Isolation and Identification of MDR *Solmonella Typhi* from Different Food Products in District Peshawar, KPK, Pakistan

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Abstract

Salmonella enteric typhi is a fatal human pathogen that causes typhoid fever. The most common source of the infection is drinking water. In the present study, 100 samples from different sources (water, chicken, meat, milk, and fruits) were screened for *S. typhi,* and 68 samples were found positive. Gram staining and different biochemical tests were carried out to identify strains of *S. typhi*. These isolates were further tested for antibiotic resistance using the disc diffusion method. The prevalence of *S. typhi* was 100% in chicken meat samples, 76% in milk samples, 24% in drinking water samples, and 72% in fruits and vegetable samples. Among the isolated strains of *S. typhi*, 39.7% showed resistance to Chloramphenicol, 29.41% to Ciprofloxacin, 100% to Amoxicillin, 0% to Imipenem, 100% to Cefotaxime, 85.29% to Streptomycin, 100% to Erythromycin, 95.58% to Doxycycline, 100% to Rifampicin and 0% resistance to Meropenem. Based on our findings, Imipenem (87% susceptibility) and Meropenem (78% susceptibility) were the most effective antibiotics against various strains of *S. typhi*.

Keywords: typhoid fever; *Salmonella enteric typh;,* antibiotics;

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INTRODUCTION

Typhoid fever affects about 17 million people annually, resulting in the deaths of about 600,000 people worldwide. Drinking water tainted by feces and urine of infected individuals is the most common source of infection. The causative agent, *S. enterica typhi*, also referred to as *S. typhi,* is a gram-negative, aerobic, spore-forming, and motile rod. The fever, if not treated, results in mortality rates ranging from 12-30%.In humans and animals, Salmonellaare important pathogens[1,2] and over

and above 1400 serovars can cause essentially a self-limited disease in humans, which is called gastroenteritis. However, *S.typhi* and *S. paratyphi* A, B, and C elicit typhoid, and a few *Salmonella* serovars can cause a serious systemic infection with soaring death rates [3, 4].

The major reservoir of this pathogen is food and animal[5]sources such as poultry meat, beef, milk, and egg which have been proven to be the carrier of these pathogens[6]. These pathogens are a major source of human illness, recognized to be caused by poultry products[7,8] and human infection is reported where eggs were the source of several outbreaks in some places[9,10]. Humans are at high risk from raw or undercooked eggs[11]. Food borne outbreaks in the entire world are the most frequently isolated serovar from *S. enterica* serovar *typhimurium* and *S. enterica* serovar *enteritidis*[12]. Across the globe, healthcare concern is antimicrobial resistance. In contrast to Western countries, the resistance of various bacterial and fungal pathogens is notable in Asia due to geographic variations in the continent

There are uneven policies of antimicrobial treatment and public hygiene standards between different countries in Asia. In Europe and USA, community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA)[13],vancomycin-resistant *enterococci* (VRE)[14],*Klebsiellapneumoniae*carbapenemase (KPC) producing *K. pneumoniae*[15] and carbapenem and fluoroquinolone-resistant *Pseudomonas aeruginosa*[16] all these have spread all over the hospital settings and in the community. In addition, the Indian subcontinent has *S. typhi* strains, which are more prone to show resistance to common first-line antibiotics and nalidixic acid[17,18]. Two approved and well-tolerated typhoid vaccines protect *S. typhi* in older people but cannot protect children below 15 years against paratyphoid[19]. Threlfall and colleagues (2002) propose that in India and Pakistan, nalidixic acid-resistant *S. typhi* with decreased ciprofloxacin sensitivity are endemic. Since late 1989, multidrug-resistant *S. typhi* and resistance for chloramphenicol, co-trimoxazole, and ampicillin have occurred in the sub-continent. In the year 1989 to 1991, in hospitals in both countries, there was a greater than before occurrence of typhoid cases with multidrug-resistant *S. typhi*. In 1991, 157 (94%) of 167 S. typhiisolates were multidrug-resistant. The rate of decrease from 94% (1991) to 28.9% (1998-99) was shown in multidrug resistance among typhoid and paratyphoid *bacilli*[20].

METHODS

Sample Collection

A total of 100 different samples were collected from various sources in other locations in the Peshawar district. The sources included chicken meat, milk (open and packed), fruits, vegetables, and drinking water. The site and other information are presented in Table 1.

Chemicals and reagents

Analytical grade chemicals and reagents (Merck and Oxoid) were used in the experimental work. Bismuth sulphite agar (Merck) was used as selective media for the growth of *S. typhi*. Triple Sugar Iron (TSI) (Oxoid) and Sulfide Indole Motility (SIM) media (Neogen) were used for the identification of isolates. *Kovac's reagent* (Oxoid)was used to determine Indole splitting to Tryptophan by isolates. Other culture media, Nutrient agar, Nutrient broth, and Muller-Hinton (Oxoid), were used to refresh bacterial culture(s). Barium chloride, HCl, and ethanol (Merck) were used throughout the experimental work. Different types of antibiotic discs (Oxoid) were usedto determine the sensitivity of bacterial isolates.

Preparation Of Media(S)

Preparation of bismuth sulphite (selective medium for isolation of S. typhi)

100 ml media was prepared by adding 4.75g bismuth sulphite agar into 100 ml of distilled water in a volumetric flask. The medium was mixed and homogenized by heating. After mixing, the medium was poured into sterilized Petri-plates in a laminar airflow hood and allowed to solidify. Plates were incubated at 37°C for 24 hr to check their sterility. The composition of bismuth sulphite is given in Table 2.

Preparation of nutrient agar (for refreshing culture)

100 ml of nutrient agar media was prepared by adding 2.8g of nutrient agar into 100 ml of distilled water in a volumetric flask. The medium was thoroughly mixed and

S.No	Chicken	Milk	Fruit, Vegetable	Drinking water
1	Shop 2, board bazaar	Peshawar Sadar	Board bazaar	Phase 1 Hayatabad
2	Shop 4 boardbazaar	Abdali market phase 2	Phase 3 chowk	Phase 2 Hayatabad
4	Bilal market, phase 1	Bilal market, phase 1	Bilal market, phase 1	Phase 3 Hayatabad
5	Super market phase 1	Super market phase 1	Supar market phase 1	Phase 4 Hayatabad
6	Khattak Market phase 3	Khattak market phase 3	Khattak market phase 3	Phase 5 Hayatabad
7	People market phase 5	People market phase 5	People market phase 5	Phase 6 Hayatabad
8	Yousafzai market phase 3	Yousafzai market phase 3	Yousafzai market phase3	Phase 7 Hayatabad
9	Basharat market phase 3	Basharat market phase 3	Basharat market phase3	Student Teacher Centre, UOP
10	Rohila market phase 3	Rohila market phase 3	Rohila market phase 3	COFFEE SHOP, UOP
11	Afridi market phase 4	Afridi market phase 4	Afridi market phase 4	Lalazar, UOP
12	PDA market HMC Phase 4	PDA market HMC Phase 4	PDA market HMC Phase 4	COBAM, UOP
13	Insaf market phase 4	Insaf market phase 4	Insaf market phase 4	Environmental Sciences, UOP
14	Shama market phas1	Shama market phas.1	Shama market phas 1	Physics Department, UOP
15	Dabgari garden	Dabgari garden	Dabgari garden	History Department, UOP
16	Jehangirabad	Jehangir Abad	Jehangir Abad	Botany Department, UOP
17	Tehkal	Shaheen town	Tehkal	KTH, lawn
18	Civil quarter	Civil quarter	Gulberg	KTH, Canteen
19	Sheikh abad	(Milk pack) STC	Sheikh Abad	KTH, med ward
20	Gulbahar	(Haleeb) Board bazaar	Gulbahar	Sub Unit, COBAM
21	Shaheen town	(Olper) PDA	Shaheen town	Distal water
22	Namakmandi	(Tea mix) Town	Sabzeemandai	Mineral water
23	Karkhano market	(Tarang) Madina market phase 3	Karkhano market	IBGE, AUP
24	Coffee shop	(Candia milk) phase 3 chowk	Nawab market, phase 6	KMC, UOP
25	Forest bazaar	Coffee shop	Forest bazaar	UET, Peshawar

Table 1. List of samples collected from different sources and locations of Peshawar.

homogenized by heating and then autoclaved at 121°C for 15 min at 20 psi. After autoclaving, the medium was poured into sterilized Petri-plates in a laminar airflow hood and allowed to solidify. Plates were incubated at 37°C for 24 hr to check the sterility of the media. The composition of nutrient agar is given in Table 2.

Preparation of nutrient broth

The composition of nutrient broth is given in Table 2. 100 ml of nutrient broth was prepared by adding 0.5g of peptone and 0.3g of yeast extract into 100 ml of distilled water in a volumetric flask. Medium is thoroughly mixed and then autoclaved at 121°C for 15 min at 20 psi. After autoclaving, the medium was poured into autoclaved test tubes and incubated at 37°C for 24 hours to check the media's sterility. The turbid test tubes were considered contaminated, and transparent test tubes were further used in the experimental work.

Table 2. Composition of different Media used in the experimental work.

S.No	Media	Composition Gram per Litter (g/L)
1	Bismuth Sulphite Agar	Peptone (5.0) , glucose (5.0), Lab-lemco powder (5.0), Di-sodium phosphate (4.0), Ferrous sulphate (0.3), Bismuth sulphite indicator (8.0), Brilliant green (0.016) and Agar (12.7)
2	Triple Sugar Iron (TSI)	Peptone casein (15.0), Peptone meat (5.0), Meat extract (3.0), Yeast extract (3.0), Sodium chloride (5.0), Lactose (10.0), Sucrose (10.0), D-glucose (1.0), Ammonium iron (0.5), Citrate (0.5), Agar (12.0), Thiosulphate (0.3) and Phenol red (0.024),
3	Sulfide Indole Motility (SIM)	Pancreatic digest of casein(20.0), Peptone (6.0), Ferrous Ammonium Sulphate (0.2), Sodium Thiosulphate (0.2), and Agar (3.5)
4	Mannitol Agar	Meat peptone (10.0), Meat extract (7.0), Sodium chloride (3.0), Disodium hydrogen phosphate (2.0), D-Mannitol (15.0), Water blue (0.625), Metachrome yellow (1.875), Pril (2.0) and Agar (13.0)
5	Nutrient agar	Peptone (15.0), Yeast extract (3.0), Sodium chloride (6.0) and Agar (12.0)
6	Nutrient Broth	Peptone (15.0), Yeast extract (3.0), and Sodium chloride (6.0)
7	Muller Hinton Agar	Meat infusion (6.0), Casein Hydrolysate (17.5), Starch (1.5) and Agar (10.0)

Innoculation

Inoculation on Bismuth Sulphite Agar media (chicken)

3g of chicken meat was added to the 10 ml of normal saline and shacked for 5 min. Autoclaved cotton bud was dipped into the normal saline containing chicken meat. After that, the cotton bud was removed. The selective media plates were streaked in Laminar Flow Hood (LFH) and incubated for 24-48 hr at 37oC. The results were recorded after the incubation period. The same process was repeated for the other 24 samples of chicken meat.

Inoculation on Bismuth Sulphite Agar media (milk)

5 ml of milk was taken in an autoclaved beaker. Autoclaved cotton bud was dipped in a beaker. After that, the cotton bud was removed, and the plates containing media were streaked with it inside LFH. After streaking, plates were incubated for 24-48 hr at 37°C. The plates were examined,

and results were noted after a given time. The same procedure was followed to screen the remaining milk samples (open and pack).

Inoculation on Bismuth Sulphite Agar media (fruits and vegetables)

Coat(s) of different fruits and vegetables were dipped in 10 ml of normal saline and shaken for 5 min. Autoclaved cotton bud was dipped into the normal saline-containing sample. The cotton bud was removed and plates containing Bismuth Sulfite Agar media were streaked inside LFH. After streaking, plates were incubated for 24-48 hr at 37°C. Black-brown colonies were observed in the plates after the incubation period. The same process was repeated for the remaining samples of fruits and vegetables.

Inoculation Bismuth Sulfite Agar media (water)

Approximately 5 ml of drinking water was taken in an autoclaved beaker. Autoclaved cotton bud was dipped into the beaker. After that, the cotton bud was removed, and the plates of selective media were streaked with it inside LFH. After streaking plates were incubated for 24-48 hr at 37°C. After a given time, black-brown colonies were observed. This process was repeated for the other 24 samples of water.

Maintenance Of Bacterial Isolates

Isolated bacterial strains were purified and preserved for further studies. For short-term storage, the bacterial isolates were cultured on nutrient agar slants/Petri plates and maintained at 4°C. These isolates were sub-cultured every month for routine use.

Identification Of Isolates

All isolates were identified by microscopic examinations, gram staining, and biochemical tests, per the procedure given below:

Gram staining

The following procedure was followed for the Gram staining of isolates. A small drop of normal saline was placed on a clean, sterile glass slide using the dropper bottle. Aseptically a bacterial colony was taken with the help of a sterilized loop from the agar surface and mixed with normal saline over a glass slide. A thin smear was formed over slid by spreading the entire bacterial colony. The slide was passed through the flame of a Bunsen burner 3-4 times to heat fix the microorganism. The smear was stained with crystal violet dye for 1-2 min, and after 2 min, the remaining dye was washed out gently with water. The slide was flooded with Gram's iodine for 1-2 min and then washed with water. The slide was decolorized with ethanol for 5-6 min. The slide was thoroughly washed with water to remove the stains without any delay. The slide was flooded with safranin counterstain for2 min and washed with water. The slide was air-dried and observed under a microscope using oil immersion.

Triple Sugar Iron (TSI)

The composition of TSI is given in Table 2. The media was prepared by adding 6.75 g in 100 ml distilled water. The media was homogenized by heating. 6-7 ml of media was poured into test tubes, plugged, and autoclaved. The slants were prepared, and the media was initially red. These

slants were incubated overnight at 37°C for a sterility check. The non-contaminated slants were then streaked with isolates and incubated overnight at 37°C. The test was considered positive if the red color of the media was entirely changed into yellow and negative if there was no change in media color. We get red color in slop and yellow in the butt in our case. So this test confirms the presence of *S. typhi*.

Sulfide Indole Motility (SIM) Media

SIM media was used to measure isolates' motility, indole, and H2S production. The composition of SIM media is given in Table 2.3 g of media was added to 100 ml of distilled water and homogenized by heating. 6-7 ml of media was poured into test tubes, plugged with cotton, and autoclaved. The media was initially yellowish. Kovac's reagent was added to 3 test tubes for the Indole test. The isolates were streaked after a sterility check and incubated at 37°C for 24 hr. After 24 hr of incubation, the results were recorded. Change in color means positive results for *S. typhi* and vice versa. In some test tubes, the color changes from yellow to partially black, which indicates that partially H2S is produced and confirms the presence of *S. typhi*.

Mannitol Agar Media

The composition of Mannitol agar media is given in Table 2.5.45g of media was added to 100ml of distilled water and homogenized by heating and autoclaved. The media was poured into autoclaved Petri-plates The blue water indicator was added to the media and then incubated for 24 hr at 37°C for sterility check. After incubation, samples were streaked on media plates and incubated again for 24 hours at 37°C. The results were recorded after the incubation period. If the blue color of media changed into blue green, it confirms *S. typhi*.

Determination Of Antibiotic Resistance

Disc diffusion method of Kirby and Bauer was used to determine antibiotic resistance [21]. Fresh broth media was inoculated with bacterial culture and incubated for 18-24 hr at 37°C. This culture was compared with McFarland's turbidity standard using sterile normal saline water dilution of the sample. Then the fresh broth culture was spread uniformly on the sterile Muller-Hinton agar plate with the help of a sterile cotton swab using sterilized forceps. Antibiotic discs were placed carefully on inoculated Muller-Hinton agar plates. Positive and negative control plates were also prepared. All these plates were incubated at 37°C for 24 hr.

The next day after completing the incubation period, zone(s) of inhibition were measured in millimeters (mm) to obtain the results.

RESULTS AND DISCUSSION

Salmonella strains in raw meat products, fruits, and water have relevant public health implications. Thus monitoring food safety is a key point in preventing and controlling the spread of *Salmonella* and providing healthier food products. In this survey, it was evident from the bacteriological analysis of the investigated samples that *S. typhi* contaminates many food products. Since there are no standard operating protocols (SOPs) for maintaining the hygienic environment in the slaughterhouses and where raw food is processed, we got more prevalence of S. typhi than in the more developed countries. The current study was carried out to determine the prevalence and antibiotic resistance of *S. typhi* from different sources.

Screening of samples

The collected samples were screened on selective media (Bismuth Sulphite Agar) to observe the growth of *S. typhi*. The results showed that out of 100 samples, 68 samples were found positive for *S. typhi* as indicated by black, brown colonies that appeared on Bismuth Sulphite Agar media plates. Representative pictures are given in Figure 1. The results showed that all of the chicken meat samples were positive for the presence of *S. typhi*. Nineteen samples (76%) of milk were positive for *S. typhi*. Drinking water gave 24% (6 samples) and fruits and vegetables showed 72% (18 samples) positive growth on selective media. So a total of 68 samples (68%) showed growth on particular media, indicating the presence of *S. typhi*. The results obtained are given in Table 3. These isolates were maintained at -4°C and cultured on nutrient agar and nutrient broth media for further experimental work.

Table 3. Percentage occurrence of *Salmonella typhi* from different sources.

Source	Sample Collected	Positive Sample	Percentage
Chicken Meat	25	25	100
Milk		19	76
Drinking Water		06	24
Fruits and Vegetables		18	72

Figure 1. *Salmonella typhi* streaks on Bismuth sulphite medium

Identification

The bacterial isolates obtained on selective media were further subjected to microscopic studies and different biochemical tests to identify *S. typhi* among the bacterial isolates. Gram staining of

the isolates was performed and then observed under a microscope for the presence of Gram–ive rods. The microscopic observations confirmed that these bacterial isolates were *S. typhi*. The results are shown in Table 4.

Table No 4. Identification of bacterial isolates

S.No	Sample NO.	Bismuth Sulphite Agar	Gram Staining	S. No	Sample NO.	Bismuth Sulphite Agar	Gram Staining
1	1	Black brown colonies	G^{-ive} (rod)	35	41	Black brown colonies	G^{-ive} (rod)
2	2	Black brown colonies	G^{-ive}(rod)	36	42	Black brown colonies	G^{-ive}(rod)
3	3	Black brown colonies	G^{-ive} (rod)	37	43	Black brown colonies	G^{-ive} (rod)
4	4	Black brown colonies	G^{-ive}(rod)	38	44	Black brown colonies	G^{-ive}(rod)
5	5	Black brown colonies	G^{-ive} (rod)	39	45	Black brown colonies	G^{-ive} (rod)
6	6	Black brown colonies	G^{-ive}(rod)	40	46	Black brown colonies	G^{-ive}(rod)
7	7	Black brown colonies	G^{-ive} (rod)	41	47	Black brown colonies	G^{-ive} (rod)
8	8	Black brown colonies	G^{-ive}(rod)	42	48	Black brown colonies	G^{-ive}(rod)
9	9	Black brown colonies	G^{-ive} (rod)	43	49	Black brown colonies	G^{-ive} (rod)
10	10	Black brown colonies	G^{-ive}(rod)	44	50	Black brown colonies	G^{-ive}(rod)
11	11	Black brown colonies	G^{-ive} (rod)	45	51	Black brown colonies	G^{-ive} (rod)
12	12	Black brown colonies	G^{-ive}(rod)	46	54	Black brown colonies	G^{-ive}(rod)
13	13	Black brown colonies	G^{-ive} (rod)	47	55	Black brown colonies	G^{-ive} (rod)
14	14	Black brown colonies	G^{-ive}(rod)	48	56	Black brown colonies	G^{-ive}(rod)
15	15	Black brown colonies	G^{-ive} (rod)	49	58	Black brown colonies	G^{-ive} (rod)
16	16	Black brown colonies	G^{-ive}(rod)	50	59	Black brown colonies	G^{-ive}(rod)
17	17	Black brown colonies	G^{-ive} (rod)	51	61	Black brown colonies	G^{-ive} (rod)
18	18	Black brown colonies	G^{-ive}(rod)	52	62	Black brown colonies	G^{-ive}(rod)
19	19	Black brown colonies	G^{-ive} (rod)	53	63	Black brown colonies	G^{-ive} (rod)
20	20	Black brown colonies	G^{-ive}(rod)	54	64	Black brown colonies	G^{-ive}(rod)
21	21	Black brown colonies	G^{-ive} (rod)	55	66	Black brown colonies	G^{-ive} (rod)
22	22	Black brown colonies	G^{-ive}(rod)	56	67	Black brown colonies	G^{-ive}(rod)
23	23	Black brown colonies	G^{-ive} (rod)	57	68	Black brown colonies	G^{-ive} (rod)
24	24	Black brown colonies	G^{-ive}(rod)	58	69	Black brown colonies	G^{-ive}(rod)
25	25	Black brown colonies	G^{-ive} (rod)	59	70	Black brown colonies	G^{-ive} (rod)
26	27	Black brown colonies	G^{-ive}(rod)	60	71	Black brown colonies	G^{-ive}(rod)
27	28	Black brown colonies	G^{-ive} (rod)	61	72	Black brown colonies	G^{-ive} (rod)
28	30	Black brown colonies	G^{-ive}(rod)	62	75	Black brown colonies	G^{-ive}(rod)
29	31	Black brown colonies	G^{-ive} (rod)	63	80	Black brown colonies	G^{-ive} (rod)
30	33	Black brown colonies	G^{-ive}(rod)	64	81	Black brown colonies	G^{-ive}(rod)
31	34	Black brown colonies	G^{-ive} (rod)	65	87	Black brown colonies	G^{-ive} (rod)
32	38	Black brown colonies	G^{-ive}(rod)	66	93	Black brown colonies	G^{-ive}(rod)
33	39	Black brown colonies	G^{-ive} (rod)	67	98	Black brown colonies	G^{-ive} (rod)
34	40	Black brown colonies	G^{-ive}(rod)	68	99	Black brown colonies	G^{-ive}(rod)

The biochemical tests performed to identify bacterial isolates include Indole, glucose, Mannitol, and H_2S gas production. Triple Sugar Iron media was used for the identification of bacterial isolates. The results obtained from these biochemical tests are given in Table 5. In positive results, the red color of the media changes to yellow at the butt and red color at the slope. In our case, we got red color at the slop and yellow at the butt. So this test confirmed the presence of *S. typhi*. The production of H_2S further confirmed the presence of *S. typhi*.

The red color on the slant region shows the glucose utilization by *S. typhi*. Since the amount of glucose in TSI is less than the other two sugars, i.e., lactose and sucrose, a weak acid is produced after the fermentation of glucose. The presence of air above the slant region alkalinize the medium and thus makes it red, while the remaining butt position remains yellow because of the absence of air. Representative pictures are given in Figure 2. Results for the mannitol test are shown in Table 5. Mannitol Agar medium contains mannitol which is degraded by *S. typhi* to acid in water blue (indicator). As a result, deep blue to green color appears in the acidic. In an alkaline environment, the media is colorless. The bacterial isolates gave strong positive results for the mannitol biochemical test by degrading mannitol. The degradation of mannitol to acid makes the environment acidic in the media, and the color of the media changes from deep blue to green. The *S. typhi* appears as blue-green colonies with ragged, irregular borders and radial striation.

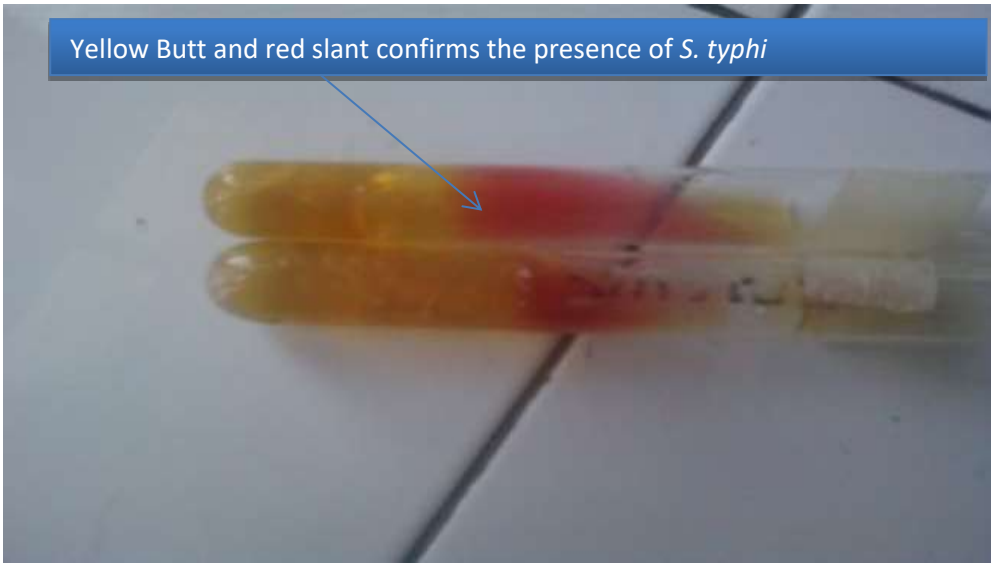

Figure 2. The slant of TSI medium after inoculation and incubation of bacterial isolates.

SIM media was used to measure isolates' motility, indole, and H2S production. Testing for indole production is important in the identification of *enterobacteria*. The test organism is cultured in a medium that contains tryptophan. Indole production is detected by Kovac's reagent, which includes 4 (*p*)-dimethyl amino benzaldehyde. This reacts with the indole to produce a red-colored compound. As *S. typhi* is indole –ive, therefore no color was observed.
S. typhi showed positive results for motility as the whole media was turbid after growth. The black color of the medium suggested that *S. typhi* species were able to produce weak H_2S in the medium.

Table 5. Results of different biochemical test performed for the identification of bacterial isolates.

S. No	Sample	Manitol	Indole	TSI				S. No	Sample	Manitol	Indole	TSI			
				H_2S	Glucose	Slope	Butt					H_2S	Glucose	Slope	Butt
1	1	+	-	weak^{+ive}	+	Red	Yellow	35	41	+	-	weak^{+ive}	+	red	yellow
2	2	+	-	weak^{+ive}	+	Red	Yellow	36	42	+	-	weak^{+ive}	+	red	yellow
3	3	+	-	weak^{+ive}	+	Red	Yellow	37	43	+	-	weak^{+ive}	+	red	yellow
4	4	+	-	weak^{+ive}	+	Red	Yellow	38	44	+	-	weak^{+ive}	+	red	yellow
5	5	+	-	weak^{+ive}	+	Red	Yellow	39	45	+	-	weak^{+ive}	+	red	yellow
6	6	+	-	weak^{+ive}	+	Red	Yellow	40	46	+	-	weak^{+ive}	+	red	yellow
7	7	+	-	weak^{+ive}	+	Red	Yellow	41	47	+	-	weak^{+ive}	+	red	yellow
8	8	+	-	weak^{+ive}	+	Red	Yellow	42	48	+	-	weak^{+ive}	+	red	yellow
9	9	+	-	weak^{+ive}	+	Red	Yellow	43	49	+	-	weak^{+ive}	+	red	yellow
10	10	+	-	weak^{+ive}	+	Red	Yellow	44	50	+	-	weak^{+ve}	+	red	yellow
11	11	+	-	weak^{+ive}	+	Red	Yellow	45	51	+	-	weak^{+ve}	+	red	yellow
12	12	+	-	weak^{+ive}	+	Red	Yellow	46	54	+	-	weak^{+ve}	+	red	yellow
13	13	+	-	weak^{+ive}	+	Red	Yellow	47	55	+	-	weak^{+ve}	+	red	yellow
14	14	+	-	weak^{+ive}	+	Red	Yellow	48	56	+	-	weak^{+ve}	+	red	yellow
15	15	+	-	weak^{+ive}	+	Red	Yellow	49	58	+	-	weak^{+ve}	+	red	yellow
16	16	+	-	weak^{+ive}	+	Red	Yellow	50	59	+	-	weak^{+ve}	+	red	yellow
17	17	+	-	weak^{+ive}	+	Red	Yellow	51	61	+	-	weak^{+ve}	+	red	yellow
18	18	+	-	weak^{+ive}	+	Red	Yellow	52	62	+	-	weak^{+ve}	+	red	yellow
19	19	+	-	weak^{+ive}	+	Red	Yellow	53	63	+	-	weak^{+ve}	+	red	yellow
20	20	+	-	weak^{+ive}	+	Red	Yellow	54	64	+	-	weak^{+ve}	+	red	yellow
21	21	+	-	weak^{+ive}	+	Red	Yellow	55	66	+	-	weak^{+ve}	+	red	yellow
22	22	+	-	weak^{+ive}	+	Red	Yellow	56	67	+	-	weak^{+ve}	+	red	yellow
23	23	+	-	weak^{+ive}	+	Red	Yellow	57	68	+	-	weak^{+ve}	+	red	yellow
24	24	+	-	weak^{+ive}	+	Red	Yellow	58	69	+	-	weak^{+ve}	+	red	yellow
25	25	+	-	weak^{+ive}	+	Red	Yellow	59	70	+	-	weak^{+ve}	+	red	yellow
26	27	+	-	weak^{+ive}	+	Red	Yellow	60	71	+	-	weak^{+ve}	+	red	yellow
27	28	+	-	weak^{+ive}	+	Red	Yellow	61	72	+	-	weak^{+ve}	+	red	yellow
28	30	+	-	weak^{+ive}	+	Red	Yellow	62	75	+	-	weak^{+ve}	+	red	yellow
29	31	+	-	weak^{+ive}	+	Red	Yellow	63	80	+	-	weak^{+ve}	+	red	yellow
30	33	+	-	weak^{+ive}	+	Red	Yellow	64	81	+	-	weak^{+ve}	+	red	yellow
31	34	+	-	weak^{+ive}	+	Red	Yellow	65	87	+	-	weak^{+ve}	+	red	yellow
32	38	+	-	weak^{+ive}	+	Red	Yellow	66	93	+	-	weak^{+ve}	+	red	yellow
33	39	+	-	weak^{+ive}	+	Red	Yellow	67	98	+	-	weak^{+ve}	+	red	yellow
34	40	+	-	weak^{+ive}	+	Red	Yellow	68	99	+	-	weak^{+ve}	+	red	yellow

Zone of Inhibition and Antibiotics Resistance

The zone of inhibition for a different antibiotic was measured against the bacterial isolates is given in Table 6. The percentage of resistance, intermediate resistance, and susceptibility of *S. Typhi* against other antibiotics were different and are shown in Table 7.

After identifying bacterial isolates, the antibiotic resistance was measured against different antibiotics by the disc diffusion method[21]. The antibiotics used to determine the resistance of *S. typhi* includes; Chloramphenicol (30mcg), Ciprofloxacin (5mcg), Amoxicillin (30mcg), Imipenem (10mcg), Cefotaxime (30mcg), Streptomycin (10mcg), Erythromycin (15mcg), Doxycycline (30mcg), Rifampicin (5mcg) and Meropenem (10mcg).

Chloramphenicol

The addition of chloramphenicol to growing bacterial cultures results in an almost immediate cessation of protein biosynthesis and hence stops the growth of *S. typhi* [22].

However the current studies showed that the bacterial isolates, when subjected to chloramphenicol, were resistant to it. The results obtained revealed that 31 (45.58%) samples showed intermediate resistance**.** The intermediate resistance range for chloramphenicol was 13-17 mm. Only 10 (14.70%) samples out of 68 (100%) samples were susceptible and the susceptibility range for chloramphenicol was ≥18 mm. Against this antibiotic, 27 (39.70%) samples showed resistance and the resistivity range was ≤12 mm. Due to the development of resistance to chloramphenicol, its use has declined significantly (Table 6-7). There is a lot of controversy regarding the resistivity of *S. typhi* to chloramphenicol, as 63.6 and 33% resistance was shown in the reported literature[23,24]. A study conducted in Dubai on antibiotic sensitivity showed resistance of 37.5%, which is nearly similar to our results of 39% resistance[25]. The reason may be that most of the labor class in Dubai is from developing countries, and they frequently travel from their countries to Dubai.

Ciprofloxacin

The isolates were subjected to ciprofloxacin and showed that 35 (51.47 %) samples were intermediate resistant. The intermediate resistance range for ciprofloxacin was 16-20 mm. Only 13 (19.11%) out of 68 (100%) samples were susceptible and the susceptibility range for ciprofloxacin was ≥21 mm. The resistivity range for ciprofloxacin was found to be ≤15 mm; the resistance was shown by 20 (29.40%) samples (Table 6-7). A study conducted in Oman on resistance in clinical isolates to *S. typhi* showed 17% **[26],** while our current study indicated resistance of 29.40%. This variation may be due to unhygienic conditions observed in the area where food materials are processed in Pakistan and the frequent use of antibiotics [27].

Amoxicillin

All of the bacterial isolates were found resistant when subjected to amoxicillin**.** So the resistance was 100% (Table 6-7). The resistivity range for amoxicillin was ≤13mm. Our results follow a study conducted in Nigeria on amoxicillin sensitivity [28].

Imipenem

Only 09 (13.23%) isolates showed intermediate resistance when subjected to Imipenem. The intermediate resistance range for Imipenem was 14-15 mm. The remaining 59 (86.76%) isolates were susceptible to Imipenem and the susceptibility range was ≥18 mm. The resistivity range for imipenem was ≤13 mm, and none of the isolates showed resistance to it (Table 6-7). A project in India to determine antibiotic resistivity of *S. typhi* against Imipenem revealed that nearly all isolates were sensitive to Imipenem[29] where about 87% of isolates were found susceptible in our study to this antibiotic. The remaining strains were intermediately resistant to Imipenem. This shows that we are prone to more resistant species of *Salmonella* in Pakistan due to a lack of check and balance of controlling authorities.

Cefotaxime

The antibiotic resistance of the bacterial isolates was also determined against Cefotaxime, indicating that all isolates were resistant to it**.** So the resistance percentage was 100 %. The resistivity range was ≤14mm (Table 6-7). The study of Steve Harakeha *et al.* (2005) showed that 74% of *salmonell*a species were resistant to cefotaxime[30]. The variation in results may be due to Pakistan's inefficient use of antibiotics [27].

Streptomycin

All the isolates were subjected to streptomycin to measure antibiotic resistance. The results showed that 10 (14.70%) isolates were intermediately resistant to streptomycin. The intermediate resistance range for streptomycin was 12-14 mm. The susceptibility rate of bacterial isolates against the antibiotic was 0%. The susceptibility range for streptomycin was ≥15mm. The resistivity range for streptomycin was found to be ≤11 mm, 58 (85.29%) isolates showed resistance to streptomycin (Table 6-7). The literature reported that. *typhi* isolates collected from different sources subjected to streptomycin, approximately 93.1% showed resistance to it [31] which shows a little more resistance in *S. typhi* in Gusau than in Peshawar, i.e., 85.29% resistance. The reason is that they measured the resistance by direct MICs (Minimum Inhibitory Concentration) method.

Erythromycin

Erythromycin was also used to determine antibiotic resistance of the bacterial isolates. The results obtained showed that all of the isolates (100%) were resistant to erythromycin. The resistivity range for the antibiotic was ≤13mm (Table 6-7). In the current study, 100% of isolates were resistant to erythromycin, similar to Steve Harakeha *et al.* (2005) **[30]**. But on the other side, in a study conducted on sensitivity to erythromycin, all isolates were found sensitive, which is the opposite of our findings**[32]**. One possible reason for this conflict is that antibiotic use in our country is inappropriate, which can be deduced from other studies conducted on antibiotic resistance. The previous research on the antibiotic resistance of *S. typhi* shows that the resistance to antibiotics in developing countries is more than in developed countries[27]. In Dhaka, a study was conducted on chicken eggs, and their finding was 62.5% resistance to erythromycin[33]. This may be due to the inappropriate use of erythromycin in the local community[34].

Doxycycline

Results obtained for the bacterial isolates against doxycycline are shown in Tables 6-7. The results showed that 3 (4.41%) isolates were intermediate resistance. The intermediate resistance range was 13-15mm. The susceptibility rate remained 0% when isolates were subjected to doxycycline and the susceptibility range was ≥16mm. The resistivity range for doxycycline was ≤12 mm; 65 (95.50%) isolates were found resistant to the antibiotic. According to Ahmed *et al.* (2011), 37.5% of isolates were resistant to doxycycline compared to 95.50%[33].

Rifampicin

All (100%) isolates were found to be resistant to rifampicin**.** The susceptibility and intermediate resistance rates were measured at 0% (Table 6-7). This antibiotic was used for gram-positive bacteria, especially Mycobacterium species. It is now confirmed that rifampicin showed no activity against *S. typhi*[34].

Meropenem

Among the isolates, 15(22.5%) showed intermediate resistance to Meropenem**.** The intermediate resistance range for Meropenem was 14-15 mm. Out of 68 (100%) isolates, 53 (78%) isolates were susceptible**.** The susceptibility range for Meropenem was ≥18 mm. The resistivity range for Meropenem was found to be ≤13 mm, and resistance against the tested antibiotic was 0%, as shown in (Table 6-7). *S. typhi* showed 100% susceptibility towards Meropenem[25]. **I**n our study, it was only 78%. The decline in the susceptibility and rise in resistance is possibly due to constant use of Meropenem without the prescription of a physician and poor transportation and improper refrigeration in Peshawar[27]**.**

Table 7. Percent resistivity, Intermediate Resistivity, and Susceptibility of bacterial isolates against different antibiotics.

S. No	Antibiotic	Concentration Per Disk(mcg)	% Resistance	% Intermediate Resistance	% Susceptibility
1	Chloramphenicol	30	39.70	45.58	14.70
2	Ciprofloxacin	5	29.41	51.47	19.12
3	Amoxicillin	30	100	0.0	0.0
4	Imipenem	10	0.0	13.23	86.77
5	Cefotaxime	30	100	0.0	0.0
6	Streptomycin	10	85.29	14.71	0.0
7	Erythromycin	15	100	0.0	0.0
8	Doxycycline	30	95.58	4.42	0.0
9	Rifampicin	5	100	0.0	0.0
10	Meropenem	10	0.0	22.0	78.0

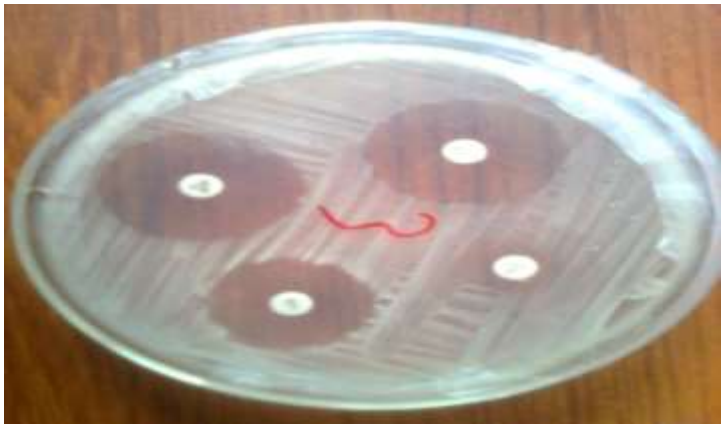

Figure 11. Representative pictures of antibiotic sensitivity tests for *S. typhi*

Table 6. Zone(s) (mm) of inhibitions of different antibiotics against the bacterial isolates.

Sam. No.	Zones of Inhibition of Different Antibiotic against Bacterial Isolates										Sam. No.	Zones of Inhibition of Different Antibiotic against Bacterial Isolates									
	CHL	CIP	AMC	IPM	CTX	STR	ERM	DOX	RIF	MEM		CHL	CIP	AMC	IPM	CTX	STR	ERM	DOX	RIF	MEM
1	14	17	2	15	4	12	5	8	0	14	**41**	7	17	9	21	5	6	0	10	7	17
2	15	14	9	14	0	11	6	8	0	15	**42**	11	16	6	19	9	9	5	12	5	18
3	12	17	11	14	0	11	6	9	0	19	**43**	13	19	0	18	9	6	5	9	5	16
4	14	19	4	22	5	10	3	8	2	12	**44**	11	20	7	19	0	11	7	7	6	21
5	15	19	6	19	7	10	6	8	2	18	**45**	13	25	7	19	0	11	10	7	5	23
6	13	20	3	16	3	11	7	10	4	18	**46**	10	22	5	20	6	10	13	6	6	20
7	12	19	6	21	5	12	9	7	9	15	**47**	13	20	0	21	0	7	9	9	9	19
8	7	15	9	19	4	9	4	12	3	18	**48**	7	19	6	19	5	12	11	11	8	18
9	11	14	5	18	4	6	9	11	5	14	**49**	11	17	4	20	9	10	8	12	6	20
10	10	17	5	17	5	12	5	9	3	16	**50**	15	19	0	21	0	11	11	10	7	22
11	10	15	6	16	4	8	3	4	8	17	**51**	14	14	0	21	0	12	9	13	9	16
12	9	13	7	15	0	0	4	6	8	16	**54**	16	17	5	17	8	11	5	7	6	14
13	0	21	6	23	6	7	0	0	6	19	**55**	19	15	5	18	9	7	0	8	5	17
14	6	17	5	18	0	5	6	9	5	21	**56**	17	14	6	21	11	11	11	12	0	15
15	8	14	7	20	4	7	4	11	0	19	**58**	14	15	0	19	0	9	4	7	8	17
16	12	19	7	16	5	10	9	6	0	18	**59**	13	19	6	20	7	6	7	9	6	18
17	13	20	5	18	0	11	10	7	7	22	**61**	18	17	8	19	0	8	6	8	7	19
18	15	21	8	19	7	12	11	8	6	19	**62**	11	13	0	18	5	11	9	12	7	19
19	16	22	7	20	4	9	12	9	0	21	**63**	12	14	0	17	0	10	11	12	7	21
20	13	20	4	21	0	11	11	7	5	20	**64**	13	17	5	18	4	12	9	11	8	19
21	14	19	8	20	6	12	9	6	7	21	**66**	13	14	6	17	5	12	13	12	7	17
22	11	20	7	22	0	9	7	11	6	22	**67**	11	16	4	21	6	11	11	11	6	19
23	17	18	6	23	5	10	8	12	0	21	**68**	13	20	0	17	0	10	10	13	4	19

24	9	13	8	17	0	9	11	7	5	20	**69**	16	19	5	19	0	11	4	11	7	20
25	14	19	6	18	4	12	8	9	0	18	**70**	14	17	0	20	5	9	7	9	5	19
27	11	26	0	17	5	9	9	10	5	20	**71**	11	23	6	18	0	13	8	12	4	21
28	14	10	3	17	0	12	9	13	6	12	**72**	13	21	0	22	0	10	6	8	6	22
30	15	15	0	16	7	9	8	12	7	17	**75**	17	19	5	21	7	12	11	9	7	23
31	19	14	0	23	5	8	10	12	8	13	**80**	17	25	7	17	5	12	9	7	0	23
33	16	19	4	15	9	7	8	13	7	14	**81**	19	23	5	21	7	11	10	9	6	21
34	11	15	0	19	5	4	10	11	8	20	**87**	20	21	8	21	6	9	7	11	7	22
38	8	14	8	18	6	15	0	9	5	17	**93**	17	23	4	20	0	12	6	6	0	23
39	7	14	5	19	8	10	0	12	6	18	**98**	18	24	7	19	6	11	7	6	5	23
40	11	16	5	17	7	10	7	11	6	21	**99**	17	21	6	21	5	10	11	8	0	22

CHL (**Chloramphenicol**), CIP (**Ciprofloxacin**), AMC (**Amoxicillin**), IPM (**Imipenem**), CTX (**Cefotaxime**), STR (**Streptomycin**), ERM (**Erythromycin**), DOX(**Doxycycline**), RIF (**Rifampicin**) and MEM (**Meropenem**)

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