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Prevalence and Antibiogram Studies of *Salmonella Enteritidis* Isolated from Human and Poultry Sources

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ABSTRACT

A total of 615 samples including poultry eggs (240), poultry meat (85), poultry droppings (100), bakery products (65) and stool from human diarrhea cases (125) were collected from different areas of Faisalabad, Pakistan. The samples enriched in Tetrathionate broth showed turbidity in 206 (33.5%) samples, and typical Salmonella colonies were found on differential agar media by using traditional methods in all these cases. Polyvalent antisera were used to identify Salmonella serovars. Results revealed that overall serovar entertidis prevalence rate in 206 Salmonella positive samples was 75.24% (155). Out of 58 isolates of Salmonella recovered from human stool samples, 44 (75.86%) were *S. enteritidis*. Isolation frequency of *S. enteritidis* from total isolates (148/206) in poultry sources was 111/148 (75.00%) which indicated the zoonotic potential of *S. enteritidis*. Antimicrobial susceptibility tests were performed by using disk diffusion and concentration methods. *S. enteritidis* isolates showed 100% resistance against bacitracin, erythromycin and novobiocin. These results indicated possible role of infected poultry and poultry products as a source of human infection with multiple drug resistant *S. enteritidis* strains.

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INTRODUCTION

Non-typhoidal salmonellosis is a food borne disease of primary concern in developed, as well as developing countries. The spread of this disease is favored by a variety of animal reservoirs and a wide commercial distribution of both animals and food products. This disease is among one of the major public health problems in terms of socio-economic impact (Mushtaq-ul-Hassan *et al*., 2008; Razzaque *et al*., 2009).

Global surveillance data indicates that incidence of gastrointestinal infections caused by *Salmonella enteritidis* has increased massively during the last decade. Salmonella serovars which cause human salmonellosis have been demonstrated to be transmitted through infected poultry flocks, meat and eggs (Holt *et al*., 1994). *Salmonella enteritidis* isolates have been isolated predominantly from poultry and are the most frequent cause of human salmonellosis. *S. enteritidis* was reported to be responsible for 380 salmonellosis out-breaks in USA between 1985 and 1991, involving 13056 illnesses and 50 deaths (Mishu *et al*., 1994).

Salmonella enteritidis causes gastroenteritis and other acute infections in human. There is however, little information on their prevalence and antibiotic susceptibilities in Pakistan, which would help to prevent the spread of infection and provide data about the best choice for treatment. This paper describes the prevalence of *S. enteritidis* in poultry and human and in vitro susceptibility of the isolates to various antibiotics.

MATERIALS AND METHODS

A total of 615 samples, including poultry egg shells (120), egg-interior (120), poultry meat (85), poultry droppings (100), bakery products (65) and stool from human diarrhea cases (125; Table 1) were collected from different areas in Faisalabad, Pakistan. The human stool specimens were investigated from people with diarrhea presented as outdoor patients in Allied Hospital and District Headquarters Hospital Faisalabad, Pakistan. The isolation and biochemical identification of Salmonella was carried out according to standard laboratory methods (Henzlar *et al.*, 1994). Each sample was added to 225 ml of sterile lactose broth (Oxoid 0985385), incubated for 60 min and transferred 1 ml into Tetrathionate broth (Oxoid 235780) for Salmonella enrichment before streaking onto Brilliant Green agar, MacConkey's agar and Salmonella

Shigella agar (Difco 0285-17-7) and incubated aerobically at 37°C for 24 h. Salmonella suspected colonies were identified by Gram staining, motility, triple sugar iron agar, indole, methyl red and citrate utilization tests and finally by serotyping using group specific antisera.

Antibiotic susceptibility tests for selected 14 isolates were performed according to the Kirby Bauer method (Bauer *et al.*, 1996). The Mueller Hinton agar was used as growth medium for standard disc diffusion test and growth was spread on plates with the help of a sterilized cotton swab to form a smooth bacterial lawn. Commercially prepared standard susceptibility test discs impregnated with known agent and strength were dispensed on the agar surface. Within 15 minutes of application of the discs, plates were incubated overnight at 37°C. Characterization of strains as sensitive or resistant was based on the size of inhibition zone around the disc compared with the interpretation standards provided by the manufacturers.

The antimicrobial drugs, including ampicillin, bacitracin, chloramphenicol, erythromycin, gentamycin, kanamycin, novobiocin, penicillin, spectinomycin, streptomycin, tetracycline and trimethoprim were used. Different dilutions of each drug (100 µg/10 µl, 50 µg/10 µl, 25 µg/ 10 µl, 12.5 µg/10 µl, 6.25 µg/10 µl, 3.125 µg/10 µl) were prepared. Mueller Hinton agar was used as growth media for all MICs testing. Plates were poured and incubated over night to see their sterility. A 100 µl of an over night culture was spread on the plates with heat sterilized glass spreader to form a smooth bacterial lawn. The sterilized blank discs were placed on the surface of the medium. The distance between the discs was kept approximately 2 cm.

The 10 µl of each antimicrobial drug dilution was poured per disc in descending order of concentration. The plates were inoculated over night at 37°C and the diameter (mm) of inhibition zones was recorded the next day. All solutions and dilutions of antimicrobials were made fresh and all handling was done using sterile equipment under sterile conditions.

RESULTS

The study included 615 samples from poultry eggs, poultry droppings, meat, bakery products and human stool samples collected from different areas of Faisalabad (Table 1). To address the hypothesis that consumption of raw or undercooked eggs and poultry products was the primary risk factor for domestically acquired cases, clinical signs and history were obtained from human gastro-enteritis patients. The clinical symptoms in human gastroenteritis patients included abdominal pain (89.7%), fever (82.2%), diarrhea (99.8%), vomiting (42.7%), chills (35.7%), headache (59.6%), joint pain (63.7%), nausea (59.5%) and blood in stool (22.7%). Investigation of food consumed by gastroenteritis patients during the period of study revealed that 45 (36%) consumed poultry eggs (in raw or undercooked form), 28 (22.5%) consumed the poultry meat, 10 (8.0%) consumed bakery products, 12 (9.6%) consumed contaminated water, 8 (6.4%) patients had consumed contaminated milk, 12 (9.6%) patients had poor household cooking conditions and 10 (8.0%) patients were infected with unknown cause.

From 615 samples, 206 Salmonella serovars were isolated. In poultry samples, Salmonella positive rates were, egg-shell 40%, egg interior 8.33%, poultry droppings 55%, meat 30%, and bakery products 13.85%. Involvement of Salmonella in human diarrhea cases included in this study was 46.4% in the same locality (Table 1). Serotyping of total 206 Salmonella isolates showed high percentage of serovar enteritidis among poultry 75% (111/148) and human samples 75.86% (44/58). Occurrence rates of other serovars, *S. typhimurium*, *S. paratyphi* B, *S. pollorum* and non-typable salmonellae was less than 25% of the total isolates (Table 1).

Table 1: Isolation frequency of Salmonella from different sources

Sampling source	Sample collected	Positive for Salmonella	Percentage	Serovars isolated	% of serovar
Egg-shell	120	48	40.00	*S. enteritidis* (40)	83.33
				S. typhimurium (7)	14.58
				S. pullorum (1)	2.08
Egg interior	120	10	8.33	*S. enteritidis* (8)	80.00
				Others (2)	20.00
Poultry droppings	100	55	55.00	*S. enteritidis* (35)	63.63
				S. pullorum (12)	21.81
				Others (8)	14.54
Poultry meat	85	26	30.00	*S. enteritidis* (22)	84.00
				S. typhimurium (2)	7.70
				S. pullorum (1)	3.85
				Others (1)	3.85
Bakery products	65	09	13.85	*S. enteritidis* (6)	66.66
				S. typhimurium (2)	22.22
				Others (1)	11.11
Human stool	125	58	46.40	*S. enteritidis* (44)	75.86
				S. typhimurium (8)	13.79
				S. paratyphi B (4)	6.89
				Others (2)	3.44
Total	615	206	35.12	*S. enteritidis* = 155	--

The results of the standard disc diffusion tests and antibiogram of selected isolates are given in Table 2. Antibiogram studies revealed that *S. enteritidis* isolates were totally resistant to bacitracin, erythromycin and novobiocin. Isolates were highly sensitive to chloramphenicol (100%) and ampicillin (92.85%). *S. enteritidis* isolates showed poor susceptibility to gentamycin, kanamycin, penicillin and streptomycin.

Percentages of resistant isolates of *S. enteritidis* against different concentrations of antibiotics are shown in Table 3. *S.* e*nteritidis* isolates were totally resistant to all concentrations of bacitracin, erythromycin and novobiocin. More than 60% strains showed resistance at higher concentration (100 µg/10 µl) to spectinomycin and trimethoprim. Resistnnce to ampicillin and chloramphenicol was also observed by the isolates at higher concentration (100 µg/10 µl).

DISCUSSION

Several years ago, pandemic spread of *S. enteritidis* in human populations of Europe and the America was suggested to be associated with modern intensive egg production. *Salmonella enteritidis* may colonize the ovaries and peri-ovarian tissue of laying hens, and thus it has the potential for vertical transmission from breeders to layers and then to eggs sold for human consumption. In the present study, percentages of salmonellae recovered from egg-shell and egg interior were 40 and 8.33, respectively. Salmonellae do not cause gastroenteritis in such a small numbers as isolated from egg interior if such eggs are refrigerated at proper temperature. Improper storage provides opportunity to bacteria to multiply in the contaminated whole eggs or foods containing such eggs (including bakery products) to infective numbers. Outbreak investigations and other studies have indicated that a principal cause of *S. enteritidis* infection is consumption of raw or undercooked eggs or dishes contaminated with raw eggs (Rabsch *et al.*, 2001).

Persistence of Salmonella in the environment is an important characteristic in its prevalence. *Salmonella enteritidis* strains can survive for long periods of time in water and in dry materials such as dust, faeces and animal feed. Low numbers of *S. enteritidis* surviving in the environment in a dormant state can multiply rapidly if suitable conditions are present. Bacteriological analysis of poultry droppings in this study revealed highest score of Salmonella (55/100), as compared to other sources of sampling. From 55 strains of Salmonella in poultry dropping, 35 (63.63%) were *S. enteritidis* when typed by single factor antisera (O9, 12; g,m). The presence of *S. enteritidis* in the environment of laying flocks is generally accepted as a sensitive and relevant indication that contaminated eggs might be produced (Henzlar *et al*., 1994). One of the characteristic features observed during the study was that human as well as the poultry and poultry derivatives shared most of the serovars, indicating the potential hazard of interspecies sharing of this organism.

The results of in vitro susceptibility by standard disc showed that all isolates were highly resistant to bacitracin, erythromycin and novobiocin, followed by streptomycin and penicillin to which 92.85 and 85.71% isolates were resistant, respectively. The overall frequencies of resistance to gentamycin and trimethoprim were 78.57 and 71.42%, respectively. In another study, Verma and Gupta (1992) demonstrated the susceptibility of various Salmonella serovars to several antimicrobial drugs and reported high resistance to kanamycin, followed by trimethoprim, sulphametho- xazole and tetracyline. High susceptibility was shown to chloramphenicol, ampicillin and tetracycline by *S. enteritidis* isolates. The majority of strains of *S. enteritidis* continues to be fully sensitive to antimicrobial drugs and of 18,968 isolates reported by Laboratory of Enteric Pathogen in 1996, only eight were resistant with less than 0.5% resistance to four or more drugs (Ward and Threlfall, 1997). Antimicrobial resistance typing can be used in conjunction with serotyping, phage typing, protein analysis and genetic characterization of resistance plasmid for epidemiological purposes (Trepka *et al*., 1997). Under such circumstances, antibiogram should continuously be monitored to keep up to date with changes in drug resistance pattern.

The 12 antimicrobial drugs used against 14 selected *S. eenteritidis* isolates by serial dilution method showed different resistance patterns. The data demonstrated very high level of resistance to erythromycin, bacitracin and novobiocin, as 100% of the isolates showed resistance at all concentrations of these drugs (Table 3). Previous study on 86 strains of *S. enteritidis* isolated from poultry and poultry environment by Singer *et al.* (1992) has also demonstrated 100% resistance to bacitracin. The development and spread of antimicrobial resistance to bacitracin may be linked to selection pressure caused by excessive use of this drug. Salmonella resistance at varying concentrations of penicillin, streptomycin, spectinomycin and erythromycin has also been reported by Sultana *et al*. (1995). The higher resistance rates (64.28%) of *S. enteritidis* to the highest concentrations of spectinomycine and trimethoprim mandate the consideration of other therapeutic options and suggest the limited use of therapeutic potentials of these antimicrobial agents.

Kanamycin has been previously recommended as a drug of choice against Salmonella (Ikram, 1993). However, our studies as well as those of Mansoor (1997) show that kanamycin MICs for all of the Salmonella isolates are relatively higher. These findings indicate that kanamycin should not be recommended in our populations against Salmonella infection.

In summary, the results of both standard disc and serial dilution methods indicate the limited therapeutic value of bacitracin, erythromycin, kanamycin, streptomycin and spectinomycin against *S. enteritidis*. The need for continued surveillance is emphasized to determine local antimicrobial susceptibility data to identify changing pattern of resistance. Such data is essential for developing appropriate treatment of salmonellosis. Moreover, the prevalence of highly susceptible *S. enteritidis* strains suggests the limited use of antibiogram as an epidemiological marker.

Table 2: Antibiogram studies by disc diffusion method

Antimicrobial drugs	Complete resistance (%)	Intermediate resistance (%)	Susceptible (%)
Ampicillin (5 µg/disc)	-	7.14 (1)	92.85 (13)
Bacitracin (10 units/disc)	100 (14)	-	-
Chloramphenicol (15 µg/disc)	-	-	100 (14)
Erythromycin (15 µg/disc)	100 (14)	-	-
Gentamycin (10 µg/disc)	78.57 (11)	14.28 (2)	7.14 (1)
Kanamycin (10 µg/disc)	42.85 (6)	42.85 (6)	14.28 (2)
Novobiocin (5 µg/disc)	100 (14)	-	-
Penicillin (10 units/disc)	85.71 (12)	-	14.28 (2)
Spectinomycin (15 µg/disc)	50 (7)	7.14 (1)	42.85 (6)
Streptomycin (10 µg/disc)	92.85 (13)	-	7.14 (1)
Tetracycline (10 units/disc)	28.57 (4)	7.14 (1)	64.28 (9)
Trimethoprim (5 µg/disc)	71.42 (10)	-	28.57 (4)

Note: No. of isolates resistant are given in the parenthesis.

Table 3: Percentage of resistant isolates of selected 14 *S. enteritidis* against different concentrations of antimicrobial drugs

Antimicrobial drug	Antimicrobial drug concentration (µg/ disc)					
	100	50	25	12.5	6.25	3.12
Ampicillin	21.42(3)	7.14(1)	14.28(2)	21.42(3)	7.14(1)	-
Bacitracin	100(14)	100(14)	100(14)	100(14)	100(14)	100(14)
Erythromycin	100(14)	100(14)	100(14)	100(14)	100(14)	100(14)
Chloramphenicol	35.71 (5)	7.14 (1)	-	-	-	-
Gentamycin	-	7.14(1)	14.28(2)	7.14(1)	28.57(4)	28.57(4)
Kanamycin	28.57(4)	14.28(2)	7.14(1)	21.43(3)	21.43(3)	7.14(1)
Novobiocin	100(14)	100(14)	100(14)	100(14)	100(14)	100(14)
Penicillin	28.57(4)	7.14(1)	21.42(3)	21.42(3)	7.14(1)	-
Spectinomycin	64.28(9)	21.42(3)	5.26(1)	-	5.26(1)	-
Streptomycin	10.52(1)	21.42(3)	0.50(7)	21.42(3)	-	-
Tetracycline	28.57(4)	35.71(5)	28.57(4)	-	-	-
Trimethoprim	64.28(9)	-	7.14(1)	-	14.28(2)	-

Note: No. of isolates resistant are given in the parenthesis.

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