. (a) Oxidase test (b) Catalase test (c) Urease test (d) MR test (e) Indole test (f) H_2S production test (g) Starch hydrolysis test (h) VP test (i) Citrate utilization test (j) Motility test.

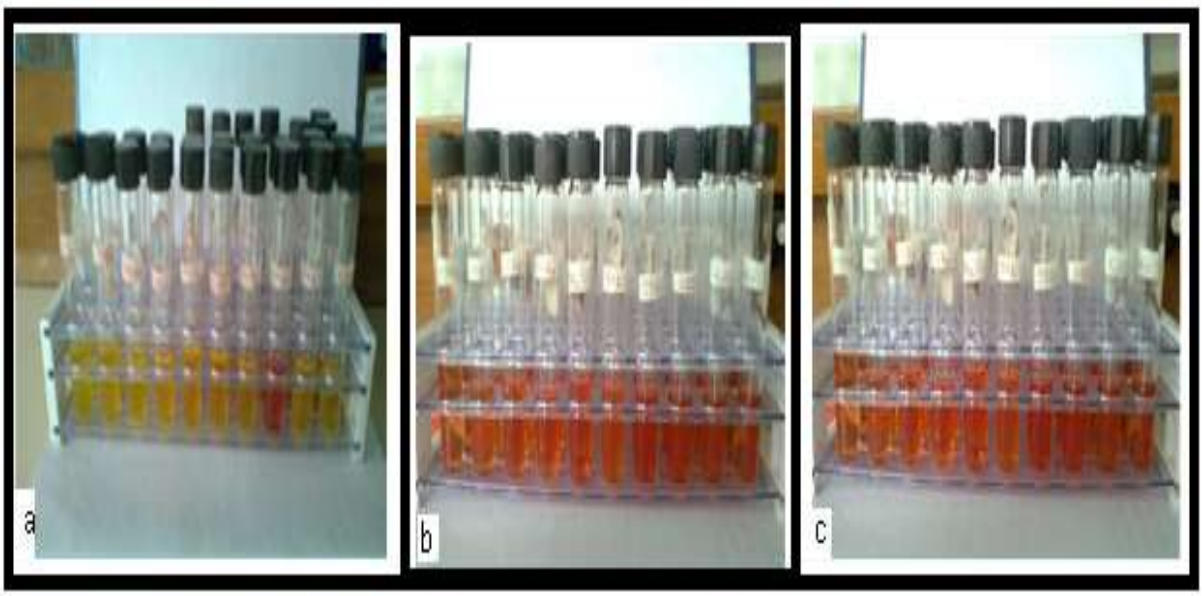

Fig. 8. Sugar fermentation test (a) Glucose (b) Sucrose (c) Lactose.

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