

Plasmid profile and drug resistance pattern of zoonotic *Salmonella* isolates from Indian buffaloes

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Abstract

Background: Buffalo is the major source of animal protein in south-east Asia, including India; therefore, the presence of multiple drug resistance in *Salmonella* strains of buffalo meat and milk products is of immense public health concern.

Methodology: Forty-six strains of *Salmonella enterica* subspecies *enterica* belonging to eight serovars (*S. Anatum*, 13; *S. Weltevreden*, 13; *S. Rostock*, 6; *S. Typhimurium*, 5; *S. Gallinarum*, 5; *S. Stockholm*, 1; *S. Dublin*, 1; and *S. Orion*, 2), isolated from buffalo meat and diseased buffaloes were studied for their antibiotic sensitivity and plasmid profile.

Results: All except six strains of *Salmonella* had one or more plasmids. Virulence plasmid of ~35Mda was present in 39 isolates while 19 strains had one to six additional plasmids with molecular weight ranging from 1 Mda > 35 Mda. A plasmid-free *S. Anatum* strain was resistant to seven drugs including fluoroquinolones, while strains having six to seven plasmids were resistant to fewer antimicrobial drugs. One *S. Anatum* isolate, resistant to 11 antibiotics, had only one plasmid. Eight serovars of *Salmonella* could be divided into 28 resistotypes on the basis of antimicrobial sensitivity assay. Most strains were resistant to streptomycin (84.8%) followed by kanamycin (58.7%), gentamicin (52.2%), ampicillin (50%) and oxytetracycline (50%). Few strains were resistant to cefotaxime (2.2%), amoxicillin (2.2%) and newer fluoroquinolones (6.5%).

Conclusion: Multiple drug resistance was common among *Salmonella* isolates of buffalo origin, particularly against aminoglycosides, oxytetracyclin, ampicillin and cephalosporins. Presence of plasmids is not mandatory for occurrence of multiple drug resistance in *S. enterica* strains.

Key words: buffaloes, *Salmonella enterica*, Typhimurium, Weltevreden, MDR, plasmids

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Introduction

Buffalo farming is a major contributor to the agriculture and livestock industry in many Asian countries through the production of good quality milk, meat and farmyard manure [1]. Therefore, the pathogens either causing disease in buffaloes and their progeny or transmitted through their produce are important because they affect milk production and overall livestock production. In buffaloes, *Salmonella* is both a major pathogen causing calf diarrhoea [2-6] leading to early-age calf-mortality [7-9] and a foremost pathogen transmitted through animal products. More than 10% of buffalo meat samples have been reported as contaminated with *Salmonella* in northern India [10]. Antibiotics are generally used as growth-promoting substances for livestock in the developing world. After the use of growth antibiotics in animals, *S. enterica* serovar Weltevreden emerged

as the most common *Salmonella* serovar globally [11], and is reported to constitute more than 20% of *Salmonella* isolates of buffalo origin in India [12]. *Salmonella* Weltevreden has not only emerged in animals in India, but also in humans, and more than 29% of *Salmonella* isolates of human origin belonged to single serovar [13]. Some other commonly reported *Salmonella* serovars in buffaloes include *S. Typhimurium*, *S. Dublin*, *S. Chester*, *S. Bareilly*, *S. Lagos*, *S. Orion*, *S. Saintpaul*, *S. Stanley*, *S.I. 4,12:i:-*, *S.I. 3,10:r:-* and rough non-typable strains [12,14-16].

While buffalo have long been identified as a source of *Salmonella*, little is known about multi-drug-resistance (MDR) and plasmids of *Salmonella* isolates of buffaloes; therefore, this study was undertaken to determine antimicrobial drug sensitivity and plasmid profiles of *Salmonella* strains

of buffalo origin to gain a better understanding of salmonellae of buffaloes.

Materials and Methods

Salmonella strains

All 46 strains of *Salmonella enterica* subspecies *enterica* isolated from buffalo meat and diseased buffaloes in India (during 2002-2005), belonging to eight serovars (namely, *S. Anatum*, 13; *S. Weltevreden*, 13; *S. Rostock*, 6; *S. Typhimurium*, 5; *S. Gallinarum*, 5; *S. Orion*, 2; *S. Stockholm*, 1; and *S. Dublin*, 1) were obtained from stocks from the National *Salmonella* Centre (Vet), Indian Veterinary Research Institute, Izatnagar, repository. All the strains were submitted from different veterinary institutions in India for serotyping to the National *Salmonella* Centre [12] with information about the source of isolation and date of isolation. As the isolates were always endorsed by the respective heads of microbiology departments, we never questioned the accuracy of the information provided with the strains. All isolates were revived and confirmed through biochemical and serological methods [17]. For serotyping, anti O and anti H group and factor-specific serum (Difco BBL, USA) were used to perform the standard tube agglutination test with O and H antigens prepared from each of the strains [17]. Except for *S. Typhimurium* (2), *S. Weltevreden* (13) and *S. Stockholm* (1), which were isolated from fresh buffalo meat samples, all other strains (30) were isolated from faecal samples of buffalo calves with clinical diarrhoea in the last three years [12].

Plasmid profiling

The method of Hames and Higgins [18] was followed for isolation of plasmids using bacteria pelleted from 10 ml of overnight grown culture in Luria Bertani (LB) broth using the phenol:chloroform extraction method. Plasmid DNA was dissolved in 50 µl sterile distilled water containing 0.1mg RNase-A ml⁻¹ and stored at -20°C. Plasmid preparations were electrophoresed on 0.7% w/v agarose (SRL, India) gel. The plasmid DNA bands were visualized and photographed under a UV-transilluminator and gel documentation system (UVP, England). The plasmid ladder used was extracted from the *E. coli* (E381) reference strain having 7 plasmids of 1.79 MDa, 2.03 MDa, 2.63 MDa, 3.39 MDa, 3.69 MDa, 5.19 MDa and 35.8 Mda.

Antimicrobial sensitivity assay

Antimicrobial sensitivity of all *Salmonella* isolates was determined in triplicate by the disc diffusion method [19] on Muller Hinton agar No. 4 against 16 antimicrobials belonging to seven classes namely, penicillins (ampicillin 10 µg, amoxicillin 10 µg); chloramphenicol 30 µg; cephalosporins (cefotaxime 30 µg, cephalexin 30 µg); fluoroquinolones (ciprofloxacin 5 µg, enrofloxacin 5 µg, lomefloxacin 2 µg, norfloxacin 30 µg); aminoglycosides (gentamicin 10 µg; kanamycin 30 µg; streptomycin 10 µg); cotrimoxazole 25 µg; quinolone (nalidixic acid) 30 µg; nitrofurantoin 300 µg; and oxy-tetracycline 30 µg discs (Hi-media, Mumbai, India). Based on the zone of inhibition, isolates were classified as sensitive or resistant [19]. The strains resistant to three or more groups of drugs were classed as multi-drug-resistant (MDR) strains. A reference *E. coli* K12 strain (E-382), sensitive to all antimicrobials, was used as the control.

Results

All strains had one or more plasmid except five strains of *S. Anatum* and one each of *S. Orion* and *S. Gallinarum*; those had no plasmids including ~35 MDa plasmids. All seven plasmid-free strains were isolated from buffalo calves with diarrhoea, while, all strains of buffalo meat (buffen) origin had one or more plasmid.

A comparatively heavier plasmid (> 35.8 MDa) was also present in six strains, including two strains each of *S. Anatum* and *S. Weltevreden* and one strain each of *S. Orion* and *S. Rostock*. In addition to the ~35 MDa plasmid, ~ 1 Mda (3 strains), 2 Mda (3 strains), 3 Mda (2 strains), 5 Mda (1 strain), 8 Mda (1 strain) and 12 Mda (3 strains), plasmids were also detected in a number of strains. A comparatively heavier plasmid (molecular weight > 35.8 MDa) was also present in six strains, including two strains each of *S. Anatum* and *S. Weltevreden* and one strain each of *S. Orion* and *S. Rostock*.

Salmonella strains of buffalo origin were resistant to comparatively fewer antimicrobials (1-8 drugs) than those from calf diarrhoea (1-11 drugs). *Salmonella* Typhimurium and *S. Stockholm* from buffalo were resistant to tetracycline only; however, *S. Weltevreden* of buffalo origin were resistant to three to eight drugs. The majority of the *Salmonella* isolates from buffaloes were resistant to streptomycin (39), followed by kanamycin (27), gentamicin (24), ampicillin (23), oxytetracycline (23), cephalexin (19),

Table 1. Antimicrobial drug resistance pattern of *Salmonella* strains of buffalo origin

S.No.	Resistant to No. of drugs	Drugs resisted (Number of strains)	Number of strains	Number of group of drugs resisted
1	1	Na (1)	<i>S. Rostock</i>	1
		T (5)	<i>S. Stockholm</i> (1), <i>S. Gallinarum</i> (2), <i>S. Typhimurium</i> (2)	1 1 1
		S (2)	<i>S. Rostock</i>	1
		A, K, S (3)	<i>S. Typhimurium</i>	2
2	3	A, Na, S (1)	<i>S. Dublin</i>	3
		A, Cp, S (2)	<i>S. Rostock</i>	3
		Nf, S, T (2)	<i>S. Anatum</i>	3
		Na, S, T (2)	<i>S. Weltevreden</i>	3
		Cp, Nf, S, T (3)	<i>S. Weltevreden</i>	4
3	4	A, C, G, K, S (1)	<i>S. Rostock</i>	4
		A, G, K, S, T (1)	<i>S. Anatum</i>	3
4	5	A, G, K, Nf, S (2)	<i>S. Orion</i>	3
		G, K, Nf, S, T (1)	<i>S. Anatum</i>	3
		A, Na, No, S, T (1)	<i>S. Weltevreden</i>	5
		C, Cp, G, K, S (3)	<i>S. Gallinarum</i>	3
		A, G, K, No, S, T (1)	<i>S. Anatum</i>	4
		A, C, G, K, No, S (1)	<i>S. Weltevreden</i>	4
5	6	A, Cp, G, K, S, T (1)	<i>S. Anatum</i>	4
		C, Cp, G, K, Nf, S (3)	<i>S. Weltevreden</i>	4
		Cp, G, K, Nf, S, T (1)	<i>S. Anatum</i>	4
		A, Cp, G, K, Nf, S (2)	<i>S. Weltevreden</i>	4
		A, C, G, K, Lo, No, Na (1)	<i>S. Anatum</i>	5
		A, Cf, Cp, En, G, K, No, S (1)	<i>S. Anatum</i>	4
6	7	A, Co, Cp, G, K, Nf, S, T (1)	<i>S. Weltevreden</i>	6
		A, C, Co, G, K, No, S, T (1)	<i>S. Anatum</i>	6
		A, Cf, Co, En, G, K, Na, No, S, T (1)	<i>S. Anatum</i>	6
7	8	A, Ce, Co, Cp, G, K, Na, No, S, T (1)	<i>S. Anatum</i>	7
		A, Am, Cf, Co, Cp, G, K, Lo, No, S, T (1)	<i>S. Anatum</i>	6

A, ampicillin; AM, amoxicillin; C, chloramphenicol; Ce, cefotaxime; Co, cotrimoxazole; Cp, cephalixin; Cf, ciprofloxacin; En, enrofloxacin; G, gentamicin; K, kanamycin; Lo, lomefloxacin; Na, nalidixic acid; Nf, nitrofurantoin; No, norfloxacin; S, streptomycin; T, oxy-tetracycline

nitrofurantoin (15), chloramphenicol (10), norfloxacin (8), nalidixic acid (6), cotrimoxazole (5), ciprofloxacin (3), enrofloxacin (2), lomefloxacin (2), amoxicillin (1), and cephotaxime (1).

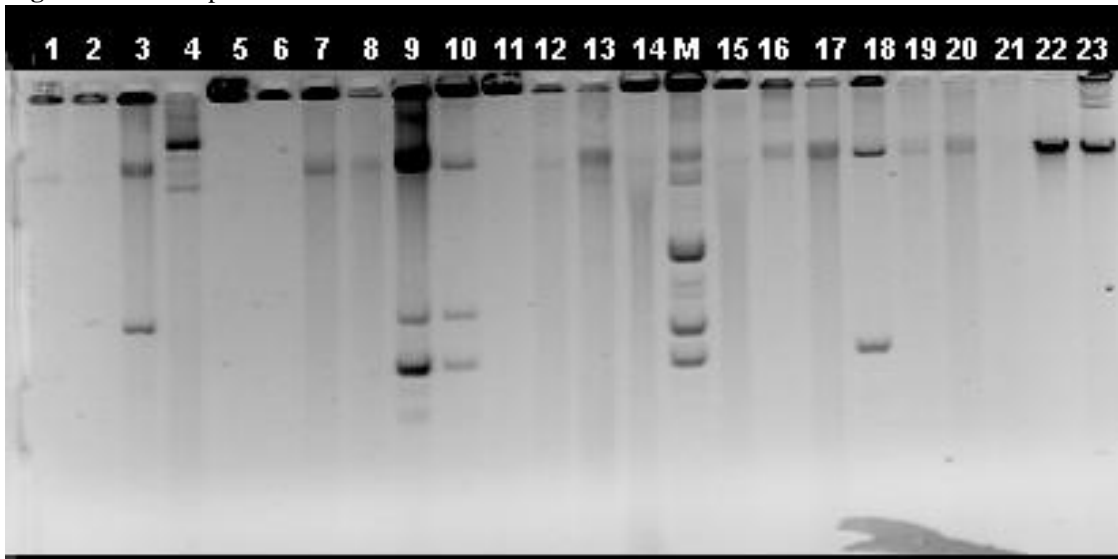
Except eight strains resistant to only one group of antimicrobial drugs (1 to nalidixic acid, 2 to streptomycin, and 5 to oxytetracycline) and three strains that were resistant to two groups of antimicrobials, the remaining 35 strains were resistant to three to seven groups of antimicrobials (Table 1), and were named as multi-drug resistant

(MDR) strains. On the basis of their resistance patterns, strains of 8 serovars could be grouped into 28 resistance groups (resistotypes). All 26 strains of *S. Weltevreden* and *S. Anatum* were MDR (Table. 1). A plasmid-free strain of *S. Anatum* was resistant to seven drugs from five different groups, including fluoroquinolones, while three strains of *S. Weltevreden* that had six (2) or seven (1) plasmids were resistant to only three (2) or four (1) antimicrobial drugs. An isolate of *S. Anatum* resistant to 11 antibiotics had only one plasmid. Drug

resistance was common among *Salmonella* against streptomycin, kanamycin, oxytetracyclin, ampicillin, and cephalexin. The study indicated that the presence or number of plasmids is not associated with resistant phenotype in *S. enterica* strains of buffalo origin.

Except for *S. Gallinarum*, most of the *Salmonella* serovars studied were of known zoonotic potential. *Salmonella* Orion, *S. Stockholm*, *S. Dublin*, *S. Typhimurium* and *S. Weltevereden* strains have long been known to cause septicemia and foodborne

Figure 1. Plasmid profiles of strains of *Salmonella enterica* serovars isolated from buffaloes.



Electrophoresed on 0.7% agarose gel, lane M contains the preparation from reference strain containing 7 plasmids of molecular weight (1.79 MDa, 2.03 MDa, 2.63 MDa, 3.39 MDa, 3.69 MDa, 5.19 MDa and 35.8 Mda). In the remaining lanes numbered with Arabic numerals, plasmid preparation of *S. Anatum* (lanes 1-13), *S. Rostock* (lanes 14-19), *S. Stockholm* (lane 20), *S. Orion* (lanes 21, 23) and *S. Dublin* (lane 22) were loaded.

Discussion

Although seven strains of *Salmonella* from buffaloes had no detectible virulence plasmid, including a heavy plasmid considered important for virulence in *Salmonella*, it can not be inferred that the strains were nonpathogenic because plasmids may either be atypical in size [20] or absent as previously reported in pathogenic *S. Paratyphi B* [21]. Furthermore, it is also possible that plasmid-free *Salmonella* strains might have virulence genes in the chromosome [22].

MDR *Salmonella* from buffalo calves and buffen are of public health importance because their zoonotic potential and ability to spread through contaminated buffalo products, direct contact, or through contamination of the environment with the excretions of sick buffaloes that can result in contaminated food and water. However, in India, there is no available report regarding the use of antimicrobial drugs in buffaloes. It is not feasible, therefore, to discuss the antimicrobial drug resistance pattern of salmonellae from buffaloes with relation to antimicrobial drug uses.

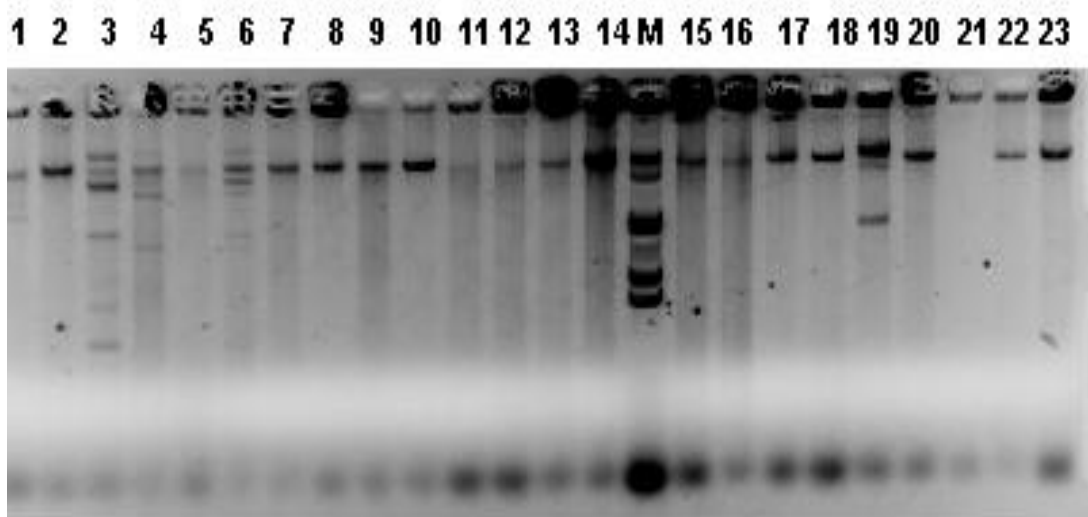
illness in humans [13,23-28]. Although *S. Anatum* is primarily considered as a duck and waterfowl pathogen causing keel disease [29], it was one of the most common isolates from buffalo calves with diarrhoea and resistant to the most number of antimicrobials among all *Salmonella* isolates of buffalo origin under study. *Salmonella* Anatum, mostly a cause of enteritis, septicemia, and arthritis in ducklings, was first recognized to cause infection in human infants in 1948 [30]. Since then it has emerged as an important zoonotic pathogen and has caused widespread outbreaks. The most devastating international *S. Anatum* outbreak was associated with infant milk formula affecting many European countries, and the causative agent was resistant to ampicillin and sulphonamides [31]. In the current study, several strains of *S. Anatum* were also resistant to ampicillin as well as sulphonamide (Co-trimoxazole).

Association of various MDR *Salmonella* serovars with buffalo calf diarrhoea might be expected as many of the *Salmonella* strains that commonly occur in India are MDR with similar resistance patterns, as

observed in the present study [3,9,12]. However, strains of serovar *S. Gallinarum* and *S. Anatum*, the two serovars primarily adapted to birds [29], are of special significance as a cause of diarrhoea in buffalo calves, but their antibiotic resistance patterns are not much different from those isolated earlier in India from birds, animals, and human patients [32-33]. Observations indicated that even host adapted *Salmonella* serovars might have some other important host or niche in ecosystem. The observations concur with earlier reports of the isolation of *S. Typhi*, primarily a human host adapted serovar, from camels [12]. Thus further studies on so-

chromosomes [21]. However, rapid spread and persistence of zoonotic salmonellosis in food animals and pet animals is associated with emerging multiple drug resistance often mediated by R-plasmids and plasmids having integrons carrying R-genes [27,36,37], but MDR strains without plasmids can not be considered less dangerous. In recent years, chromosomal genes responsible for the efflux mechanism are found to be a major cause of antimicrobial resistance in freshly isolated *Salmonella* strains, even in the absence of R-plasmid genes and integrons, and such strains might be associated with difficult-to-cure infections [26,34,

Figure 2. Plasmid profiles of strains of *Salmonella enterica* serovars isolated from buffaloes.



Electrophoresed on 0.7% agarose gel, lane M contains the preparation from reference strain containing 7 plasmids of molecular weight (1.79 MDa, 2.03 MDa, 2.63 MDa, 3.39 MDa, 3.69 MDa, 5.19 MDa and 35.8 Mda). In the remaining lanes numbered with Arabic numerals, plasmid preparation of *S. Weltevreden* (lanes 1-13), *S. Typhimurium* (lanes 14-18), *S. Gallinarum* (lanes 19-23) were loaded.

called host-adapted or host-restricted serovars may reveal the important patho-biomolecules associated with widening host adaptability in *Salmonella*.

Typical MDR resistance to streptomycin+ tetracycline+ chloramphenicol, common in India in human typhoid strains [26,34], was rare in *Salmonella* strains of buffalo origin and was detected only in one strain of *S. Anatum*. However, resistance to fluoroquinolones, though only in few strains, and resistance to nalidixic acid, are of public health concern because these are commonly used drugs for infectious diarrhoea, foodborne infections and non-typhoidal salmonellosis in humans [34] and animals [35].

Absence of a correlation between antimicrobial drug resistance and the presence of plasmids in isolates of *Salmonella* of buffalo origin might be due to the presence of drug resistance genes on

38]. Therefore, MDR strains of *Salmonella* of buffalo origin might be important for both public and personal health, as well as for epidemiologists monitoring the spread of MDR in zoonotic pathogens. Further studies on plasmid-free strains for MDR may also reveal the alternate strategy of bacteria to maintain R genes.

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