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# Anti-microbial resistance and molecular characterization of *Salmonella* serovars from man and animals*

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ABSTRACT

Varied samples (1,132) comprising poultry meat (212), poultry eggs (49), poultry droppings (60), autopsied poultry tissues (60), pork (156), pig faeces (189), cattle dung (105), buffalo dung (103), sheep faeces (11), goat faeces (31), deer faeces (2) and human stool (154) were screened for isolation of *Salmonella* spp. Total 50 *Salmonella* isolates were recovered from samples, viz. poultry meat (2), poultry eggs (1), poultry droppings (6), autopsied poultry tissues (16), pork (2), pig faeces (9), cattle dung (6), buffalo dung (3), sheep faeces (4) and human stool (1). The recovered *Salmonella* isolates belonged to 10 different serovars with most prevalent serovar *S*. Typhimurium (21) followed by *S.* Weltevreden (12), *S.* Ughelli (5), *S.* Essen (3), *S.* Elisabethville (2), *S.* Lagos (2), *S.* Drogana (2), *S.* Enteritidis (1), *S.* London (1) and un-typable *Salmonella* (1). *Salmonella* isolates showed extreme variation in resistance (0–100%) against anti-microbial agents. The maximum resistance was observed among *Salmonella* isolates against sulphamethizole and furazolidone (100% each) followed by kanamycin (50%), gentamicin (44%), nalidixic acid (14%), tobramycin (10%), amikacin, ampicillin, streptomycin and tetracycline (8% each), amoxicillin/clavulanic acid (6%), norfloxacin (4%), ciprofloxacin and cefotaxime (2% each) and chloramphenicol (0%). A high level of sensitivity in *Salmonella* isolates was observed against chloramphenicol (100%) and tetracycline (92%). A high multiple antibiotic resistance (MAR) index in a range of 0.133–0.666 was observed in *Salmonella* serovars. Molecular characterization of *Salmonella* was performed by targeting amplification of *inv*A gene primer fragment for confirmation of *Salmonella* spp. in which all isolates were found positive for this gene. Moreover, this gene encodes virulence factor in *Salmonella* spp. which was exhibited by all *Salmonella* isolates of the present study.

**Key words**: Animals, Anti-microbial resistance, Man, Molecular characterization, *Salmonella* serovars

*Salmonella*, a major cause of food-borne diseases throughout the world, is generally transmitted to humans through consumption of contaminated foods of animal origin mainly meat, poultry, eggs and milk. Human cases also occurr when individuals come into contact with infected animals including pet animals. The use of anti-microbial drugs in the animals has exposed animals, irrespective of their health, to frequently sub-therapeutic concentrations of anti-microbials (Dupont and Steele 1987). The increase of antibiotic resistance in developing countries is due to the widespread use for human treatment (Cohen *et al.* 1986). Resistance to 2 or more classes of drugs (MDR phenotype) is becoming increasingly widespread in *Salmonella* species. Multidrug-resistant (MDR) strains of *Salmonella* are now encountered frequently and the rates of multidrug-resistance have increased considerably in recent years. Keeping these in view the present study was undertaken to— isolate and identify *Salmonella* organisms from varied sources using conventional culture method followed by molecular characterization of isolates by targeting *inv*A gene primer fragment using PCR-based molecular technique; and to know the anti-microbial resistance in these isolates including determination of multiple antibiotic resistance (MAR) index to assess the degree of resistance in *Salmonella* serovars of public health importance as well as health and production in animals.

## MATERIALS AND METHODS

Samples (1,132) comprising poultry meat (212), poultry eggs (49), poultry droppings (60), autopsied poultry tissues (60), pork (156), pig faeces (189), cattle dung (105), buffalo dung (103), sheep faeces (11), goat faeces (31), deer faeces (2) and human stool (154) were aseptically collected from Pantnagar, Nagla, Jawahar nagar, Lalkuan and Rudrapur and processed for isolation of salmonellae using conventional culture method. All the samples collected in sterile containers

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following all possible aseptic conditions and brought to the laboratory as soon as possible following cold chain.

*Salmonella* organisms were isolated from human stool and animal faecal samples as per ISO 6579: (2002) and CDC/WHO (2003). Pork, poultry meat and eggs were subjected to the isolation of *Salmonella* organisms as per USDA/FSIS (2002) and Andrews and Hammack (2003). *Salmonella* organisms were identified on the basis of cultural, morphological and biochemical characters (Old 1996). Further, these isolates were subjected to agglutination reaction by using O antiserum poly A-I and Vi. *Salmonella* isolates were confirmed by PCR technique by targeting *inv*A gene fragment as per Chiu and Ou (1996). The agarose gel electrophoresis of amplified PCR products was carried out according to Sambrook and Russell (2001) using 1.5% agarose gel in 0.5×TBE buffer in horizontal submarine gel electrophoresis system. *Salmonella* identified on the basis of cultural, morphological, biochemical, serological and molecular characters were sent for serotyping to *Salmonella* Typing Center, Division of Bacteriology and Mycology, IVRI, Izatnagar.

Anti-microbial susceptibility testing by disc diffusion on Mueller-Hinton agar was performed as per Bauer *et al*. (1966) and CLSI (2011). All *Salmonella* isolates were subjected to the anti-microbial susceptibility testing using commercially available anti-microbial discs. The anti-microbial discs used were ampicillin (10 mcg), amoxycillin/clavulanic acid (20/10 i.e. 30 mcg), amikacin (30 mcg), norfloxacin (10 mcg), ciprofloxacin (5 mcg), cefotaxime (30 mcg), gentamicin (10 mcg), kanamycin (30 mcg), streptomycin (10 mcg), tobramycin (10 mcg), tetracycline (30 mcg), chloramphenicol (30 mcg), nalidixic acid (30 mcg), furazolidone (50 mcg) and sulphamethizole ((300 mcg). The isolates were interpreted as sensitive, intermediate or resistant as per the interpretation chart provided by the manufacturer.

The multiple antibiotic resistance (MAR) index was also calculated to know the degree of resistance using the formula "MAR index = a/b", where 'a' is the number of antibiotic(s) to which an isolate was resistant and 'b' is the number of antibiotic(s) to which the isolate was exposed. As per the above formula, the range of MAR index of *Salmonella* serovars was calculated as follows:

O antiserum poly A-I and Vi was procured and used for serological identification of *Salmonella* spp. The primer sequence for *inv*A genes used in present study were as follows: forward primer "ACAGTGCTCGTTTACGACC TGAAT" and reverse primer "AGACGACTGGTACTGA TCTAT". The biologicals required for molecular works using PCR were procured commercially.

## RESULTS AND DISCUSSION

The anti-microbial resistance is recognized by the World Health Organization as a major emerging problem of public health (CDC 1994). Resistance to combination of several classes of anti-microbial agents led to the emergence of multidrug-resistant strains that may pass from food animals to humans (O'Brien 2002). The emergence and spread of multi-drug resistance in *Salmonella* organisms have reinforced the need for epidemiological studies describing the prevalence and resistance pattern of *Salmonella* organisms.

*Salmonella* isolates (50) were recovered from varied samples comprising poultry meat (2), poultry eggs (1), poultry droppings (6), autopsied poultry tissues (16), pork (2), pig faeces (9), cattle dung (6), buffalo dung (3), sheep faeces (4) and human stool (1). However, *Salmonella* isolates could not be recovered from goat and deer faecal samples which might be due to good hygienic and managemental practices, good immune status of animals and small number of samples from these animals processed in the present study. On serotyping, these isolates were identified as 10 different serovars of *Salmonella* with most prevalent serovar was *S*. Typhimurium (21) followed by *S*. Weltevreden (12), *S*. Ughelli (5), *S*. Essen (3), *S*. Elisabethville (2), *S*. Lagos (2), *S*. Drogana (2), *S*. Enteritidis (1), *S*. London (1) and un-typable *Salmonella* (1). These serovars recovered from different animal species pose a serious threat to human and animal health as well as reduced production in the animals due to serious suffering from this organism.

In the present study, 15 anti-microbial agents were used to observe the resistance pattern among *Salmonella* isolates (Table 1). The maximum resistance was observed among *Salmonella* isolates against sulphamethizole and furazolidone (100% each) followed by kanamycin (50%), gentamicin (44%), nalidixic acid (14%), tobramycin (10%), amikacin, ampicillin, streptomycin and tetracycline (8% each), amoxicillin/clavulanic acid (6%), norfloxacin (4%), ciprofloxacin and cefotaxime (2% each) and chloramphenicol (0%). The anti-microbial resistance was observed in a range of 0–100% that is extreme variation in the sensitivity of *Salmonella* isolates against different anti-microbial agents. Aarestrup *et al*. (2003) observed low frequency of resistance against ampicillin (1.8%), chloramphenicol (1.6%), tetracycline (4.0%), streptomycin (4.4%), sulfphamethoxazole (4.2%) and nalidixic acid (1.6%). This finding is almost similar to the observations of the present study. Jaulkar *et al*. (2011) noticed resistance in *Salmonella* isolates against each of ampicillin and amoxicillin/clavulanic acid (81.81%) followed by tetracycline (63.63%), streptomycin (18.18%) and nalidixic acid (9.09%) which is comparatively higher resistance than the findings of the present study might be due to frequent exposure as well as sub-therapeutic doses of these anti-microbial agents used for prevention or control of infection.

A high level of sensitivity in *Salmonella* isolates was observed against chloramphenicol (100%) and tetracycline (92%) which are not commonly in use for past few years due to availability of new classes of anti-microbial agents. This high level of sensitivity could be attributed to the fact that for a long period of time, these antibiotics have not been in use which might have resulted in development of anti-microbial sensitivity in *Salmonella* isolates.

Table 1. Anti-microbial sensitivity/resistance pattern in *Salmonella* isolates

| *Salmonella* serovars | AMP | AMC | AK | NX | CIP | CTX | GEN | K | S | TOB | TE | C | NA | FR | SM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *S*. Typhimurium | S | S | I | S | S | S | I | I | S | I | S | S | S | R | R |
| *S*. Typhimurium | S | S | S | S | S | I | S | I | S | I | S | S | S | R | R |
| *S*. Typhimurium | S | S | I | S | I | I | I | R | I | I | S | S | S | R | R |
| *S*. Typhimurium | S | S | I | S | S | I | I | R | I | I | S | S | S | R | R |
| *S*. Typhimurium | S | S | I | S | S | I | I | I | I | I | S | S | S | R | R |
| *S*. Typhimurium | S | S | S | S | S | I | I | I | I | I | S | S | S | R | R |
| *S*. Typhimurium | S | S | I | I | S | I | R | R | I | R | R | S | R | R | R |
| *S*. Typhimurium | S | S | I | S | S | I | I | R | I | I | S | S | S | R | R |
| *S*. Typhimurium | S | S | I | S | S | I | I | R | I | I | S | S | I | R | R |
| *S*. Typhimurium | S | S | I | S | S | I | I | R | I | I | S | S | I | R | R |
| *S*. Typhimurium | S | S | I | S | S | I | I | R | I | I | S | S | S | R | R |
| *S*. Typhimurium | S | S | I | S | S | I | I | R | I | I | I | S | I | R | R |
| *S*. Typhimurium | S | S | R | S | S | I | R | R | I | R | I | S | R | R | R |
| *S*. Lagos | S | S | I | I | S | I | I | R | R | R | S | S | R | R | R |
| *S*. Typhimurium | S | S | I | I | S | I | I | R | I | R | S | S | S | R | R |
| *S*. Typhimurium | S | S | R | S | S | I | R | R | I | R | S | S | I | R | R |
| *S*. Typhimurium | S | S | S | S | S | S | S | S | S | S | S | S | S | R | R |
| *S*. Typhimurium | S | S | I | I | S | I | I | R | I | I | I | S | S | R | R |
| Un-typable *Salmonella* | S | S | S | S | S | S | I | I | I | I | S | S | S | R | R |
| *S*. Weltevreden | S | S | I | S | S | S | R | R | I | S | S | S | S | R | R |
| *S*. Weltevreden | S | S | I | S | S | S | I | R | I | I | S | S | S | R | R |
| *S*. Ughelli | S | S | I | S | S | S | R | R | I | I | S | S | S | R | R |
| *S*. Weltevreden | S | S | I | S | S | S | R | R | I | I | S | S | R | R | R |
| *S*. Weltevreden | S | S | I | S | S | I | R | R | I | I | S | S | S | R | R |
| *S*. Ughelli | S | S | I | S | S | I | R | I | I | S | S | S | I | R | R |
| *S*. Elisabethville | S | S | I | S | I | S | R | R | R | I | S | S | I | R | R |
| *S*. Ughelli | S | S | R | S | S | S | R | R | I | I | S | S | S | R | R |
| *S*. Essen | S | S | I | S | S | S | R | I | I | S | S | S | S | R | R |
| *S*. Weltevreden | S | S | S | S | S | S | R | I | I | S | S | S | S | R | R |
| *S*. Elisabethville | S | S | I | S | S | S | I | I | S | S | S | S | S | R | R |
| *S*. Ughelli | S | S | S | S | S | S | R | I | I | I | S | S | S | R | R |
| *S*. Weltevreden | S | S | I | S | S | S | R | R | I | I | S | S | S | R | R |
| *S*. Weltevreden | S | S | I | S | S | S | S | I | I | I | S | S | S | R | R |
| *S*. London | S | S | I | S | S | S | R | R | I | I | S | S | S | R | R |
| *S*. Weltevreden | S | S | I | S | S | S | R | R | I | I | S | S | S | R | R |
| *S*. Weltevreden | S | S | S | S | S | S | S | S | S | S | S | S | S | R | R |
| *S*. Weltevreden | S | S | S | S | S | S | R | I | S | S | S | S | S | R | R |
| *S*. Weltevreden | S | S | S | S | S | S | R | I | I | S | S | S | S | R | R |
| *S*. Ughelli | S | S | I | S | S | S | R | R | S | S | S | S | S | R | R |
| *S*. Typhimurium | R | R | R | R | R | S | R | R | R | S | R | S | R | R | R |
| *S*. Typhimurium | R | R | S | R | I | S | R | R | R | S | R | S | R | R | R |
| *S*. Lagos | R | R | S | I | I | S | R | I | I | S | R | S | R | R | R |
| *S*. Typhimurium | S | S | S | S | S | I | S | I | S | I | S | S | S | R | R |
| *S*. Weltevreden | S | S | S | S | S | I | S | S | S | S | S | S | S | R | R |
| *S*. Essen | S | S | S | S | S | I | S | I | I | I | S | S | S | R | R |
| *S*. Typhimurium | S | S | S | S | S | S | S | S | S | S | S | S | S | R | R |
| *S*. Drogana | S | S | S | S | S | S | S | S | S | S | S | S | S | R | R |
| *S*. Enteritidis | S | S | S | S | S | S | S | S | S | S | S | S | S | R | R |
| *S*. Essen | S | S | S | S | S | S | S | S | S | S | S | S | S | R | R |
| *S*. Drogana | R | S | S | S | S | R | I | I | I | I | S | S | S | R | R |

AMP, ampicillin; AMC, amoxicillin/clavulanic acid; AK, amikacin; NX, norfloxacin; CIP, ciprofloxacin; CTX, cefotaxime; GEN, gentamicin; K,kanamycin; S, streptomycin; TOB, tobramycin; TE, tetracycline; C, chloramphenicol; NA, nalidixic acid; FR, furazolidone; SM, sulphamethizole; S, sensitive; I, intermediate; R resistant.

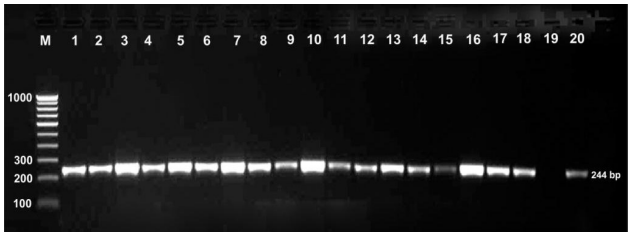


Fig. 1. Agarose gel electrophoresis showing amplified PCR products of *inv*A gene; Lane M: 100 bp Marker, Lane 1–18: *Salmonella* isolates, Lane 19: Negative control, Lane 20: Positive control (*Salmonella* Typhimurium).

In the present study, all *Salmonella* isolates (100%) exhibited resistance against 2 or more anti-microbial agents. The prevalence of such a large number of MDR *Salmonella* isolates (100%) indicated widespread and extensive use of anti-microbial agents in these areas which indicated negative impact on economy related to animal production, and pose serious threat to the animal and human health. Gautam *et al.* (2002) reported an increase in multidrug resistance (MDR) from 53.6 to 63.9% from 1997 to 2001 in Northern India. Out of 113 isolates recovered from poultry slaughterhouses, 65% were found to be multiple resistant to antibiotics including tetracycline, neomycin and streptomycin in Spain (Carraminana *et al.* 2004).

In the present study, some *Salmonella* isolates showed resistance against up to 10 anti-microbial agents of 15 antimicrobial agents used. Such a high degree of resistance in *Salmonella* isolates may pose serious health hazards in animal and human beings. The multiple antibiotic resistance (MAR) index of salmonellae was found in a range of 0.133–0.666. However, Jaulkar *et al*. (2011) reported comparatively lower MAR index in a range of 0.06–0.53. The high MAR index against *Salmonella* organisms observed in the present study indicated injudicious use of several anti-microbial agents for preventive as well as therapeutic purposes.

The molecular characterization of *Salmonella* isolates was performed by targeting amplification of *inv*A gene primer fragment for confirmation of *Salmonella* spp. The *inv*A gene is genus specific for *Salmonella* organism. All *Salmonella* serovars were found positive for the presence of *inv*A gene. Some of the *Salmonella* serovars showing amplification with this gene are given in Fig. 1.

It may be concluded that the wide variations in the prevalence of multi-drug resistance in the *Salmonella* organisms might be due to the use of anti-microbial agents in different frequencies in different geographical areas. Moreover, the use of anti-microbial agents in sub-therapeutic doses might have resulted in the development of multi-drug resistance in the organisms. This high degree of resistance in *Salmonella* serovars may pose serious threat to animal as well as public health and affect the production in the animals.

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