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Antibiogram and Multiple Antibiotic Resistance index of *Salmonella enterica* isolates from Poultry

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Antimicrobial resistance analysis by 16 different antibiotics was done on 24 isolates of *Salmonella enterica* belonging to six different serotypes viz *S*. Virchow (1), *S*. Heidelberg (5), *S*. Kentucky (1), *S*. Agona (1), *S*. Haifa (14) and two rough strains. The results revealed that the *Salmonella* isolates were sensitive to ciprofloxacin, chloramphenicol and norfloxacin, nalidixic acid and co-trimoxazole, co-trimazine, gentamicin, amikacin and streptomycin and cephalexin in the decreasing order of frequency. All the strains were found resistant to ampicillin and 75% isolates were resistant to nitrofurantoin and colistin. Twenty isolates exhibited multiple drug resistance against 3 to 9 antibiotics with Multiple Antibiotic index ranging from 0.06 to 0.56. MAR index of 0.37 was found in maximum number of isolates (8) which were resistant to six different antimicrobials. The dendrogram based on resistotyping pattern differentiated 24 isolates into 19 resistance patterns (resistotypes) indicating the validity of this test for differentiating the strains to a level of 97.8%. All 24 *Salmonella* isolates revealed 65.9% average genetic similarity and level of similarity ranged between 20% to 100%.

Key words: Antimicrobial-resistance, *Salmonella*, Multiple Antibiotic Resistance Index, Dendrogram, Resistotypes.

In the past few decades, emergence of antibiotic resistance among different species of bacteria is on the rise (Davies and Davies, 2010). The problem arises due to the non-judicious usage of antibiotics for prophylaxis, therapeutic or growth promotion reasons. Poultry is an important food animal in which usage of antibiotics is high as prophylactic measures, treatment protocols or growth promoters. This has resulted in high selective pressure on various bacterial species resulting in emergence of multi-drug resistant bacterial species (Van den Bogaard & Stobberingh, 1999). The problem is compounded when it occurs in pathogenic bacteria like *Salmonella enterica* having public health significance. The emergence of multi-drug resistant strains leads to intractable infections leading to treatment failures and loss of money on drugs. Poultry harbours a number of serovars which are zoonotic and can acquire resistance easily in poultry environment due to the selective pressure put on them by non-judicious usage of drugs. Such strains can easily pass to humans resulting in complicated cases of human gastroenteritis which are difficult to control. (White *et al*., 2002).

The present study was carried as very little work has been done to explore the status of antibiotic burden in Poultry farms of Bikaner region. As such, we investigated the antibiogram profile of *Salmonella enterica* isolated from different poultry farms of Bikaner to evaluate and monitor the extent of resistance in the isolates of our region. We also calculated the Multiple Antibiotic Resistance (MAR) index which has been shown to be a cost effective and valid method of bacteria

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source tracking. MAR analysis has been used to differentiate bacteria from different sources using antibiotics that are commonly used for human therapy. Compared to other methods of bacteria source tracking such as genotypic characterization, the MAR indexing method is cost effective, rapid and easy to perform. MAR index values greater than 0.2 indicate high risk source of contamination where antibiotics are often used.

MATERIALS AND METHODS

Bacterial strains

The study was conducted on 24 isolates of *Salmonella enterica* previously isolated by Khan *et al.* (2015) from different species of poultry. The isolates consisted of *S.* Haifa (14) followed by *S.* Heidelberg (5), *S.* Virchow (1), *S.* Agona (1), *S.* Kentucky (1) and two Rough strains. The strains were isolated from different species of poultry from India. All the isolates were revived in Luria Bertani broth from Nutrient broth containing foetal calf serum (1.5%) and 50% (vol/vol) glycerol kept at -80 °C. After 24h incubation at 37°C, cultures were streaked on XLD agar and confirmed again

Antimicrobial susceptibility testing (Resistotyping)

All isolates were tested for antibiotic resistance by the disc diffusion method as described by Bauer *et al.* (1966) with Mueller-Hinton agar (Difco) plates. Sixteen antimicrobial agents viz. Amoxyclav (10mcg), Cephalexin (30mcg), Cephotaxime (30mcg), Chloramphenicol (30mcg), Ciprofloxacin (10mcg), Co-Trimoxazole (25mcg), Gentamicin (10mcg), Norfloxacin (10mcg), Amikacin (10mcg), Ampicillin (10mcg), Cephaloridine (30mcg), Colistin (10mcg), Co-Trimazine (25mcg), Nalidixic Acid (30 mcg), Nitrofurantoin (300mcg) and Streptomycin (10mcg) were used. These plates were incubated at 37°C for 24 hours. Around the antibiotic disc, zone of inhibition was measured and matched with standard table and interference was recorded accordingly to manufacturer of antibiotic discs (Hi Media, India).

Cluster analysis of *Salmonella* isolates

Cluster analysis of *Salmonella* isolates on the basis of their various sensitivity/resistance pattern was carried out following guidelines of Rohlf (1987) using NTSYS pc (Numerical Taxonomy Systems). Cluster analysis for the genetic distance was then carried out using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering method. The genetic distances obtained from cluster analysis through UPGMA were used to construct the dendrogram, depicting the relationship with clones using computer programme NTSYS pc version 1.7 (Rohlf, 1987).

Table 1. Percent antibiotic sensitivity patterns of *Salmonella* isolates of poultry origin

S. No.	Antibiotic	*Salmonella* isolates (%) Resistant	Intermediate	Sensitive
1.	Amoxcylav	62.5	12.5	25
2.	Cephalexin	50	4.16	45.83
3.	Cephotaxime	12.5	62.5	25
4.	Chloramphenicol	4.16	0	95.83
5.	Ciprofloxacin	0	4.16	95.83
6.	Co-Trimoxazole	8.33	0	91.66
7.	Gentamicin	8.33	8.33	83.33
8.	Norfloxacin	4.16	0	95.83
9.	Amikacin	0	37.5	62.5
10.	Ampicillin	100	0	0
11.	Cephaloridine	62.5	33.33	4.16
12.	Colistin	75	25	0
13.	Co-Trimazine	8.33	4.16	87.5
14.	Nalidixic acid	8.33	0	91.66
15.	Nitrofurantoin	75	12.5	12.5
16.	Streptomycin	0	37.5	62.5

MAR (Multiple Antibiotic Resistance) Index Study:

The MAR Index of an isolate is defined as a/b, where 'a' represents the number of antibiotics to which the isolate was resistant and 'b' represents the number of antibiotics to which the isolate was subjected (Jayaraman *et al.*, 2012).

Identification of MDR (Multi Drug Resistance):

Multi Drug Resistance is defined as resistance to more than two classes of antibiotics among all the tested antibiotics. The Multi Drug Resistance (MDR) characters of the isolates were identified by observing the resistance pattern of the isolates to the antibiotics.

RESULTS AND DISCUSSION

The detailed results of antibiotic sensitivity test of *Salmonella* isolates are given in table 1. The results of antibiogram showed 100% of the isolates were resistant to ampicillin, followed by colistin and nitrofurantoin (75%), amoxyclav and cephaloridine 62.5% and cephalexin 50%. The high resistance to beta–lactam antibiotics is *at par* to Diarra *et al*. (2014) who reported in their study similar pattern of resistance against beta-lactam antibiotics. Growing resistance towards beta-lactam antibiotics has been prevalent worldwide among members of *Enterobacteriaceae* from animal origin especially in *Salmonella* sp. Colistin resistance has not been much reported earlier but the increased trend in the resistance is linked to

Table 2. Multiple Antibiotic Resistance index of *Salmonella enterica* isolates

S. No.	Serotype	Isolate No.	No.of antibiotics to which isolate is resistant(a)	MAR index=a/b
1.	Virchow	VW1	08	8/16=0.50
2.	Heidelberg	HD1	05	5/16=0.31
3.	Heidelberg	HD2	05	5/16=0.31
4.	Heidelberg	HD3	06	6/16=0.37
5.	Heidelberg	HD4	06	6/16=0.37
6.	Heidelberg	HD5	06	6/16=0.37
7.	Agona	AG1	02	2/16=0.12
8.	Kentucky	KT1	09	9/16=0.56
9.	Rough	RG1	01	1/16=0.06
10.	Rough	RG2	03	3/16=0.18
11.	Haifa	HF1	06	6/16=0.37
12.	Haifa	HF2	06	6/16=0.37
13.	Haifa	HF3	03	3/16=0.18
14.	Haifa	HF4	02	2/16=0.12
15.	Haifa	HF5	03	3/16=0.18
16.	Haifa	HF6	02	2/16=0.12
17.	Haifa	HF7	07	7/16=0.43
18.	Haifa	HF8	03	3/16=0.18
19.	Haifa	HF9	03	3/16=0.18
20.	Haifa	HF10	03	3/16=0.18
21.	Haifa	HF11	07	7/16=0.43
22.	Haifa	HF12	06	6/16=0.37
23.	Haifa	HF13	06	6/16=0.37
24.	Haifa	HF14	06	6/16=0.37

Table 3. MAR index values with Number of isolates

S.No.	MAR index	No. of isolates
1.	0.56	1
2.	0.50	1
3.	0.43	2
4.	0.37	8
5.	0.31	2
6.	0.18	5
7.	0.12	3
8.	0.06	1

mis-sense mutations in two genes *pmrA* and *pmrB* genes which encode a regulator and sensor of a two-component regulatory system of outer membrane (Sun *et al.*, 2009).

Many *Salmonella* isolates were resistant to nitrofurantoin which is also reported by Dias *et al.* (2005). Resistance to nitrofurantoin may be attributed as it is often added into feed of poultry birds and used to control infection in young chicks as a cheap antibacterial agent, creating a selective pressure for development of nitrofurantoin resistant strains (Agarwal *et al.*, 2005).

The percent sensitivity of the isolates were 95.83% for chloramphenicol, ciprofloxacin and norfloxacin, followed by co-trimoxazole and nalidixic acid 91.66%, co-trimazine 87.5%, gentamicin 83.33% and amikacin and streptomycin 62.5%. One isolate among the 24 isolates showed resistance to chloramphenicol and norfloxacin and intermediate resistance against ciprofloxacin while rest of isolates was sensitive. Thus ciprofloxacin is the drug of choice for salmonellosis of poultry origin in this area.

High sensitivity of *Salmonella* isolates to ciprofloxacin, chloramphenicol, norfloxacin, nalidixic acid, co-trimoxazole, co-trimazine and gentamicin suggested that they could be used for effective prevention and control of salmonellosis in poultry birds. This might be attributed to the fact that these antibiotics were seldom used in poultry birds for therapeutic, prophylactic and growth enhancement purpose in our region. High degree of sensitivity of *Salmonella* isolates to these antibiotics has been reported earlier by other workers (Purushothaman *et al.*, 2003 and Tsai and Hsiang, 2005).

These differences in the incidence of resistance among *Salmonella* isolates mainly attributed by exchanging of resistance genes efficiently among bacterial populations and even passed to mammalian cells and at little extent by managemental conditions of farms.

Multiple drug resistance was recorded in 20 (83.33%) out of the 24 isolates which showed resistance against 3 to 9 antibiotics (table 2). Multiple antibiotic resistance (MAR) has been attributed to the presence of plasmids which contain one or more resistance genes, each encoding a single antibiotic resistance phenotype (Daini *et al.*, 2005). The multiple drug resistance Index calculated ranged from 0.06 to 0.56 with 0.37 being the predominant MAR index in 8 isolates (table 3). The isolates belonged to *S.* Haifa (5) and *S.* Heidelberg (3). One isolate of *S.* Kentucky was found to have highest MAR index of 0.56 which was resistant to 9 antibiotics from 16 antibiotics tested. The serotype is known to cause human salmonellosis in travelers. Dissemination of such type of resistant clones can have serious public health concerns (Le Hello *et al.*, 2011).

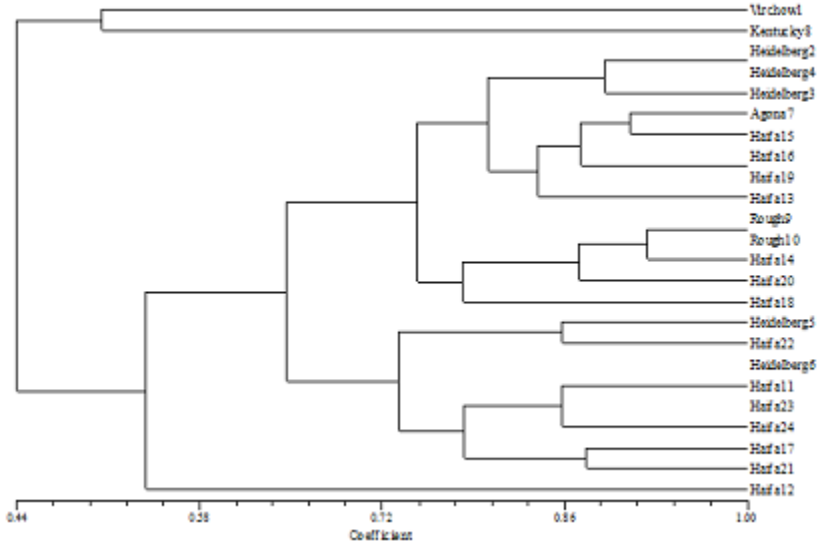

Fig. 1. Dendrogram showing relationship among *Salmonella* isolates using UPGMA generated by antibiotic sensitivity patterns (resistotyping)

Multiple drug resistance against 6 to 8 drugs has been reported in several *Salmonella* serotypes of animal and human origin in India by several workers (Ashwini *et al*., 2013; Singh *et al*., 2012). Emergence of multiple drug resistant *Salmonella* strains has often been held responsible for frequently occurring outbreaks and hyperendemicity of salmonellosis in India (Singh *et al*., 2006). India is said to be home for multiple drug resistant strains of different *Salmonella* serotypes. The phenomena namely the localization of resistance genes on conjugative plasmids, cointegrate formation which gave rise to new plasmids and the localization of resistance genes on transposons has led to the efficient spread of multiple drug resistance in *Salmonella* (Helmuth, 2000).

The dendrogram computed on 24 *Salmonella* isolates based on antibiotic sensitivity patterns, clearly divided the accessions into nineteen resistotypes (Fig. 1). Five strains of *S.* Heidelberg divided into four resistotypes and fourteen strains of *S.* Haifa divided into twelve resistotypes. More than one strain of *Salmonella* were included only in four resistotypes. Strains of these four resistotypes include same serotype or different serotypes and show 100% genetic similarity (0% heterogeneity) within a resistotype.

Five strains of *S.* Heidelberg have shown average genetic similarity 81.4% (average genetic diversity 18.6%). The level of similarity of *S.* Heidelberg strains ranged between 66% to 100%. Average genetic similarity among fourteen strains of *S.* Haifa was 68.1% and level of similarity ranged between 40% to 100%. All 24 *Salmonella* isolates showed 65.9% average genetic similarity (average genetic diversity 34.1%) and level of similarity ranged between 20% to 100%. Discriminatory ability of resistotyping system was 0.978 (97.8%).

In present investigation all 24 *Salmonella* isolates showed nineteen resistotypes using 16 antibiotics, discriminatory index of resistotyping was 97.8% and ciprofloxacin considered as the drug of choice for salmonellosis of poultry origin. Nevertheless, Purushothaman *et al*. (2003) obtained 57 resistotypes using 33 different antibiotics and antibacterials agents against 65 *Salmonella* isolates, discriminatory index of test was 89.69%.

Antimicrobial resistance in *Salmonella* strains is generally encoded by plasmids, which has been acquired as consequence of antibiotic pressure in humans and veterinary medicine, in whatever manner due to the fluidity of resistant plasmids and transposons, antimicrobial drug resistance pattern cannot be recorded as satisfactory method for discrimination within serovar (Olsen *et al.*, 1992).

The present investigation indicates the need and importance of changing the choice of chemotherapy from the commonly prescribed antibiotics to the drugs of higher potentiality for effective treatment and control of salmonellosis in poultry birds.

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