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CHARACTERIZATION OF SALMONELLA ISOLATED FROM PIGS OF SADAR UPAZILLA OF MYMENSINGH DISTRICT

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ABSTRACT

Characterization of Salmonella isolated from pigs was conducted between January to November, 2008 in the district of Mymensingh. Out of 23 pigs examined, only 4 (17.39) pigs had Salmonella infection. Genome analysis using PCR revealed that ST gene of enterotoxin was absent in some strains of pigs Salmonella but it's not clear whether the serovars belongs to *Salmonella* (S.) *choleraesuis*, *S. typhimurium* or *S. derby*. The isolates were found to be highly sensitive to ciprofloxacin, kanamycin, nalidixic acid, co-trimoxazole and cephalexin but highly resistant to chloramphenicol.

Key words: Salmonella, Isolation, Characterization, PCR, ST gene, Pig, Antibiogram

INTRODUCTION

Salmonellae are Gram negative, short plump shaped rods, nonsporeforming, noncapsulated, aerobic and facultatively anaerobic organisms and classified under the family Enterobacteriaceae (Freeman, 1985). The genus Salmonella includes a large group of serologically and biologically related bacilli and as a rule they are motile by means of peritrichous flagella with the exception of *Salmonella pullorum and Salmonella gallinarum*. More than 2500 serovars exist based on 67 "O" antigens (for non motile species) and the numerous "H" antigens (for motile species) recognized so far (Blood *et al*., 2003). Salmonella infection remain a serious problem to livestock and public health significance throughout the world (Tabaraie *et al*., 1994) and cause substantial economic loss resulting from mortality, morbidity, poor growth of infected animals, poultry and human beings; hazardous of transmitting food poisoning with gastroenteritis to human and represents a serous problem for the food industry (Todd, 1990; Barrington *et al*., 2002 and Khan *et al*., 2007). Among domesticated animals, pigs, constitute one of the most important reservoirs of Salmonellae and are susceptible to disease caused by a wide variety of serotypes. Most important of these is *Salmonella choleraesuis* which is relatively species specific for pigs, and is the cause of pig paratyphoid. This organism is disseminated by symptomless carriers and infection occurs from ingestion of contaminated material. The most susceptible age is approximately 4-8 weeks after weaning. The members of the genus Salmonella are being isolated, identified and characterized by using various cultural, biochemical, serological and molecular studies. The reliable methods for isolation require the use of media which encourage the growth of Salmonella and inhibit that of other enteric organisms. Antibiogram study, serum agglutination test, pathogenicity test, ELISA, PCR (Polymerase chain reaction), DNA-DNA hybridization, Pulsed field gel electrophoresis (PFGE) are widely being used to identify and characterize *Salmonella spp.* in the laboratories (Deighan *et al*., 2000; Veling *et al*., 2000 and Buerfeind *et al*., 2001). Molecular biology based detection techniques especially PCR assays have been reported for the rapid, specific and sensitive detection of microorganisms in different clinical samples (Baumler *et al*., 1997). PCR is the best known and most successfully implemented nucleic acid detection technology to date (Nissen and Sloots, 2002).

MATERIALS AND METHODS

A total number of 23 field samples comprising rectal swab and faeces samples from apparently healthy and diarrhoeic pigs were aseptically collected and carried to the laboratory in Bacto selenite broth (BSB) for the characterization of Salmonellae. The samples were then cultured on to Salmonella-Shigella (SS) agar, MacConkey (MC) agar, Brilliant Green agar (BGA) and Eosin Methylene Blue (EMB) agar. Individual colonies were picked up and stained with Gram stain for morphological study and subjected to biochemical tests (sugar fermentation tests, Voges Proskauer test, Indole test and Methyl red test). Salmonella polyvalent antiserum (Poly "O" and Poly "H") was used for the serological identification of *Salmonella* spp. The isolated *Salmonella* spp. of pig and previously isolated Salmonella of chicken and human were preserved in 20% glycerin. The isolated Salmonella preserved in 20% glycerin were placed in ice box and transported to ICDDR,B for performing molecular characterization by PCR.

DNA template preparation for PCR

DNA template was prepared by inoculation a single colony from LA plate to 3 ml of LB broth and incubated at 37^0C with agitation (1200 rpm). Then 1.5 ml of sample was taken in an eppendorf tube and centrifuged at 13000 rpm for 10 min. After then supernatant was discarded and pellet was dissolved in the sterile normal saline and again sample was centrifuged at 13000 rpm for 10 minutes. The supernatant was discarded and the pellet was dissolved in 200µl of normal saline. The samples was boiled for 10 minutes and cooled in ice for 30 minutes. Then the sample was centrifuged at 13000 rpm for 10 minutes and supernatant was transferred to a fresh eppendorf tube and stored at -20^0C for use template DNA in PCR (Mohan *et al*., 1995).

Amplification of ST gene of enterotoxin of *Salmonella* spp. by PCR

PCR amplification was performed in a final volume of 20µl containing 3µl of DNA template, Tap polymerase 0.2µl, dNTP 10mM, F primer (GCCTGA GCG AGA AGGT) 1.0µl, R primer (CAG TCC CACCCA CTT C) 1.0µl, $MgCl_2$ 50mM and Deionized Distilled water 8.8µl. The required number of PCR tubes were labeled and kept on ice. Then 17µl reaction mixture was dispensed into each of the PCR tubes and 3µl of DNA from each sample was added to that respective tube and mixed properly with the help of the micropipette. The tubes were placed in peltier thermocycler. The initial denaturation was at 95^0C for 5 minutes, 95^0C for 1 minute, annealing at 60^0C for 1 minute and extension at 72^0C for 10 minutes for total 7 cycles and held for 4^0C for maximum 18 hours. After completion of PCR, PCR products were kept in 0^0C for Agarose Gel Electrophoresis (Haque *et al*., 1999).

Image analysis

The fingerprint in the gel was analyzed using a computer software package, Quantity one version 3.0 (Bio-Red, USA). After background subtraction and gel normalization, the fingerprint patterns were subjected to typing based on banding similarity and dissimilarity. Two methods for similarity measuring, one based on binary data of occurrence of the band (band- based) calculated using the Dice coefficient and another method based on overall densitometry profile (curve-based) of the banding pattern calculated using Pearson's product moment correlation were compared.

Antibiogram study

Eight different antibacterial discs, manufactured by Span Diagnostics Limited, India were selected for the antibacterial sensitivity study against isolated Salmonella that were marketed by Stimulus Speciality Diagnostics, 173-B, New Industrial Estate, Udhna-394210 (Suart) India. The antibacterial agents corresponding eight different discs are commonly used in the field condition for the treatment of salmonellosis.

RESULTS AND DISCUSSION

The research work was conducted to isolate and characterize the *Salmonellae* spp. of pig from Mathorpotty, Sadar upazilla, Mymensingh and compare these with the isolates of chicken and human by the application of molecular tool, PCR. Antibiotic sensitivity of the isolates was also studied. Specific enriched media and biochemical tests were used for the isolation and identification of Salmonella as previously suggested by a number of researchers (Buxton and Fraser, 1977; Mallinson and Scherrer, 1991; Sharma and Katock, 1996; Dhruba *et al*., 1999 and Habrun *et al*., 2006). In this study, colony characteristics of *Salmonella* spp. on MC agar, SS agar and BG agar were similar to the findings of other authors (Shaffer *et al*., 1964; Merchant and Packer, 1967 and Buxton and Fraser, 1977). In this study, colony characteristics of Salmonella spp. on MC agar, SS agar and BG were similar to the findings of other authors (Shaffer *et al*., 1964; Merchant and Packer, 1967 and Buxton and Fraser, 1977). In Gram's staining the isolated Salmonella exhibited Gram negative, small rod shaped, single or paired in arrangement under microscope which was supported by other researchers (Freeman, 1985 and Gene, 2002). In motility test, all the isolates have shown swinging movement which differentiates the motile bacteria from non-motile and also from E. coli which has forward movement (Merchant and Packer, 1967 and Buxton and Fraser, 1977).

Differentiation of Salmonella into species level was difficult to identify based on their sugar fermentation pattern (Freeman, 1985). In sugar fermentation test, all the isolated Salmonellae fermented dextrose, maltose and mannitol and produced acid and gas but did not ferment sucrose and lactose which satisfy the statement of Buxton and Fraser (1977). Again, all the isolates were positive to methyl red test and negative to indole test. Reaction in TSI agar slant with all the isolates produced red slant, yellow butt with the production of H_2S gas which strongly supports the observation of Merchant and Packer (1967). Out of 23 samples, 4 samples (17.39%) were positive for salmonellosis in pig (Table 1).

Table 1. Results of isolation of Salmonella from the gastro-intestinal tract of pigs in Mymensingh

S/N	Pig farm	No. of animal tested	Animal positive		Total	
			Cultural test	Biochemical test	No.	%
1	Ramadas Harijan	6	1	1	1	16.67
2	Nondolal Harijan	7	1	1	1	14.29
3	Haridas harijan	10	2	2	2	20.00
Total		23	4	4	4	17.39

S/N = Serial Number, No. = Number, % = Percent

Slide agglutination test was performed with commercially available agglutinating polyvalent antiserum which is very sensitive (Avakian *et al*., 1988). The isolates were agglutinated with both Poly "O" and Poly "H" antisera which indicated that the isolates were of *Salmonella* spp.

Polymerase chain reaction is used for the rapid detection of Salmonella, by sequencing Salmonella enterotoxin gene (stn) which is able to detect *Salmonella typhi, Salmonella paratyphi* A and B and *Salmonella typhimurium* (Riyaz-UI-Hassan *et al*., 2004). Some strains of *Escherichia coli* produce enterotoxin. The ability to produce enterotoxin can be transmitted by conjugation from some strains to other deficient strains of *E. coli* and also to some strains of *Salmonella typhimurium* and *S. choleraesuis.*

Table 2. Antibiotic sensitivity pattern of isolated Salmonella from pig

S/N	Salmonella isolates	No. of isolate tested	CI	KA	NA	CT	CP	ER	AX	CK
1	Ramadas Harijan	6	+++	+++	+++	+++	+++	+	+	-
2	Nondolal Harijan	7	+++	++	+++	+++	++	++	+	-
3	Haridas harijan	10	+++	+++	++	++	+++	+	+	-

S/N = Serial Number, No. = Number, CI = Ciprofloxacin, KA = Kanamycin, NA = Nalidixic acid, CT = Co-trimixazole, CP = Cephalexin, ER = Erythromycin, AX = Amoxicillin, CK = Chloramphenicol, +++ = Highly sensitive, ++ = Moderately sensitive, + = Less sensitive, - = Resistant

Three PCR primer pairs were determined to be suitable for application of PCR amplification of Salmonella in faecal samples - 16S rDNA, Salmonella enterotoxin gene (stn), and histidine transport operon. The authors were evaluated their sensitivity of detetion of Salmonella in faecal samples (Ziemer and Steadham, 2003 and Murugkar *et al*., 2003).

PCR was performed to characterize the genomic organization of pig Salmonella with the isolates of chicken and human. From the results of PCR it could be concluded that ST gene of enterotoxin was present in some strains of human salmonella but absent in some strains of pig and chicken Salmonella (Figure 1). Using PCR with ST gene of enterotoxin it could not be confirmed whether the organism was *Salmonella choleraesuis, S. typhimurium* or *S. derby.*

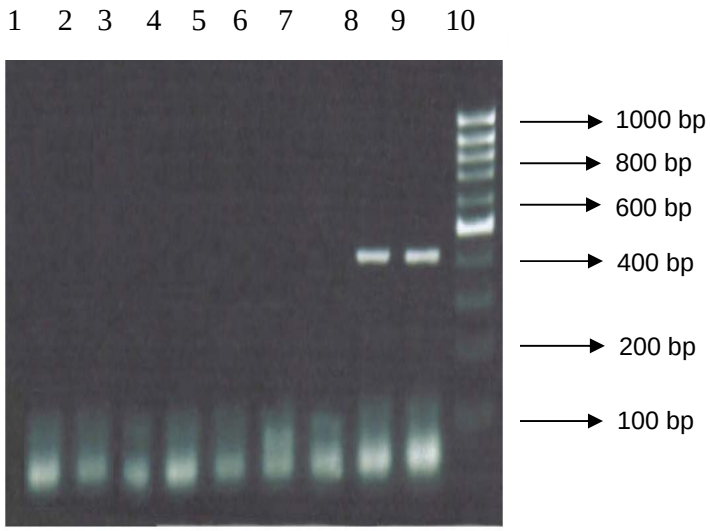

Fig. 1. PCR for *Salmonella* spp. of pig, chicken and human. Lane 1-3: isolates from pig; Lane 4-6: isolates from chicken; Lane 7-8: isolates from human; Lane 9: G5Fb, strain of *Escherichia coli* used as control and Lane10: 10-100 bp Marker

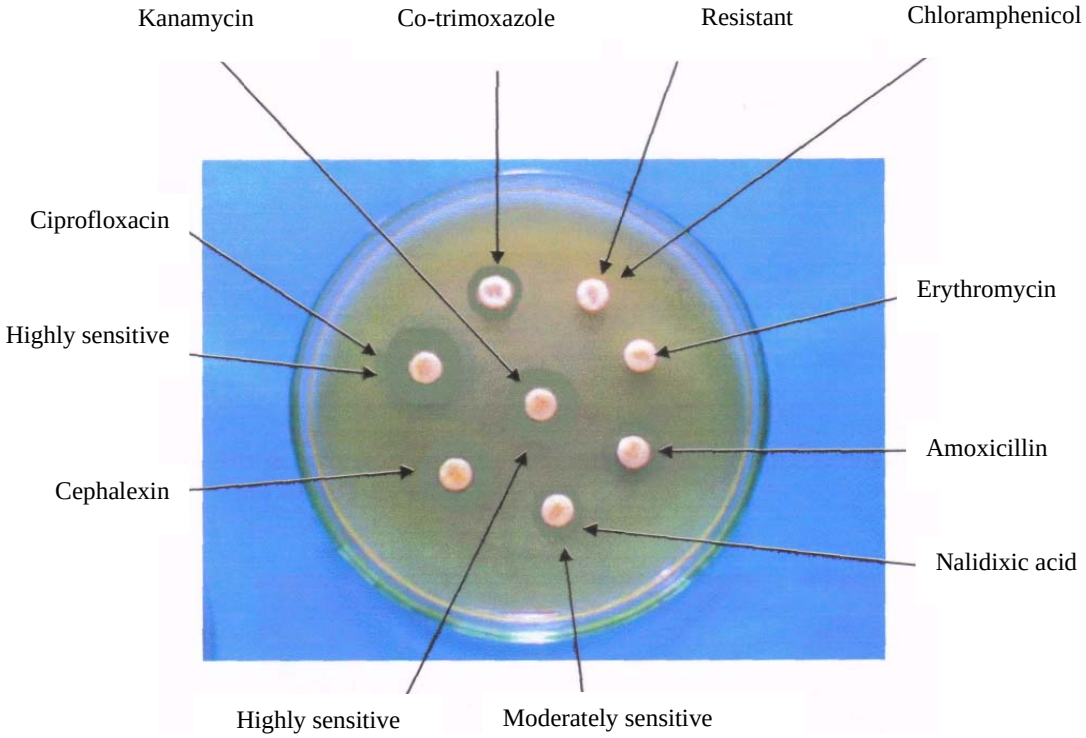

Fig. 2. Antibiotic sensitivity and resistance pattern of isolated *Salmonella* spp. to different antibiotics

The study also opined that the isolated Salmonellae were highly sensitive to ciprofloxacin, kanamycin, nalidixic acid, co-trimoxazole and cephalexin, moderately sensitive to kanamycin, nalidixic acid, co-trimoxazole, cephalexin, amoxicillin and erythromycin. These are less sensitive to erythromycin, amoxicillin and chloramphenicol while resistant to chloramphenicol (Table 2, Figure 2). This finding satisfy the result of Chugh and Suheir (1983), Banani *et al*. (2003), Zhang *et al*. (2006) and Kobayashi *et al*. (2007). The antibacterial resistance observed here in the isolated *Salmonella* might be due to indiscriminate use of those antibacterial agents in field condition in study areas and/or rapid chromosomal mutation and presence of specific plasmid DNA. The antibiotic sensitivity tests were performed by disc diffusion method using eight different antibiotic discs

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