ANTIBIOGRAM STUDIES OF SALMONELLA ENTERITIDIS PHAGE TYPE 4 ISOLATES FROM POULTRY AND MEAT

Nusrat Shaheen, Naheed Fatima[*], Saraj-ud-Din Sajid[**], Abdus-Salam Khan Gandapur

Woman Medical College Abbottabad, [*]Department of Biological Sciences Quaid-i - Azam University Islamabad, [**]Frontier Medical College Abbottabad.

Background: Human infection with *Salmonella enteritidis phage type 4* has increased worldwide since last decade and has been shown to be related mainly with the consumption of poultry meat and eggs. The public health significance and economic importance of this serovar underscores the need to generate base line data on the antimicrobial susceptibilities and protein profile of indigenous *S. enteritidis.* This study was performed to investigate the antimicrobial susceptibilities of *S. enteritidis PhageType4* isolates from poultry and meat. **Method:** This study was carried out in the department of biological sciences Quaid-i-Azam University Islamabad during 1998-2000. A total of nineteen quinolone sensitive isolates of *S. enteritidis* from poultry meat and eggs collected during1994-1998 were characterized. The isolates were serotyped and phage typed at federal institute of consumer Health and Veterinary Medicine, Wernigerode Germany. Antimicrobial susceptibility tests were performed in accordance to the method of Bauer et al. (1966). **Results:** The results of the standard disc diffusion test showed 100% resistance against bacitracin, erythromycin and novobiocin. All (100%) isolates were highly sensitive to chloramphenicol. The results of minimum inhibitory concentration (MICs) tests using serial dilution of antimicrobial drugs revealed that 100% of the isolates were resistant against bacitracin, erythromycin and novobiocin at various levels of concentrations. Kanamycin, streptomycin and spectinomycin, all had very poor activity against serovar Enteritidis. **Conclusion:** These findings suggest the limited therapeutic potential and low typability of this serovar.

Key Words: Antibiogram, *Salmonella Enteritidis*, Phage Type 4.

INTRODUCTION

Non-typhoidal salmonellosis is a foodborne disease of primary concern in developed as well as developing countries. The spread of this disease is favored by wide array of animal reservoir and by the wide commercial distribution of both animals and food products and is one of the major public health problems in terms of socio-economic impact[1]

Global surveillance data indicates that incidence of gastrointestinal infections caused by *S. enteritidis* has increased massively during the last decades. Among the *S.enteritidis* isolates, those assigned to phage type 4 (PT4) and phage type 8 (PT8) have been isolated predominantly from poultry and are the most frequent cause of human salmonellosis. Similar increase in *S. enteritidis* infections have also been reported in Europe, where phage type 4 has emerged as the predominant phage type, spreading rapidly through both poultry and human populations and virtually replacing all other phage types.[2] *S. enteritidis* has been reported to be responsible for 380 salmonellosis out-breaks in USA between 1985 and 1991, involving 13056 illnesses and 50 deaths.[3]

The most prevalent phage type isolated from humans in Europe is PT4, while the most common PTs isolated in North America are PT8 and PT13a.[4] According to Rubino et al the most prevalent phage types present in *S. typhimurium* strains isolated in 1994 were DT104 followed by DT's 124, 173, 193, 135, 12 and 99.[5] Terajima et al reported the distribution of phage type of *S. enteritidis* isolates associated with the outbreak from human source in Japan during the year 1994 and 1995.[6]PT1 and PT4 predominated with prevalence of 43.2% and 32.6% for 1994 and 35.3% and 31.8% for 1995, respectively. Ten different PTs were found among the 302 isolates phage typed PT4 was the predominant PT in both human (73.9%) and poultry (76.2%) isolates, followed by PT1 (8.0%), 8 (3.6%) and 7a (2.2%) in human isolates and by PT7a (4.9%), 1 (3.7%) and 12 (2.4%) in poultry isolates.[7]

Salmonella is an important food and water-borne pathogen around the world. It causes acute gastrointestinal illness. The infective dose can be as low as 15-20 cells.[8]

Despite improved sanitation, human infections with *S. enteritidis* have been increased worldwide since 1980 and shown to be related mainly to the consumption of poultry meat and eggs.[7]

S. enteritidis has been shown to cause gastroenteritis and other acute infections. There is however, little information on the antimicrobial susceptibilities and epidemiology of indigenous *S. enteritidis* PT4 isolates, which

would help to prevent the spread of infections and provide data about the best choice for treatment. Our objectives in the present study were to investigate the antimicrobial susceptibilities of this organism. The study will have therapeutic, epidemiological and economic implications.

MATERIAL AND METHODS

This study was carried out in the department of biological sciences Quaid-i-Azam University Islamabad during 1998-2000. A total of nineteen quinolone sensitive isolates of. *S enteritidis* phage type 4 collected during 1994-1998 were used in this study. The isolates were serotyped and phage typed at the Federal Institute of Consumer Health and Veterinary Medicine, Wernigerode Germany.

Chemicals used in this study were obtained from Sigma and E. Merck and were of molecular biology grade. Culture media were purchased from Oxide Ltd and Difco Laboratories.

Antimicrobial susceptibility tests were performed in accordance to the method of Bauer et al. (1966).[9] The Mueller Hinton Agar was used as growth medium for standard disc diffusion test. *Staphylococcus. aureus* ATCC 25923 strain was used in each antimicrobial assay to serve as control organism. Plates were incubated over night at 37ºC to check the sterility. Later on, 100 µl of test and control culture grown overnight in L.B broth at 37ºC were spread on plates with the heat sterilized glass spreader to form a smooth bacterial lawn. Standard susceptibility test disc 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 35ºC within 15 minutes after applying the disc. 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. An isolate was defined as resistant, if it was resistant to at least one of the tested antimicrobial agent.

The antimicrobial drugs were dissolved (100µg/10µl) in the different volumes of water and ethanol. Different dilutions of each drug (100µg/10µl, 50µg/10µl, 25µg/10µl, 12.5µg/10µl, 6.25µg/10µl, 3.125µg/10µl) were prepared. Mueller Hinton agar was used as growth media for all MICs testing. Plates were poured and incubated over night to check their sterility. 100µl 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 disc was kept approximately 2 cm. The 10µl of each antimicrobial drug dilution was poured per disc in descending order of concentration. The plates were inoculated over night at 37^0C 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 results of minimum inhibitory concentration (MICs), and antibiogram of different isolates are shown in tables 1 and 2 respectively.

Standard Disc Diffusion Tests Results

The results of the standard disc diffusion tests and antibiogram of different isolates are given in tables 3 and 4 respectively.

Table-1: Percentage resistance of 19 *Salmonella enteritidis* PT4 isolates against different concentrations of antimicrobial drugs

	Antimicrobial drug concentration µg/discs					
ANTIMICROBIAL DRUGS	100	50	25	12.5	6.25	3.125
AMPICILLIN	15.78% (3)	5.26% (1)	21.05% (4)	15.78% (3)	10.52% (2)	-
BACITRACIN	100% (19)	100% (19)	100% (19)	100% (19)	100% (19)	100% (19)
CHLORAMPHENICOL	-	-	-	-	5.26% (1)	36.84% (7)
ERYTHROMYCIN	100% (19)	100% (19)	100% (19)	100% (19)	100% (19)	100% (19)
GENTAMYCIN	-	5.26% (1)	10.52 (2)	5.26% (1)	31.57% (6)	31.57% (6)
KANAMYCIN	31.57% (6)	10.52% (2)	10.52% (2)	21.05% (4)	21.05% (4)	5.26% (1)
NOVOBIOCIN	100% (19)	100% (19)	100% (19)	100% (19)	100% (19)	100% (19)
PENICILLIN	26.31% (5)	10.52% (2)	21.05% (4)	21.05% (4)	10.52% (2)	-
SPECTINOMYCIN	68.42% (13)	21.05% (4)	5.26% (1)	-	5.26% (1)	-
STREPTOMYCIN	10.52% (2)	21.05% (4)	47.36% (9)	21.05% (4)	-	-
TETRACYCLINE	31.57% (6)	36.84% (7)	26.31% (5)	-	-	-
TRIMETHOPRIM	63.15% (12)	-	5.25% (1)	-	10.52% (2)	-

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

Table-2: Antibiogram of individual isolate of *Salmonella enteritidis* PT4

ISOLATES NO	ANTIMICROBIAL DRUG RESISTANCE PHENOTYPE
S.E 1	KN^1, SP^1, ST^4, TP^1, TE^1, AP^5, PN^5, CM^6, GM^6, NB^1, EM^1, BC^1
S.E 2	NB^1, SP^2, AP^1, CM^5, KN^1, ST^4, TE^1, PN^1, TP^1, EM^1, BC^1
S.E 3	KN^1, SP^1, ST^2, TP^1, TE^1, AP^5, PN^3, CM^6, GM^6, NB^1, EM^1, BC^1
S.E.4	TE^1 , NB^1, KN^6, ST^1, SP^3, , BC^1, EM^1
S.E 5	AP^3, NB^1, SP^1, PN^1, ST^2, KN^5, TE^3, TP^5, GM^5, EM^1, BC^1
S.E.6	SP^1, KN^4, ST^3, TP^5, TE^3, AP^2, PN^3, CM^6, GM^5, NB^1, EM^1, BC^1
S.E 7	PN^4, SP^1, AP^3, GM^5, KN^2, ST^2, NB^1, TE^2, TP^1, EM^1, BC^1
S.E 8	TP^1, TE^2, NB^1, AP^4, KN^1, ST^1, PN^5, SP^5, EM^1, BC^1
S.E 9	PN^4, SP^1, AP^1, GM^3, KN^5, ST^3, NB^1, TE^2, TP^1, EM^1, BC^1
S.E10	AP^3, NB^1, SP^1, PN^1, ST^3, KN^4, TE^2, TP^1, GM^6, EM^1, BC^1
S.E11	SP^1, KN^3, ST^4, TP^1, TE^2, AP^3, PN^1, CM^6, GM^4, NB^1, EM^1, BC^1
S.E12	TE^2, ST^3, NB^1, KN^4, SP^1, PN^3, GM^2,EM^1, BC^1
S.E13	TP^1, TE^1, NB^1, GM^5, KN^2, ST^3, PN^3, SP^1, EM^1, BC^1
S.E14	ST^3, TE^3, NB^1, GM^5, KN^4, AP^4, PN^1, SP^2, EM^1, BC^1
S.E15	SP^1, KN^3, ST^3, TP^3, TE^1, AP^4, PN^2, CM^6, GM^3, NB^1, EM^1, BC^1
S.E16	NB^1, SP^1, GM^5, CM^6, KN^5, ST^2, TE^3, PN^4, TP^1, EM^1, BC^1
S.E17	TE^2, ST^3, NB^1, KN^5, SP^2, PN^4, GM^6, EM^1, BC^1
S.E18	ST^3, TE^3, NB^1, GM^6, KN^1, TP^1, PN^2, SP^2, EM^1, BC^1
S.E19	KN^1, SP^1, ST^3, TP^1, TE^1, AP^1, PN^4, CM^6, GM^6, NB^1, EM^1, BC^1

Note: The numbers 1, 2, 3, 4, 5 and 6 represent the antimicrobial drug concentrations (µg/disc) as follows:
1 = 100 µg/disc 2 =50 µg/disc 3 = 25 µg/disc 4 = 12.5 µg/disc 5 = 6.25 µg/disc 6 = 3.125 µg/disc

TABLE-3: Antimicrobial drug susceptibility testing of nineteen *Salmonella enteritidis* PT4 isolates using standard antimicrobial drug disc

ANTIMICROBIAL DRUGS	% RESISTANT	% INTERMEDIATE	% SUSCEPTIBLE
AMPICILLIN	-	5.26 % (1)	94.73 % (18)
BACITRACIN	100% (19)	-	-
CEFOPERAZONE	-	68.42% (13)	26.31 % (5)
CHLORAMPHENICOL	-	-	100% (19)
ERYTHROMYCIN	100 % (19)	-	-
FORTUM CEFTAZIDIME	42.10% (8)	5.26% (1)	31.57% (6)
GENTAMYCIN	78.94 % (15)	10.52 % (2)	5.26 % (1)
KANAMYCIN	42.10% (8)	42.10 % (8)	15.78% (3)
NOVOBIOCIN	100% (19)	-	-
PENICILLIN	89.47% (17)	-	10.25 % (2)
SXT*	52.63 % (10)	5.26% (1)	42.10 % (8)
STREPTOMYCIN	94.73% (18)	-	5.26% (1)
TRIMETHOPRIM	68.42 % (13)	-	26.31 % (5)
TETRACYCLINE	31.57 % (6)	5.26 % (1)	63.15 % (12)

Note: No. of isolates resistant are given in the parenthesis. SXT*(Septran Co Trimoxazole)

Table-4: Antibiogram of individual resistant isolates of *Salmonella enteritidis* PT4 for standard antimicrobial drugs discs

Isolate no:	Antimicrobial drug resistance phenotype
S.E 1	EM , PN , TE , KN , ST , NB , SX, TP , GM , BC
S.E 2	EM , PN , KN , SX , GM , CA , NB , TE , BC
S.E 3	EM , SX ,CA , GM , KN , ST , TE , NB , TP , BC
S.E 4	EM , SX , PN , ST , TE , NB , CA , BC
S.E 5	EM , SX ,CA , GM , PN , ST ,TE , NB , TP , BC
S.E 6	EM , TP , PN , ST , CA, SX, GM , KN , NB , BC
S.E 7	EM , CA , TP , KN, GM , PN , ST , TE , NB , SP , SX, BC
S.E 8	EM , SX, PN , ST , NB , TP , GM , BC
S.E 9	EM , CA, PN , ST , NB , SX , TP , GM , BC
S.E10	EM , PN , ST , CA , GM , NB , BC

S.E 11	EM , PN , ST , NB , KN , GM , BC
S.E 12	EM , PN , ST , NB , GM , BC
S.E 13	EM , PN , ST , NB , GM , BC
S.E 14	EM , PN , ST , GM , NB , BC
S.E 16	EM , PN , ST , TP , NB , GM , BC
S.E 17	EM , PN , ST , KN , NB , TP , BC
S.E 18	EM , NB , KN , TP, BC
S.E 19	EM , TP , TE , SX , PN , NB , TE , ST , KN , BC

*CAZ (Fortum Ceftazidime) *SXT (Septran Co Trimoxazole)

DISCUSSION

For the antimicrobial drugs used against nineteen *S. enteritidis* isolates, the results of MICs showed nine different phenotypic patterns. The MICs data demonstrated very high level of resistance to erythromycin, bacitracin and novobiocin, as 100% of the isolates showed resistance at higher concentrations of these drugs (See table: 1). Previous studies on 86 strains of *S. enteritidis*, isolated from poultry and poultry environment by Singer et al. (1992) has also demonstrated 100% resistance to bacitracin.[10]The development and spread of antimicrobial resistance to bacitracin may be linked to selection pressure caused by excessive use of this drug.[11] *Salmonella* resistance at varying concentrations of penicillin, chloramphenicol, streptomycin, spectinomycin and erythromycin has also been reported by other workers.[12,13,14,15] The higher resistance rates of *S. enteritidis* to tetracycline, bacitracin and kanamycin 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 drug of choice against *Salmonella*,[16]however, our studies as well as those of Mansoor, shows that kanamycin MICs for all of the *Salmonella* isolates are relatively higher.[15] These finding indicates that kanamycin should not be recommended in our populations against *Salmonella* infection. The results of in vitro susceptibility by standard disc showed that all isolates were highly resistant to erythromycin (15µg/disc), novobiocin(5µg/disc) and bacitracin (10units/disc), followed by streptomycin (10µg/disc) and penicillin (10 units/disc) to which 94.73 % and 89.47 % isolates were resistant respectively (See table-1). The over all frequencies of resistance to gentamycin(10µg/disc), trimethoprim (5µg/disc) and ceftazidime (30µg/disc) were 78.9%, 68.4% and 42.10% respectively (See table:1). These levels of resistance are quite different than those reported in previous studies by Athar .[17] During six years (1978-1982) study, 105 paratyphoid organisms belonging to 32 serovars were reported with sensitivity to tetracycline (10 units/disc) and six other antimicrobial drugs, including furazolidone, gentamycin, neomycin, penicillin and erythromycin. In another study, Javed (1992) demonstrated the susceptibility of various *Salmonella* serovars to several antimicrobial drugs using 112 *Salmonella* strains and reported high resistance to kanamycin followed by trimethoprim sulphamethoxazole and tetracyline.[18]Similar trend to these antimicrobial drugs has also been reported by others .[19,20,21] The frequency of antimicrobial drugs resistance in *S. enteritidis* has been found to be low and stable.[22, 23] The majority of the strain of *S.enteritidis* continue 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.[24] The antimicrobial susceptibility and molecular epidemiology of 275 *S. enteritidis* strains isolated in Hong Kong from 1986-1996 were studied. Over 99% of these isolates were susceptible to 17 of the 19 antimicrobials tested. One isolate harbored an autotransfering plasmid that confers resistance to tetracycline, trimethoprim-sulfamethoxazole. Another isolate harbored a mobilizable plasmid that confers resistance to ampicillin and cephalothin.[25]

Antimicrobial resistance in *Salmonella* strains is generally encoded by plasmid, which has been acquired as consequence of antibiotic pressure in humans and veterinary medicine, however, due to the fluidity of resistant plasmids and transposons, antimicrobial drug resistance pattern can not be recorded as satisfactory method for discriminations within serovars.[26] This has also been observed in our study.

However antimicrobial resistance typing can be used in conjunction with serotyping, phage typing, protein analysis and genetic characterization of resistance plasmid for epidemiological purposes[27]. Under such circumstances antibiogramshould continuously be monitored to keep up to date with changes in drug resistance pattern.

In summary, both the results, i.e. standard disc and serial dilution MICs indicate the limited therapeutic value of bacitracin, erythromycin, kanamycin, streptomycin and spectinomycin (See table 1, 3) 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* PT4 strains suggest the limited use of antibiogram as an epidemiological marker as reported previously.[26]

REFERENCES

1. Gracia del portillo F, Finlay BB. Invasion and intracellular proliferation of Salmonella within non-pathogenic cells. Microbiologia. SEM 1994;10: 229-38.
2. Rampling AA. Salmonella enteritidis five years on. Lancet 1993;342:317-8.
3. Mishu B, Kohler J, Lee LA. Out break of Salmonella enteritidis infections in the United States, 1985-1991. Journal of Infections Disease 1994;169: 547-52.
4. Rodrigue DC. International increase in Salmonella enteritidis. A new pandemic?Epidemiol Infect 1990;105:21-7.
5. Rubino S, Muresu E, Santona M, Paglietti B, Azara A, Schiaffino A et al. IS 200-finger printing of Salmonella enterica. Epidmiol Infect 1998;120:215-22.
6. Terajima J, Nakamura A, Watanabe H. Epidemiological analysis of Salmonella enterica, serovar Enteritidis isolates in Japan by phage typing and pulsed field gel-electrophoresis. Epidemiol Infect 1998;120:223-9.
7. Sumalee-Bangtrakulnonth BA, Pornrunangwong S, Terajima J, Watanable H, Ichikaneko K, Ogawa M. Epidemiological analysis of Salmonella enteritidis 1998; 36(4): 971-974.
8. ChrongKhan MC, Van T, Lin W, Lin J. PCR Assay For Rapid Detection of Salomonella spp in Foods. PRA-SW, ORA, FDA, Los Angeles CA 900915. 2002.
9. Bauer KA, Sherris J, Turk M. Antibiotic Susceptibility testing by standardized single disc method. Am J Clin Pathol 1966; 45: 493-6.
10. Singer, JT, Opitz HM, Gershman M, Hall MM, Moniz IG, Rao SV. Molecular characterization of Salmonella enteritidis isolates from main poultry and poultry farm environment. Avian Dis 1992;36:324-33.
11. Parkash, Pilli. Multiple resistant S.typhi in India. APUA Newslett 1992; 10.
12. Siddique BM, Bucar E. Antibiotic sensitivity of Salmonella isolated from poultry. Arch.Roum.path.Exp. Microbiol 1985;44; 301-9.
13. Lamb VA, Mayhall CG, Spadora AC, Dalton HP. Out break of Salmonella typhimurium gastroenteritis due to an imported strain resistant to ampicillin, chloramphenicol, trimethoprim-sulphamethoxazole in a nursery. J Clin Microbiol 1984; 20;1076-9.
14. Sultana K, Bushra MA, Nafisa I. Evaluation of antibiotic resistance in clinical isolates of Salmonella typhi from Islamabad Pakistan. Zool 1995; 27(2): 185-7.
15. Mansoor A. Molecular characterization of Avian Salmonella isolates. M.Phil. Thesis. Department of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan. 1997.
16. Ikram N. Molecular epidemiology of Salmonella isolates obtained from local hospital in Islamabad. M.Phil. Thesis, Department of Biological Sciences, Quaid-i-Azam University, Pakistan. 1993.
17. Athar SM. Final report on Salmonella in poultry, poultry products, poultry feed, and feed ingredients. Directorate of poultry production and Research Sind. Karachi. Pakistan. 1982.
18. Javed T. Prevalence and pathological studies on salmonellosis in broiler breeders. Ph.D Thesis, Department of Biological Sciences, Quaid-i-Azam University. Islamabad. Pakistan. 1992.
19. Verma JC, Gupta BR. Determination of minimum inhibitory concentration of antibiotics against Salmonella organisms from animal sources by using multiple inoculation. Indian J Ani Sci 1992;62:793-6.
20. Maiorini E, Lopez EE, Marrow AL, Ramirez F, Procapio A. Fuvmanski, S et al. Multiple resistant non-typhoidal Salmonella gastroenteritis in children. Ped Infect Dis 1993;12:139-144.
21. Sajid SD. Epidemiology and molecular characterization of indigenous Salmonella isolates from poultry. Ph.D. Thesis. Deparment of Biological Sciences Quaid-i-Azam University Islamabad. 1998.
22. Ward LR, Threlfall EJ, Row B. Multiple drug resistance in Salmonella in England and Wales: a comparison between 1981 and 1988. J Clin Path 1990;43:563-6.
23. Son Ansary R, Salmah I, Maznah A. Survey of plasmids resistance factors among veterinary isolates of S .enteritidis in Malaysia. J Clin Path 1995;11:315-8.
24. Ward LR, Threlfall EJ. Human salmonellosis in England and Wales. Current situation. Symposium "Salmonella and salmonellosis" 20-22 May 1997. Plaufragan/Saint Brieve, France. Proceedings 1997; 8: 547-9.
25. Ling JM, Koo IC. Kam KM, Cheng AF. Antimicrobial susceptibility and molecular epidemiology of Salmonella enterica serotype Enteritidis isolates in Hong Kong from 1986-1996. J Clin Microbiol 1998;36:1693-9.
26. Olsen JE, Brown DJ, Baggessen DL, Bisgaard M. Biochemical and molecular characterization of Salmonella enterica serotype Berta and comparison of methods for typing. Epidemiol Infect 1992;108:243-60.
27. Threlfall EJ, Frost JA. Plasmid profile typing can be used to subdivide phage type 49 of Salmonella typhimurium outbreak investigation. Epidemiol Infect 1990;104: 243-51.

Address for correspondence:
Nusrat Shaheen, Assistant Professor, Department of Biochemistry, Women Medical College Abbottabad, Pakistan.
Email: natrasweet@yahoo.com