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Detection of drug-resistant *Salmonella* serovars and absence of *Listeria* in the associated organic farm environment

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ABSTRACT

The study was carried out to investigate two important foodborne pathogens, *Salmonella* and *Listeria* spp. in five organic farms in Uttarakhand state in the year 2018. The samples from soil, manure, water, and plants/plant parts were collected and screened for pathogens. The covered isolates were assayed for their antimicrobial susceptibility and the presence of representative beta-lactamase antibiotic-resistant gene (ARG) by PCR. A total of 2.2% (11/500) of samples tested positive for the genus *Salmonella*. However, none of the samples tested positive for *Listeria* spp. All the *Salmonella* isolates were recovered from environmental sources. On serotyping, 4 isolates were identified as serogroup Group C1, 3 as *Salmonella* Miyazaki, 2 as *Salmonella* Virchow and 1 each as *Salmonella Infantis* and *Salmonella* Gabon. Two *Salmonella* isolates, belonging to *S.* Group C1 and one *S.* Miyazaki isolate were pan-susceptible to all the antibiotics tested, while *S.* Virchow and *S.* Infantis showed multidrug-resistant. On PCR screening for three β-lactamase resistant genes (*bla*CTX-M-9, *bla*TEM, and *bla*AmpC), only three *S.* Miyazaki isolates amplified the *bla*CTX-M-9 gene. The study highlights the presence of MDR *Salmonella* in the organic farm environment warranting further studies in this direction to ensure safe organic farm produce for consumers.

Keywords: Antimicrobial resistance, Organic agricultural farms, *Salmonella*, Serotypes

The organic sector has shown an exponential increase worldwide owing to its environmental friendly nature ensuring better health benefits over conventional produce. Consumers prefer organic produce and quality food for their life. Such awareness has increased demand for organic produce. Despite this growing demand, organic produce yet has not been declared fully safe in terms of foodborne hazards. Under normal circumstances, the foodborne pathogens, in a food supply chain, can contaminate, persist, and amplify at any point of production to consumption with sources like soil, irrigation water, inadequately composted raw animal manure, insects, presence of wild and domestic animals including inappropriate human handling (Strawn *et al*. 2013, Maffei *et al*. 2016). Further, such contaminated agriculture produce at the pre-harvest stage does not undergo any kind of sanitation or 'kill step' to stop the pathogens from entering into the fresh produce or their products (Mogren *et al.* 2018).

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Numerous foodborne outbreaks have been reported with *Listeria* and *Salmonella* amidst a varied number of food pathogens. Data from Integrated Disease Surveillance Programme (IDSP) and Open Government Data Platform India (OGDPI) reported 2688 food borne outbreaks between 2009 and 2018. The numbers however, may increase as true outbreaks may go unreported (Bisht *et al*. 2021). The major contributing factor for the foodborne outbreak is the raw consumption of the yield commodities (fruits and vegetables) or with minimal processing (cereals, pulses) (Strawn *et al*. 2013). *Listeria* outbreaks have been associated with the ready to eat foods, freshly cut fruits (Oliveira *et al*. 2014) and fresh-cut vegetables (Vandamme *et al*. 2013) from agricultural farms produce. Similarly, Salmonellae have also been isolated from fruits and vegetables (Baishakhi *et al.* 2020).

Since reports are citing the presence of the foodborne pathogen in farm produce and food safety being an important health issue, the study was undertaken to study the occurrence of *Listeria* and *Salmonella* species from organic farms of Tarai region of Uttarakhand, which has entered into the organic farming, mostly as an unorganized sector. The present study was envisaged to ensure the microbial safety of such products. This study aimed to detect, identify and monitor antibiotic and MDR resistant *Salmonella* and *Listeria* present in organic farm produce.

MATERIALS AND METHODS

Sample collection: The study was conducted in five different organic farms listed in Table 1.

All the environmental samples (25 g soil and manure; 50 ml water) were collected in 100 ml sterile sample containers (Abdos, WI bags India) while the plant samples were aseptically collected in 100 ml sterile WhirlPak (Nasco, FortAtkinson). Soil samples were collected from 5-10 cm below the surface of the field, apparently from 5 locations, i.e. the four corners and one from the center of the field. Water samples were collected from the source itself (borewell and/or stream) and if available, the water accumulated in the field. Manure samples were collected from the place of its formation as compost and the place of storage before being applied to the fields. Whole plants (5 from a field) of rice/tomato/soybean (subject to availability) were collected. All the samples were collected aseptically and brought to the laboratory under cold conditions and processed at the earliest possible.

Sample preparation: The environmental samples (soil, manure, and water) were used as such while the plant samples (roots, leaves/grains) were processed for isolation of *Salmonella* and *Listeria* as described by Araujo *et al*. (2000).

Isolation and identification of Salmonella: The isolation of *Salmonella* spp. was performed as described by Singh *et al*. 2014. All presumptive *Salmonella* isolates tested positive on TSI and negative on urea slants were subjected to DNA isolation using the snap chill method (Swetha *et al*. 2015). A simplex PCR targeting the *inv*A gene (284 bp) was employed for the identification of the genus *Salmonella* (Rahn *et al.* 1992).

Isolation and Identification of Listeria: The isolation of *Listeria* spp. was attempted as per the US Department of Agriculture (USDA) method described by Mcclain and Lee (1988) with suitable modifications. Briefly, 1ml sample added in 9 ml of the University of Vermont I broth (UVM I) (1:9) incubated at 37^0C for 24 h were enriched in UVM II broth (0.1 ml of pre-enriched sample to 10 ml of UVM II). Thereafter, a loop full of the enriched sample was plated onto selective medium, i.e. Polymixin Acriflavin Lithium Chloride Ceftazidime Aesculin Mannitol (PALCAM) agar. The greenish-yellow glistening, iridescent and pointed colonies surrounded by a diffuse black zone of aesculin hydrolysis were suspected of listeriae. They were checked for motility and catalase test. The catalase-positive isolates with tumbling motility at room temperature were further subjected to DNA isolation using the snap chill method (Swetha *et al*. 2015).

A simplex PCR targeting, the *prs* gene (370 bp) was employed for identification and confirmation of the genus *Listeria* (Callejo *et al.* 2008).

The amplified PCR products (10 µl) of *Salmonella* and *Listeria* genus were electrophoresed on agarose gel (1.5%) at 85V for 1 h and visualized in a gel documentation system (AlphaImager^{R}HP, Alpha Innotech Corp., California, U.S.A.).

Serotyping of Salmonella isolates: The serotyping

Table 1. Details of samples collected

Organic farm location	Soil	Manure	Water	Rhizosphere	Roots	Leaves/ grains	Total
Pantnagar 1	89	17	15	5	5	5	136
Pantnagar 2	22	15	13	10	10	10	80
Kotabagh	43	12	19	20	20	20	134
Dhamola	37	15	6	5	5	5	73
Ramnagar	36	7	4	10	10	10	77
Total	227	66	57	50	50	50	500

Table 2. Details of the primers for antibiotic resistance genes (ARGs), amplicon size, and PCR cycling conditions

Name of antibiotic resistance gene (amplicon size)	Primer sequence	PCR cycling conditions
*bla*AmpC (346 bp)	F - CACCTCCAGCGACTTGTTAC R - GTTAGCCAGCATCACGATCC	Initial Denaturation – 95°C, 5 min Denaturation – 95°C, 30 sec Annealing – 52°C, 1 min (30 cycles) Extension – 72°C, 1 min Final Extension – 72°C, 7 min
*bla*CTX-M-9 (561 bp)	F - TCAAGCCTGCCGATCTGGT R –TGATTCTCGCCGCTGAAG	Initial Denaturation – 95°C, 5 min Denaturation – 95°C, 30 sec Annealing – 52°C, 1 min (35 cycles) Extension – 72°C, 1 min Final Extension – 7 min
*bla*TEM (800 bp)	F - CATTTCCGTGTCGCCCTTATTC R - CGTTCATCCATAGTTGCCTGAC	Initial Denaturation – 95°C, 5 min Denaturation – 95°C, 30 sec Annealing – 52°C, 1 min (35 cycles) Extension – 72°C, 1 min Final Extension – 72°C, 7 min

F, Forward; R, Reverse.

of the confirmed *Salmonella* isolates was done at National *Salmonella* Centre (NSC), ICAR-Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly, Uttar Pradesh, India.

Antibiotic susceptibility testing (AST): Antimicrobial resistance (AMR) profile of all *Salmonella* isolates was prepared by using standard Kirby-Bauer disc diffusion method (Bauer *et al*.1966). Six different classes of antibiotics, comprising eleven antimicrobial agents, namely ampicillin/cloxacillin (AX) 10 µg, cefazolin (CZ) 30 µg, cefotaxime (CTX) 30 µg, cefoxitin (CX) 30 µg, ciprofloxacin (CIP) 5 µg, gatifloxacin (GAT) 5 µg, levofloxacin (LE) 5 µg, nalidixic acid (NA) 30 µg, streptomycin (S) 10 µg, sulfisoxazole (SF) 300 µg and tetracycline (TE) 30 µg were used. The results were interpreted as per Clinical and Laboratory Standards Institute for disk-diffusion assay (CLSI 2018), using ATCC25922 *Escherichia coli* as a reference strain. The isolates showing resistance to three or more classes of antimicrobials were termed as multidrug-resistant (MDR) (Micek *et al,* 2015).

Detection of antimicrobial resistance genes (ARGs): All isolates (n=8) displaying phenotypic resistance against β lactam antibiotics were further screened for the presence of β-lactamase resistance genes (*bla*TEM, *bla*CTX-M-9*,* and *bla*AmpC) (ICMR 2015). The details of the primers and their PCR cycling condition are presented in Table 2.

RESULTS AND DISCUSSION

Detection of Salmonella: A total of 2.2% (11/500) samples were found positive for genus *Salmonella*. All these 11 *Salmonella* isolates were recovered from environmental sources (3.14%; 11/350). None of the plant samples were found positive for *Salmonella* (Table 3).

Table 3. Detection of *Salmonella* in plant and environmental samples

Sample	Source	Total samples	Isolates obtained
Environmental	Soil	227	3(1.32%)
	Manure	66	3(4.5%)
	Water	57	5(8.7%)
	Total	350	11(3.14%)
Plant	Rhizosphere	50	0(0.0)
	Roots	50	0(0.0)
	Leaves/grains	50	0(0.0)
	Total	150	0(0.0)
	Overall total	500	11(2.2)

The highest presence was found in water followed by 3 each from manure and soil samples, respectively.

The recovery of *Salmonella* isolates was highest at Ramnagar farm (6.4%) followed by Pantnagar farm-2 (3.7%) and Pantnagar farm-1 (2.2%), respectively. The Kotabagh and Dhamola farms did not yield *Salmonella* isolates (Table 4). Overall, a very low (2.2%) detection rate of foodborne pathogens {*Salmonella* (n=11) and *Listeria* (n=0)} was observed in the organic farms that were investigated. Few studies conducted on the farm produce have also reported a similar detection rate of foodborne pathogens. Denis and his co-workers have recorded an overall low prevalence (0.08% in tomatoes and 1.30% in leafy herbs) of bacterial pathogens (*Salmonella, Listeria monocytogenes, Shigella, E. coli* O157:H7*, Campylobacter,* and *E. coli*) in farm products of Canada (Denis *et al*. 2016). On the contrary, studies that have compared the organic and conventional farms, have reported a higher presence of micro-organisms in organic than conventional farms, justifying that the use of animal manure as a fertilizer might have contributed to higher microbial load in the organic farm produce (Oliveira *et al.* 2010). However, any universal consensus has not been made on the quality of one being better than the other except that both types of farms are reportedly not completely safe (Kuan *et al.* 2017).

Table 4. Serotyping results of *Salmonella* isolates

Isolate ID	Source	Antigen	Serotypes
S 15	Water	6,7	*S.* (Group C1)
S 16	Water	6,7:1, w:1, 2	*S.* Gabon
S 17	Water	6,7	*S.* (Group C1)
S 18	Soil	6,7	*S.* (Group C1)
S 19	Soil	6,7: r:1, 2	*S.* Virchow
S 20	Soil	6,7	*S.* (Group C1)
S 21	Manure	9,12:1, Z_{13}:1,7	*S.* Miyazaki
S 22	Manure	9,12:1, Z_{13}:1,7	*S.* Miyazaki
S 23	Manure	9,12:1, Z_{13}:1,7	*S.* Miyazaki
S 24	Water	6,7:r:1, 2	*S.* Virchow
S 25	Water	6,7:r:1, 5	*S.* Infantis

The presence and survival of *Salmonella* in the environment, water, soil**,** and manure (Pandey *et al.* 2018) has been extensively explored. Its survival is also influenced by temperature, soil type, and the presence of protozoa (Jacobsen and Bech 2012). The major *Salmonella* serotypes reported from organic and conventional farms including environment and farm produce were *Salmonella* Enteritidis (Kuan *et al*. 2017) and *Salmonella* Typhimurium (Peng *et al*. 2016). However, in the present study, we highlight the presence of two important serovars (*S*. Gabon and *S.* Miyazaki) for the first time in the organic farms. The organic farms in this study used cattle manure (composted) as a fertilizer. The source of contamination may be the manure used (Rohinishree *et al.* 2016) faeces of birds, domestic animals, wild animals, reptiles, etc. which may affect the quality of the environment and the produce (Hilbert *et al*. 2012).

Identification of Salmonella serotype: The serotyping details of *Salmonella* isolates (n=11) recovered from different sources are presented in Table 5. Of the 11 *Salmonella* isolates, 4 isolates (36.36%, 4/11) were typed as serogroup Group C1, 3 (27.27%, 3/11) were identified as *Salmonella* Miyazaki, 2 (18.18%, 2/11) as *Salmonella* Virchow, 1 (9.09%, 1/11) as *Salmonella* Infantis, and 1 (9.09%, 1/11) as *Salmonella* Gabon, respectively (Table 5).

According to the CDC, serotyping forms the basis of public health monitoring of *Salmonella* infections (CDC

Table 5. Antibiotic sensitivity profile and detection of representative β-lactamase resistant genes in the recovered *Salmonella* serotypes

ID	Species	Source	Resistance Pattern	Resistant to number of antibiotics	Resistant to number of classes of antibiotics	Antibiotic-resistant genes		
						blaTEM	blaCTX-M-9	blaAmpC
S15	*S.* (Group C1)	Water	CZ, CX, CTX	3	1	-	-	-
S16	*S.* Gabon	Water	CTX	1	1	-	-	-
S17	*S.* (Group C1)	Water	Susceptible	0	-	-	-	-
S18	*S.* (Group C1)	Soil	Susceptible	0	-	-	-	-
S19	*S.* Virchow	Soil	CIP, CZ, CX, CTX	4	2	-	-	-
S20	*S.* (Group C1)	Soil	CIP, CTX	2	2	-	-	-
S21	*S.* Miyazaki	Manure	Susceptible	0	-	-	+	-
S22	*S.* Miyazaki	Manure	CIP, CTX	2	2	-	+	-
S23	*S.* Miyazaki	Manure	CZ, CTX	2	1	-	+	-
S24	*S.* Virchow	Water	S, NA, CIP, CZ, CTX, TE, SF	7	5	-	-	-
S25	*S.* Infantis	Water	S, NA, CIP, GAT, CX, CTX, TE, SF	8	5	-	-	-

S, streptomycin (10 µg); NA, nalidixic acid (30 µg); CIP, ciprofloxacin (5 µg); GAT, gatifloxacin (5 µg); CX, cefoxitin (30 µg); CTX, cefotaxime (30 µg); TE, tetracycline (30 µg); SF, sulfisoxazole (300 µg).

2015). The four isolates belonging to *Salmonella* serogroup C1 could not be further serotyped and therefore were referred to as C1 in further content. The serogroup has reportedly no isolations from an organic farm particularly but holds high significance as is reported frequently from animals. It has been reviewed as a predominant serotype in dairy herds (Habing *et al*. 2012) with isolations also confirmed in humans (Kagirita *et al*. 2017) and agricultural produce (Orozco *et al.* 2008). Members of this serogroup are also observed to have high pathogenicity. Fuche *et al*. (2016) have shown serogroup C to be predominantly responsible for the NTS burden in the United States and European countries suggesting high relevance of this serogroup. In this study, two isolates of *Salmonella* Virchow (water and soil) and one isolate were of *Salmonella* Infantis (water) were recovered from the farm environment under study. Both serovars also belong to *Salmonella* Group C1. Earlier, recovery of *Salmonella* Infantis isolates has been reported from pigs and pork samples (Campos *et al*. 2019). Also, the isolation of *Salmonella* Infantis has been was reported from various animal sources with major confirmation from the poultry along with cattle, equines, sheep, and goats (Gelaw *et al.* 2018). Concerning *Salmonella* Virchow, it has been classified as a public health pathogen and is normally observed as a poultry-related serotype and due to its predominant presence in the poultry; it serves as a target serovar for breeding flock control programs (EFSA 2018). The presence of these organisms in the environmental samples in the present study might be attributable to the nearby animal dwellings.

In this study, the isolation of *Salmonella* Miyazaki from the farm manure sample has its significance as this appears to be the first report of its isolation since 1972 from anywhere in the world. Sasahara and Fukuda (1972) reported isolation of this serotype (*S*. Miyazaki) in 1966 from a patient showing symptoms of dysentery. This infection was traced back to the faeces of dogs as they isolated the same serotype from one of the lumps of the dung of dogs. Since then, except for these cases, no reports of its isolation have been heard from Japan or any other country.

Another interesting observation in this study was the detection of *S.* Gabon from water samples. A single presence of *S*. Gabon in the samples is suspected to be of a reptile origin as *S.* Gabon was first isolated from the faeces of a Gaboon viper and hence was named as *S*. Gabon (McWhorter *et al*. 1966). Recently, *S*. Gabon along with *Yersinia enterocolitica* was found to be the reason for the fever and abdominal pain in a patient which was later diagnosed with mesenteric lymphadenitis (Ogata *et al*. 2018). Apart from this, there have been no reports about the incidences or prevalence of this serovar. The intrusion of wild animals into the crop field may also introduce pathogens in the farm environment which, in turn, might serve as a potential source of pre or post-harvest contamination (VT Nair *et al.* 2018). The presence of these *Salmonella* enteric serovars in the environmental samples of the organic farm holds high public health significance as these enteric pathogens can enter the farm produce through migration.

Detection of Listeria spp.: None of the samples collected yield *Listeria* spp. isolates. Although soil provides the environmental niche for the *Listeria* organisms but the edaphic factors like soil composition, *p*H, microbial communities and macrofauna also direct the fate of *L. monocytogenes* in the soil environment, in addition to the farming practices (Vivant *et al.* 2013). As Uttarakhand comprises majorly hilly areas having different ecology than the plains, variation in the type of food pathogen occurrence may vary. Thus, survival of pathogens can be an issue, and it could be taken up for/in future studies.

Antibiotic susceptibility testing of Salmonella isolates and detection of β-lactamase resistant genes: The result of antibiotic susceptibility towards antibiotics for the

11 recovered *Salmonella* is presented in Table 5.

Of the 11 recovered *Salmonella* isolates, the β-lactamase resistant gene namely *bla*CTX-M-9 was detected only in the three *S.* Miyazaki isolates, of which two were also revealing phenotypic resistance (Table 5). None of the isolates was positive for *bla*TEM and *bla*AmpC genes. The two *Salmonella* serovars, *S.* Virchow and *S.* Infantis isolated from water samples exhibited a multidrug resistance profile. Also, all three *S*. Miyazaki isolates harbored the *bla*CTX-M-9 gene which is commonly associated with the hydrolysis cefotaxime drug. Generally, CTX-M ESBL genes are horizontally transferred via plasmids and/or transposons (VT Nair *et al*. 2018) and its presence in the recovered *S*. Miyazaki isolates can be a real threat to the organic produce.

This study highlights the presence of two rare serotypes of *Salmonella*, *S*. Gabon, and *S*. Miyazaki from organic farm plants and its environment. To the best of our knowledge, this is the first report of their isolation from the organic farms globally and also from India. *Listeria* was absent in all the samples tested while *Salmonella* serotypes were recovered from the soil, manure, and water samples of the farms. The recovered serovars majorly belonged to the *Salmonella* C group (C1, Infantis, and Virchow) whose members are known to cause invasive salmonellosis. Two isolates were found to be multidrug-resistant while three isolates of *S.* Miyazaki harbored *bla*CTX-M-9*,* β-lactamase resistant genes. The study highlights that though no plant samples were positive for either of the foodborne pathogens, their presence in the environment may serve as an important contributing factor to contaminate the organic produce during pre-or post-harvesting processes. Thus, to reach food safety goals in the 'Go Organic' era, the role of the environment and wild animals needs the utmost attention. Also, future studies in these directions are warranted to recommend strategies to improve food safety.

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