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Biochemical identification, antibiotic sensitivity and resistance pattern for salmonella typhi and salmonella paratyphi

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Salmonella typhimurium is a pathogenic gram-negative bacterium, which is found primarily in the intestinal lumen. It often causes diarrhea in infants and young children and leads to food poisoning. Drug resistance of Salmonella typhimurium presented serious complications in clinical patients. The purpose of this study was to find out the prevalence and antibiotic sensitivity pattern of Salmonella typhi and Salmonella paratyphi isolated from blood samples of typhoid suspected patients of both sexes. Samples were inoculated on MacConkey agar media and Blood agar media for primary identification of Salmonella species. Depending on colony formation, pigmentation, elevation and margins, colonies were presumably identified. All the islolate were were tested for antimicrobial susceptibility was measured in vitro by the Kirby-Bauer method. Out of total samples 1494 (76.8%) were positive for Salmonella typhi and 451 (23.2%) isolates were positive for Salmonella paratyphi. The growth positive rate in the samples of the two genders for Salmonella typhi- Male: 58% and Female: 42%, and for Salmonella paratyphi male: 74% and female: 26%. Of total isolates studied (60%) were found to be multidrug resistant (MDR) (defined as resistance to ampicillin, chloramphenicol and co-trimoxazole, Azithromycin). Sensitivity ciprofloxacin of S. Typhi was high. Concurrently, there has been an increase in the number of isolates sensitive to all antibiotics except nalidixic acid. MDR S. Typhi continues to be an important public health issue. Presence of associated low-level ciprofloxacin resistance is a concern and requires further study.

Keywords: Salmonella paratyphi, salmonella typhi, MDR S. Typhi, antibiotics, resistant, sensitive, biochemical.

INTRODUCTION

Typhoid fever is very common in the developing countries of the world like Bangladesh. It is caused by Salmonella typhi, a Gram-negative bacterium. A very similar but often less severe disease paratyphoid is caused by Salmonella paratyphi **(**Slack et al. 1998; Collee et al. 1996**).** Most of the incidents are due to ingestion of

contaminated food stuffs. Modes of infections and development of carrier states are almost the same as typhoid fever (Liang et al. 2012). Persons with typhoid fever carry the bacteria in their bloodstream and intestinal tract and can spread the infection directly to other people by contaminating food or water. Travelers visiting developing countries are at greatest risk for getting typhoid fever (Hendriksen et al. 2015). The disease is endemic in many developing countries, particularly in the Indian subcontinent, South and Central America, and Africa, with an estimated mean incidence (per 100,000 people per annum) of 150 in South America and 900 in Asia (Jemiseye et al. 2017)**.** Chloramphenicol-resistant strain was that which occurred in Mexico in 1972 **(**Kumar et al. 2017; Thamizhmani et al. 2012)**.** Acquisition of multiple resistance was described in 1976 in Germany. Salmonella Typhi resistant to chloramphenicol and ampicillin developed during unsuccessful treatment with chloramphenicol followed by ampicillin in a 9-year-old boy. In 1997 there was an epidemic of nalidixic acid-resistant S. Typhi, with decreased susceptibility to ciprofloxacin, in Tajikistan (Fatema et al. 2016). A similar outbreak has been ongoing in Viet Nam since 1993, where a poorer (Dyson et al. 2019). Decreased susceptibility to the fluoroquinolones now also appears in S. Typhi endemic in India and Pakistan. Resistance to third-generation cephalosporins has been reported in Salmonella (Threlfall et al. 2001), but to date only a single report has been published describing resistance in S. Typhi (Chinh et al. 2000). One outbreak of particular interest was that which occurred in the Philippines (metropolitan Manila) from July 1993 to April 1994, in which 252 cases of MDR S. typhi in 13 hospitals were reported (Tinya-Superable 1995)**.**The strains were not phage-typed. The strains were resistant to chloramphenicol, co-trimoxazole, kanamycin, streptomycin, and tetracyclines. The recent explosive emergence in developing countries of strains of S. typhi with resistance to trimethoprim and ampicillin has caused many problems, as since 1980 these antibiotics had been used extensively for the treatment of patients infected with chloramphenicol-resistant strains (Vala et al 2016). The situation has changed dramatically since 1990. In that year 20% of strains were resistant not only to chloramphenicol but also to trimethoprim (MIC, > 125 mg/L) and ampicillin (MIC, > 125 mg/L**) (**Singh et al. 2019)**.** The situation has worsened in the succeeding 5 years, and since 1994 about 35% of strains from patients with typhoid fever have been resistant to chloramphenicol (table 2); the majority of chloramphenicol-resistant strains also are resistant to ampicillin and trimethoprim (Rowe et al. 1995)**.**

The purpose of this study was to find out the prevalence and antibiotic sensitivity pattern of Salmonella typhi and Salmonella paratyphi isolated from collected blood samples of both males and females in kth Peshawar kpk pakistan

MATERIALS AND METHODS

2.1. Methods and working procedure

2.1.1. Sterilization:

All the media were sterilized (for 15 minutes) by using autoclave. Glass materials sterilized at 180 degree Celsius for 1 hour in a hot air oven prior to use. All solutions were sterilized under the same condition.

2.1.2 Bactec technique

After collection the bottles were put in the Bactec machine where it was incubated at 37 °C and agitated continuously. In case of unloading positive bottle; when there is any growth, both the machine and the computer will indicate the growth by alarm message on the computer screen.

2.1.3 Microbiological culture media

Two culture media were used in this study. These are MacConkey agar and Blood agar.

Table 1: Colony characteristics of Salmonella species on different agar media

Name of pathogen	Colony Characteristics on MacConkey agar media	Colony characteristics on Blood Agar media
Salmonella	Circular, low convex, smooth, translucent, colorless due to absence of lactose Fermentation.	Red colonies, some with black centers. The agar itself will turn red due to the presence of Salmonella type colonies.

2.2. Microscopic study

2.2.1 Gram staining (Morphology):

Small drop of distilled water was placed on a slide. Then one loop full isolated colony was taken and smeared over the surface of slide. The smear

was allowed to dry thoroughly. The smear was fixed quickly through the Bunsen flame three times. After cooling the smear was stained. Between each staining reagent the smear was washed under gently running tap water. Staining and reagent were applied as per following sequence: 1. Ammonium oxalate (Crystal violet) (60 sec) 2. Gram's iodine (60 sec) 3. 95% Ethanol (30 sec) 4. Safranin (45 sec) Then it was air dried and observed under a compound microscope

2.3. Isolation and Identification of Micro-organisms

Identification of bacterial isolate was carried out by different bio-chemical test such as Triple sugar Iron (TSI) test, Motility Indole Urease (MIU) test, Citrate utilization test, Oxidase test and Catalase test.

2.3.1 Catalase

To find out if the particular bacterial isolate is able to produce catalase enzyme, small inoculum of bacterial isolate is mixed into hydrogen peroxide solution (3%) and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. It was done by picking a pure colony by a sterile loop and immersing it in 2 drops of 3% H2 O2 solution in a glass slide. Production of bubbles indicated the positive results (Taylor et al.1972).

2.3.2 Oxidase

The test was done to detect the presence of cytochrome oxidase in the organism. A single colony was picked up with a sterile toothpick and rubbed on to whatman filter paper that is soaked with 2-3 drops of N, N, N', N'-tetramethyl-pphenylenediamine dihydrochloride Positive result was recognized by a dark purple color within 5-10 seconds (Jurtshuk et al 1976).

2.3.3 Motility Indole Urea (MIU)

One isolated colony was touched with a sterile wire and stabbed into semisolid agar medium very carefully down the tube, without touching the bottom. The tube was incubated at 37 °C for 18 to 24 hours. Non-motile bacteria generally give growths that are confined to the stab-line, have sharply defined margins and leave the surrounding medium clearly transparent. Motile Bacteria typically give diffuse, hazy growths that spread throughout the medium rendering it slightly opaque (Kumala et al. 2006).

2.3.4 Triple Sugar Iron (TSI) test

The test was done to determine the ability of an organism to attack a specific carbohydrate incorporated in a basal growth medium, with or without the production of gas, along with the determination of possible hydrogen sulfide (H2S) production. This test is used, in conjunction with others, for the identification of enteric pathogens. TSI agar was prepared by Lactose, Sucrose and Glucose in the concentration of 10:10:1 (i.e. 10 part Lactose (1%), 10 part Sucrose (1%) and 1 part Glucose (0.1%). One isolated colony was touched with a sterile wire and inoculated by stab-and-streak inoculation into agar very carefully. The tube was incubated at 37 °C for 18 to 24 hours (Faisal et al. 2017).

2.3.5 Citrate Utilization test

Simmons citrate agar tests the ability of organisms to utilize citrate as a carbon source. Simmons citrate agar contains sodium citrate as the sole source of carbon, ammonium dihydrogen phosphate as the sole source of nitrogen and the pH indicator bromothymol blue. If the medium turns blue, the organism is citrate positive. If there is no color change, the organism is citrate negative (Cordaro et al. 1965).

3. Determination of antibiotic Susceptibility of Salmonella isolates

Susceptibility of Salmonella isolates to different antimicrobial agents was measured in vitro by the Kirby-Bauer method which allowed rapid determination of the efficacy of drug by measuring the zone of inhibition that result from diffusion of the antimicrobial agent into the medium surrounding the disc. Commercially available antimicrobial discs were used for the test (Drew et al. 1972).

3.1. Antimicrobial agents

Antimicrobial agents used Ampicillin-10 mcg, Azithromycin- 15 mcg, Cefixime- 5 mcg, Ceftriaxone-30 mcg, Ciprofloxacin-5 mcg, Chloramphenicol30 mcg, Gentamycin-30 mcg, Nalidixic acid-30 mcg, Cotrimoxazole-25 mcg

3.1.2. Inoculation

Inoculation of the Mueller-Hinton agar plate with test organism the isolated colony from the various media was inoculated on the media by spreading technique.

3.1.3. Application of discs to inoculated agar plates a.

The antimicrobial discs were dispensed into the surface of the inoculated agar plate. Each disc must be pressed down to ensure complete contact with the agar surface. The discs must be distributed evenly so that they are not closer than 24 mm to each other. Not more than 5 discs were used in a 100 mm plate because some of the drugs diffuse almost instantaneously. A disc should not be relocated once it has come into contact with the agar surface. b. The plates were inverted and placed in an incubator set to 37 degree Celsius for overnight.

RESULTS AND DISCUSSION

Blood samples of 8756 typhoid suspect patients of both sexes were studied. Among them 1945 specimens were analyzed to observe the antibiotic susceptibility because they had significant growth of Salmonella species. The samples were inoculated on MacConkey agar media and Blood agar media for primary identification of Salmonella species. Depending on colony formation, pigmentation, elevation and margins, colonies were presumably identified. Then the presumed isolates were further tested for more confirmation. All isolates were examined through biochemical tests- TSI (Triple Sugar Iron) test, MIU (Motility Indole Urea) test, Citrate utilization test, Catalase test and oxidase test as shown in figure 2 and table 2. **(**C) Gram Staining. (D) Catalase test. (E) Oxidase Test. (F) Motility Indole Urea. (G) Citrate test. (H) Triple Sugar Iron Test. Salmonella typhi is believed to cause typhoid fever in most of the cases. This study also showed the same result because 1494 patients of 1945 patients were infected by Salmonella typhi and rest of the 451 patients was infected by Salmonella paratyphi. So the percentages of patients affected by Salmonella typhi and Salmonella paratyphi were respectively 76.8% and 23.2% as shown in table 3, 4, 5. previous studies demonstrate the percentages of patients affected by Salmonella typhi and Salmonella paratyphi were respectively 91% and 9% (Faisal et al. 2017).This study has also shown that males were affected slightly more than females. Usually males remain outside of home more than females and as a result males are exposed to the contaminated food and water more than females. The samples of Salmonella typhi were most sensitive to Ceftriaxone, Ciprofloxacin and Gentamycin followed by, Cefixime, Cotrimoxazole, Chloramphenicol and Ampicillin and was least sensitive to Nalidixic acid followed by Azithromycin. Salmonella paratyphi was found to be most sensitive to Cefixime, Ceftriaxone, Ciprofloxacin and Gentamycin followed by Chloramphenicol, Cotrimoxazole and Ampicillin and was least sensitive to both Nalidixic acid and Azithromycin. Our work is in agreement with previous reports on the susceptibility of S.typhi toward cephalosporin as there are many reports shows that the incidence of MDR salmonella Typhi may be as high as 60% and nalidixic acid resistant S.Typhi has also reported but the isolates was sensitive to cephalosporin and ceftraxione.the increase in MIC of cephalosporin has been reported in India and UK (Madhulika et al. 2017).

Table 2: Total number of suspected patients and the prevalence of typhoid in patients of both sexes

Month	Total Suspects	Total +vie	Total –vie	Male +vie	Female +vie	Total
January	758	182	576	124	58	8756
February	680	175	505	98	77	
March	820	218	602	150	68	
April	654	168	486	70	98	
May	1058	204	854	126	78	
June	858	130	728	74	56	
July	738	182	556	100	82	
August	674	142	532	90	52	
September	550	84	466	54	30	
October	630	166	464	128	38	
November	670	160	510	108	52	
December	666	134	532	80	54	
	8756	Total positive 1945	6811	1202	743	

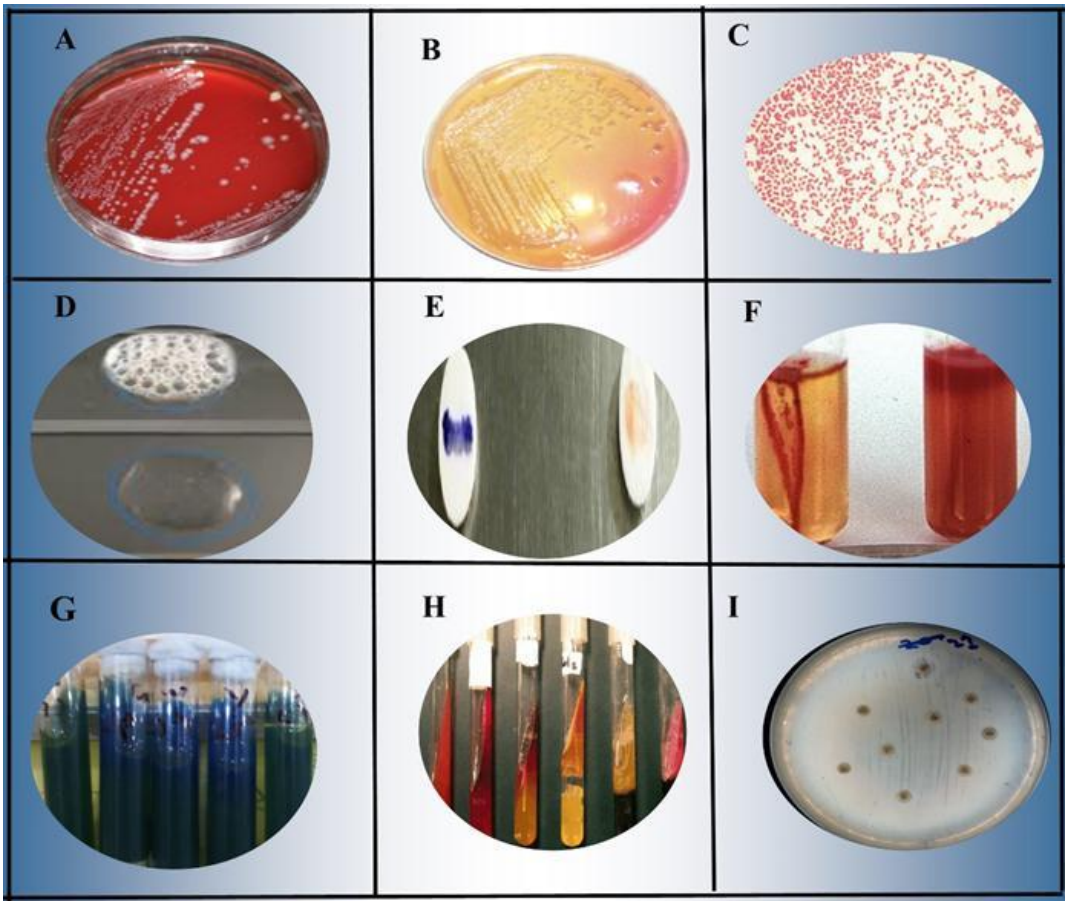

Figure: 2 (A) Growth of Salmonella typhi and Parathypi on Blood Agar (B) Growth of Salmonella typhi and Parathypi on MacConkey agar **. (C) Gram Staining. (D) Catalase test. (E) Oxidase Test. (F) Motility Indole Urea. (G) Citrate test. (H) Triple Sugar Iron Test. (I) Antibiotic Sensitivity test**

Table 3: Results of biochemical tests for Salmonella typhi and Salmonella paratyphi

Etiological agents	Catalase	Oxidase	TSI	MIU	Citrate utilization	Gram reaction
Salmonella typhil	+ve	-ve	Alkaline slant & acid with H2S & no gas produced.	M: +ve I: -ve U: -ve	-ve	-ve
Salmonella paratyphi	+ve	-ve	Alkaline slant & acid without H2S & gas produced.	M: +ve I: -ve U: -ve	-ve	-ve

Table 4: Percentage of Positive Result of Salmonella spp.

No of total specimen	No of salmonella spp. negative specimens	No of salmonella spp. positive specimens	Percentage of salmonella spp. positive specimens
8756	6811	1945	22.2%

Table 5: Percentages of Etiological Agents

Etiological Agents	No. of isolates	Percentage %
Salmonella typhi	1494	76.8%
Salmonella paratyphi	451	23.2%

Table 6: Percentages of Salmonella typhi positive patients according to sex

No of Salmonella typhi positive patient	No of male patient	% of male patient	No of female patient	% of female patient
1494	866	58%	625	42%

No of Salmonella paratyphi positive patient	No of male patient	% of male patient	No of female patient	% of female patient
451	333	74%	118	26%

Antibiotic susceptibility patterns of isolated Salmonella After 24 hour of incubation, inoculated Muller – Hinton agar plates were observed according to Kirby–Bauer method to determine the antibiotic susceptibility.

Table 7: Antibiotic Sensitivity and Resistance Pattern for Salmonella typhi and Salmonella paratyphi

Antibiotics (conc.)	Salmonella typhi					Salmonella paratyphi				
	Sample	S	%	R	%	Sample	S	%	R	%
Cefixime (5 mcg)	1494	1462	97.8	32	2.2	451	449	99.5	2	0.5
Ceftriaxone (30 mcg)	1494	1491	99.8	3	0.2	451	450	99.8	1	0.2
Ciprofloxacin (5 mcg)	1494	1487	99.5	7	0.5	451	447	99.1	4	0.9
Gentamycin (30 mcg)	1494	1486	99.4	8	0.6	451	439	97.3	12	2.7
Ampicillin (10 mcg)	1494	984	65.9	510	34.1	451	411	91.1	40	8.9
Chloramphenicol (30 mcg)	1494	1194	79.9	300	20.1	451	409	90.7	42	9.3
Cotrimoxazole (25 mcg)	1494	1239	82.9	255	17.1	451	371	82.3	80	17.7
Azithromycin (15 mcg)	1494	794	53.1	700	46.9	451	237	52.5	214	47.5
Nalidixic acid (30 mcg)	1494	681	45.6	813	54.4	451	141	31.3	310	68.7

CONCLUSION

MDR S. Typhi continues to be an important public health issue. Presence of associated low-level ciprofloxacin resistance is a concern and requires further study. This study showed the percentages of patients affected by Salmonella typhi are more than Salmonella paratyphi. The antibiotic susceptibility of Salmonella typhimurium is very high to the third and fourth generation cephalosporins. Isolates of Salmonella typhi are sensitive to nearly all antibiotics except nalidixic acid. The combined use of multi-antibiotics is a convenient and effective method to reduce Salmonella typhimurium infections.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.F.SA.S and M.T.A; methodology, S.K.MR.; software, S.S; validation, Z.A., Z.H; formal analysis, M.N.U and HS; writing—original draft preparation, SAS and S.F; writing—review and editing, R.U and S.H.A.; supervision, S.A.S., S.

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