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RESEARCH ARTICLE

PREVALENCE AND ANTIBIOTIC RESISTANCE OF *BACILLUS* STRAINS ISOLATED FROM VARIOUS FOOD STUFFS.

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ABSTRACT

The present study was aimed to explore the prevalence of *Bacillus* spp. in some commonly offered foodstuffs and to assess the antibiotic resistance and susceptibility profile of these isolated bacterial contaminants. A total of fifty-seven food samples were collected comprising of different types of foods from street vendors, restaurants, kitchens, ready-to-eat packed foods and mid-day meals from different locations of NCR and Delhi, India. These samples were subjected to the isolation of *Bacillus* strains. Various biochemical analysis were done for the identification of *Bacillus* spp. Out of fifty-seven samples evaluated, twenty-six morphologically distinct isolates were obtained. All the bacterial isolates were then evaluated for their antibiotic resistance and sensitivity against twenty commonly prescribed and commercially available antibiotics. A greater degree of variability was observed in resistance profile of isolated *Bacillus* strains. Some of the isolates were found to be 100% susceptible against a few of antibiotics such as ciprofloxacin, gentamycin, meropenem, doxycycline, leavofloxacin and gatifloxacin. Out of the selected antibiotics, some of them were observed to have moderate-to-severe antibacterial effectiveness against the isolated *Bacillus* strains. Only one species of *Bacillus* was found to have maximum MAR value i.e. 0.55; however, the least resistance was found in two isolates. Other organisms were found to be fragile within the range of 0.15 to 0.45 showing the variable sensitivities against the antibiotics used in the study.

KEYWORDS: Foodstuffs, *Bacillus,* Antibiotic, Resistance, Susceptibility, MAR Index

INTRODUCTION:

Foods are associated with a number of microbial contaminations among which different strains of *Bacillus* are responsible for various food-borne illnesses. The *Bacillus* genus is a heterogeneous group of Gram-positive, facultative anaerobic, endospore-forming bacteria and are widely distributed in nature, and also frequently associated with a multiplicity of food products such as milk and dairy products, meat and meat products, rice, pasta, and dried products such as spices. The ability to produce endospores allows *Bacillus* to withstand extreme environmental conditions as those occurring during the food processing. *Bacillus* spp., particularly *B. subtilis*, are usually found in foods such as dry cured sausages, cheeses, traditional fermented milks, sourdough, etc. in which they cooperate with other microorganisms during fermentation, releasing amylases, lipases and proteases. Traditionally these microorganisms have been associated with the spoilage of food products; however, recently they have been linked to potential food poisoning and issues pertaining to the emergence of resistance against the commonly prescribed antimicrobial used to treat various infectious diseases.

Foodstuffs can easily be targeted for microbial spoilage due to cross contamination from various sources such as utensils, knives, raw foodstuffs, flies that are sporadically landing on the foods, by vendors' bare hand serving occasionally, food handling by consumers [1, 2]. Ready-to-eat foods (street food) are processed (peeled, squeezed, cut up and/or cooked) and readily available for purchase and consumption. However, street foods have been implicated in the transmission of food-borne disease [3-5] Food-borne illness is a major international health problem and an important cause of reduced economic growth [6]. Food-borne illness of microbial origin is major cause of death in developing Countries [7, 8]. The problems of food safety in the industrialized world differ considerably from those faced by developing Countries. Whereas, in developing countries traditional methods of processing and packaging, improper holding temperature, poor personal hygiene of food handlers are still observed during food marketing and technology [9].

Antibiotics were first introduced for the treatment of microbial diseases. Since then, the greatest threat to the use of antimicrobial agents for therapy of bacterial infections has been the development of antimicrobial

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resistance in pathogenic bacteria. Antibiotic resistance has been shown to have occurred rarely in bacteria collected before the antibiotic era [10]. Shortly after the introduction of each new antimicrobial compound, emergence of antimicrobial resistance is observed [11]. The magnitude of the problem is significantly increased by the possibility of bacteria to transfer resistance determinants horizontally and by the mounting increase in the use (over-use and misuse) of antibiotics, which has created an enormous selective pressure towards resistant bacteria [12]. It has also been concluded that gene transfer occurs widely in vivo between gastrointestinal tract bacteria, and between gastrointestinal tract bacteria and pathogenic bacteria [13]. The number of antimicrobial-resistant (AMR) bacteria in the environment increases exponentially with the use of antimicrobials, as a result of increasing selective pressure on bacterial populations [14-16] and its spread between different bacterial strains in different habitats has also been demonstrated [17-19].

Food contamination with antibiotic resistant bacteria can be a major threat to public health, as the antibiotic resistance determinants can be transferred to other pathogenic bacteria potentially compromising the treatment of severe bacterial infections. The prevalence of antimicrobial resistance among food borne pathogens has increased during recent decades [20-25]. Recently many investigators have speculated that commensal bacteria may act as reservoirs of antibiotic resistance genes similar to those found in human pathogens [26, 27] and are thus very important in our understanding of how antibiotic resistance genes are maintained and spread through bacterial populations [28]. The main threat associated with these bacteria is that they can transfer resistance genes to pathogenic bacteria. Such reservoir organisms could possibly be found in various foods and food products containing high densities of non-pathogenic bacteria as a result of their natural production process [29-32].

In the present investigation, prevalence and antibiotic resistance of various strains of *Bacillus isolated* from different foodstuffs *was studied*. This is a novel study and comprises the following aspects: (a) Collection of food samples from different locations of NCR, India; (b) Isolation and identification of *Bacillus* strains; (c) Determination of susceptibility and resistance pattern against different antibiotics; (d) Determination of multiple antibiotic resistance (MAR); and (e) Interpretation of the data generated which will have a greater impact in determining the pervasiveness of resistance among microorganisms isolated from foodstuffs.

MATERIALS AND METHODS:

CHEMICALS, REAGENT AND BACTERIOLOGICAL MEDIA:

Various media and reagents used throughout the study include Mueller Hinton Agar (MHA), Nutrient Agar (NA), Mannitol Yolk Polymixin-B agar (MYP), Buffered Peptone Water (BPW), Polymixin B Supplements, normal saline, Kovac's reagent, Voges-Proskauer reagent, Hydrogen peroxide, Ethanol etc. were of analytical grade and procured from Hi-Media, Mumbai and Sigma Laboratories, India.

COLLECTION OF FOOD SAMPLES:

Wide mouth PET jars (sterilized by gamma-radiation) were used for sampling of different foodstuffs. The lid of the jar was removed by maintaining aseptic conditions. The samples were kept in an ice pack to prevent any changes in the microbial flora of the samples. The samples of food were transported in vertical position maintaining the temperature 1-4°C with ice pack enveloped conditions to the Microbiology lab for analysis. Microbiological analysis was started within 6 hrs of collection.

ISOLATION OF *BACILLUS* SPP:

For the detection of *Bacillus* spp., 25 g homogenized sample was diluted with 225 ml of Buffer Peptone Water (BPW) and then incubated at 37^{o}C for 48 hrs. Subcultured on the plates of Mannitol Yolk Polymixin Agar (MYPA) and further confirmation was done by biochemical test as per Indian Standards [33].

IDENTIFICATION OF *BACILLUS SPP.* BY BIOCHEMICAL TEST:

Isolated microbes were identified as *Bacillus* spp. biochemically by using several analytical methods as per the guidelines of Indian Standards [33]. These biochemical tests include (a) Glucose agar test, (b) Nitrate test, (c) Voges Proskauer test, (d) Catalase test, (e) Skim milk agar test, (f) Mannitol test, (g) Xylose test, (h) Indole test, (i) Citrate test, (j) Starch agar test, (k) Growth at 30^{o}C, (l) Growth at 44^{o}C and (m) Growth at 4^{o}C.

ANTIBIOTICS AND THEIR SOLUTIONS:

Twenty commonly prescribed clinically significant antibiotics i.e. azithromycin, norfloxacin, ciprofloxacin, ofloxacin, amplicillin, amoxicillin, streptomycin, cefixime, tetracycline, gentamycin, meropenem, metronidazole, cloxacillin, doxycillin, vancomycin, rifampicin, chloramphenicol, leavofloxacin, gatifloxacin, and erythromycin were used to evaluate the susceptibility and resistance pattern of *Bacillus* spp.. All these antibiotics

were obtained from local pharmacy store and they were used in 10µg/ml concentration against *Bacillus* isolates.

INOCULUM PREPARATION:

All *Bacillus* isolates were sub cultured on non selective nutrient agar slants. The bacterial cultures were incubated overnight at 37°C. 0.5 McFarland density of bacterial isolates was adjusted using normal saline (0.85% NaCl) using densitometer to get bacterial population of 1.0 x 10^8 cfu/ml.

AGAR WELL DIFFUSION ASSAY (ZONE OF INHIBITION EVALUATION):

Antibiotic susceptibility and resistance were evaluated by agar well diffusion assay [34-36]. 100µl of each of the adjusted cultures were mixed into separate 100 ml of sterile, molten, cool MHA, mixed well and poured into sterile petri plates. These were allowed to solidify and then individual plates were marked for each individual *Bacillus* isolates. Each plate was punched to make wells of 6 mm diameter with the help of sterile cork borer at different sites of the plates. 100 µl of respective antibiotic solutions were pipette into the well in assay plates. Plates were incubated overnight at 37°C. Following incubation, petri-plates were observed for the inhibition zones, diameters of which were measured by using Vernier Calipers.

RESULTS:

The results of present study are summarized in Table 1 in which susceptibility and resistance patterns of *Bacillus* isolates against selected antibiotics were shown. A total of fifty-seven food samples were collected from different locations from NCR, India. These samples were further microbiologically analyzed and twenty-six *Bacillus* isolates were morphologically and biochemically identified. These isolates were then evaluated for their resistance and susceptibility patterns against twenty commonly prescribed clinically significant antibiotics.

In the current study, results were found to be very promising as tetracycline was found to be completely ineffective (as no any zone of inhibitions were observed against any of the isolates). Data revealed that *Bacillus* isolates were found to have variable sensitivities against the antibiotics used in the study. Susceptibility patterns of these isolates against evaluated antibiotics have been shown in Figure 1. Further characterization of these isolates representing the percentage value of resistant and susceptible *Bacillus* was shown in Table 2. Metronidazole was another unproductive antibiotic and as only one isolate was having the susceptibility against this antibiotic. *Bacillus* isolates were found to have completely susceptible (100%) against a few of antibiotics such as ciprofloxacin, gentamycin, meropenem, doxycycline, leavofloxacin and gatifloxacin because of significant inhibitions as observed in agar well diffusion assay. Out of the selected antibiotics, some of them were observed to have moderate-to-severe antibacterial effectiveness against the isolated *Bacillus* strains. These antibiotics were streptomycin (96.15%), ofloxacin (92.30%), cefixime (92.30%), vancomycin (92.30%), norfloxacin (88.46%), rifampicin (84.61%), and erythromycin (76.92%). Several antibiotics such as azithromycin (69.23%), cloxacillin (50.00%), amplicillin (42.30%), amoxicillin (42.30%), and chloramphenicol (42.30%) were observed as mild-to-moderate while evaluating their efficiency against the *Bacillus* isolates.

Multiple antibiotic resistances (MAR) index were calculated on the basis of susceptibility and resistance patterns of bacterial isolates and were shown in Table 3. It has been observed that all the isolates were having a sort of susceptibility on the scale of 0-1 and none of *Bacillus* isolate was found to be 100% resistant against the evaluated antibiotics. One of the *Bacillus* sp. isolated from sample (S-55) was found to have maximum MAR value i.e. 0.55; however, the least resistance was found in two isolates viz. S-21 and S-48. Other organisms were found to be fragile within the range of 0.15 to 0.45 (Figure-2) showing the variable sensitivities against the antibiotics used in the study.

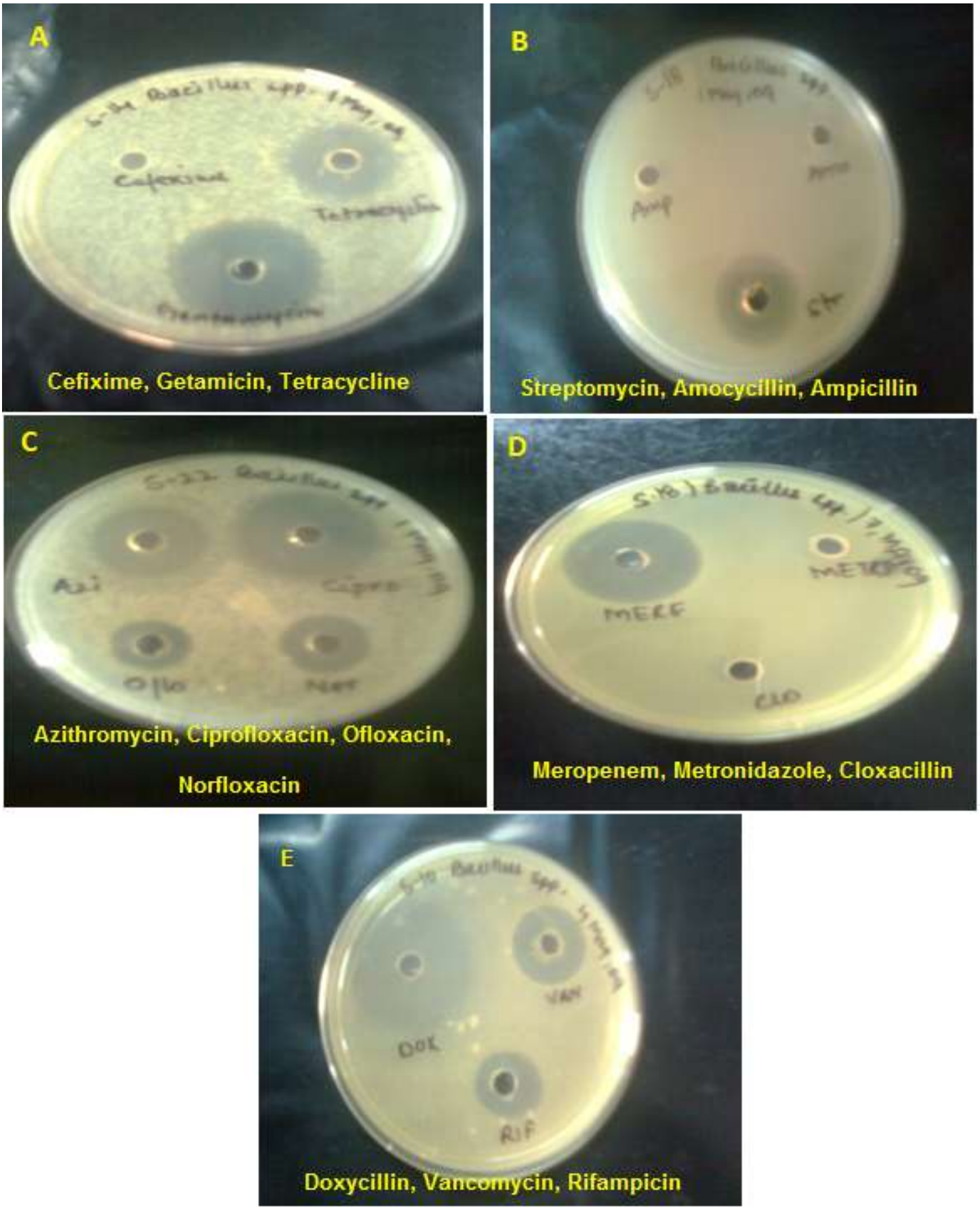

Figure 1: Zone(s) of Inhibition of different antibiotics against *Bacillus spp.*

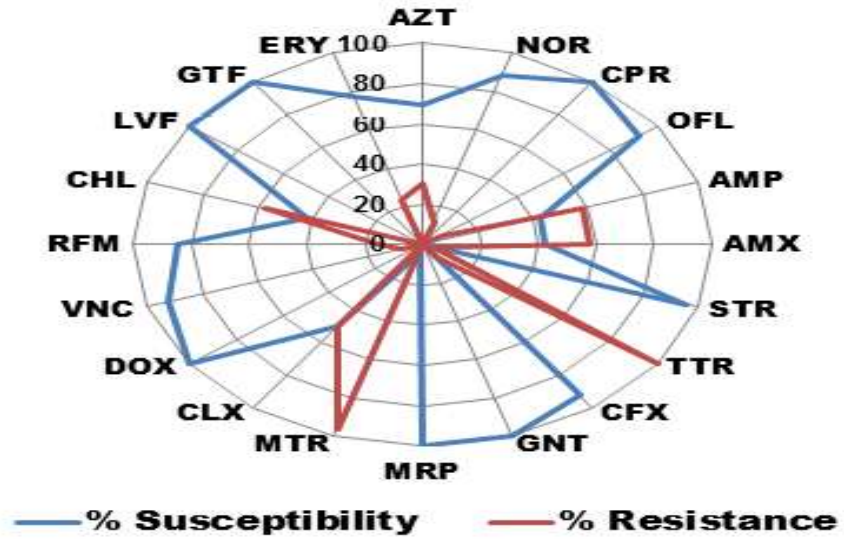

Figure 2: % Resistant and Susceptible Bacillus spp. against various Antibiotics

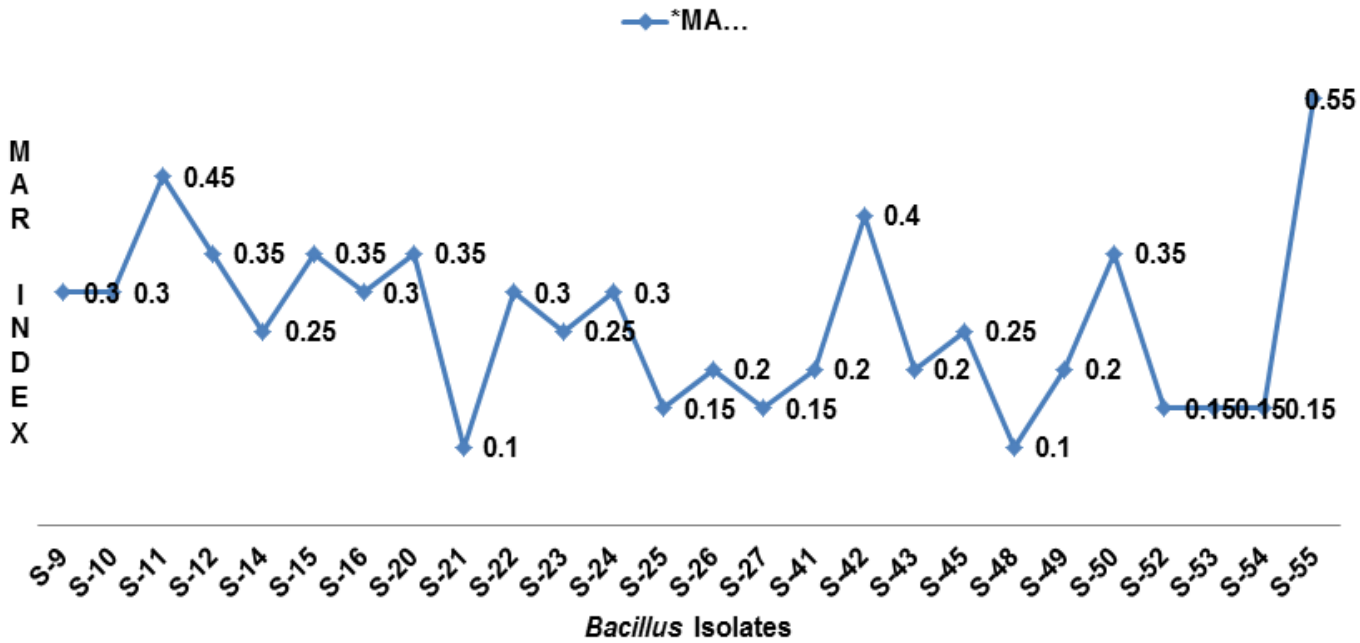

Figure 3: Multiple Antibiotic Resistance (MAR) Index of various *Bacillus* Isolates

Table 1: Zone of Inhibition of different antibiotics against *Bacillus* spp. isolated from different food samples

BACILLUS ISOLATES	AZT	NOR	CPR	OFL	AMP	AMX	STR	TTR	CFX	GNT	MRP	MTR	CLX	DOX	VNC	RFM	CHL	LVF	GTF	ERY
S-9	13.91	22.63	27.15	22.14	0	0	17.42	0	18.55	11.36	17.54	0	0	25.98	17.42	14.49	0	18.45	20.37	18.65
S-10	17.95	24.58	30.81	26.34	0	0	20.41	0	0	15.61	24.41	0	25.37	30.95	20.03	15	0	20.54	22.58	20.31
S-11	0	23.01	27.95	22.79	0	0	19.08	0	20.4	11.98	29.13	0	0	28.19	14.44	0	0	16.45	14.13	0
S-12	17.4	12.49	22.33	14.37	0	0	18.08	0	17.4	24.04	27.52	0	0	28.64	13.63	12.15	0	19.97	18.76	0
S-14	17.38	12.01	22.7	10.74	0	0	20.77	0	18.14	20.28	18.67	0	0	27.73	16.16	21.82	13.91	16.93	18.28	17.7
S-15	17.51	12.28	21.72	11.31	0	0	20.29	0	17.93	20.67	20.62	0	0	28.94	15.92	12.61	0	19.21	17.46	0
S-16	15.64	14.12	19.32	11.79	0	0	19.11	0	17.72	20.2	25.76	0	0	28.38	14.68	12.05	12.05	19.49	17.95	0
S-20	17.97	13.01	21.75	11.94	0	0	22.9	0	17.4	22.66	30.62	0	0	28.07	16.16	14.21	0	20.95	18.74	0

S-21	16.93	17.22	34.69	21.71	22.62	22.7	20.59	0	12.48	17.71	37.62	0	24.65	27.29	20.82	14.98	17.72	28.93	29.17	25.89
S-22	20.78	16.91	26.14	14.17	0	0	21.72	0	12.83	19.83	28.62	0	0	19.3	14.75	11.7	0	12.46	20.9	19.08
S-23	20.8	16.3	23.71	13.43	0	0	21.75	0	15.42	20.48	27.97	0	23.2	33.26	19.77	16.28	0	26.12	25.22	16.13
S-24	15.35	21.21	30.77	23.69	0	0	20.77	0	0	18.09	27.41	0	24.05	28.79	19.83	16.93	0	27.17	25.75	15.75
S-25	24.64	13.46	30.41	18.24	25.85	23.79	25.19	0	20.11	26.84	27.28	0	26.13	34.6	19.99	14.46	0	25.66	26.11	19.44
S-26	26.6	0	29.57	16.86	25.08	29.15	26.19	0	20.43	26.46	26.67	0	26.27	33.42	20.14	16.94	0	26.28	26.26	21.93
S-27	14.43	18.13	26.92	18.38	22.55	24.1	14.78	0	23.49	22.2	31.66	0	11.64	30.12	14.69	0	15.85	19.58	25.11	23.1
S-41	0	18.64	31.28	19.39	34.46	30.85	0	0	24.13	19.68	26.99	0	24.95	32.29	12.76	15.4	16.74	22.17	25.92	21.02
S-42	0	0	20.14	0	0	0	13.23	0	16.73	9.44	16.24	0	0	19.22	9.47	10.8	14.23	14.06	14.38	9.7
S-43	0	16.22	15.79	17.1	19.9	18.39	19.84	0	26.02	19.96	22.72	0	18.61	28.15	12.59	19.14	0	20.44	22.95	9.45
S-45	24.83	12.24	20.92	13.56	0	0	15.03	0	19.07	19.71	22.37	0	0	19.31	23.6	16.2	19.88	19.01	21.24	27.15
S-48	0	17.48	29.78	17.55	27.01	25.11	18.52	0	23.85	17.47	27.57	16.03	23.73	34.71	15.85	26.69	17.65	28.58	24.64	23.3
S-49	0	14.57	23.27	10.95	14.46	13.44	18.89	0	23.09	13.65	26.69	0	0	24.65	13.51	14.66	14.84	14.75	17.33	22.93
S-50	0	9.55	21.25	10.37	0	0	14.71	0	17.21	9.64	16.95	0	0	20.35	9.91	10.83	0	11.64	13.75	9.98
S-52	17.18	18.18	21.16	15.01	25.72	24.84	17.22	0	22.99	20.81	29.38	0	24.13	30.25	14.76	0	18.96	19.01	27.71	24.07
S-53	18.59	28.8	29.7	14.36	15.74	14.45	13.79	0	15.73	10.14	20.03	0	10.3	19.22	0	9.71	11.33	15.91	22.64	23.75
S-54	18.93	15.27	32.97	16.63	22.87	25.87	17.07	0	16.67	16.1	31.47	0	19.76	17.44	17.17	15.05	0	21.88	28.5	25.54
S-55	0	0	19.28	0	0	0	13.21	0	17.33	9.07	16.27	0	0	18.41	0	0	10	11.49	14.82	0
POSITIVE CONTROL	23.31	0	29.02	16.55	25.31	25.54	24.39	0	19.33	26.13	25.45	0	17.28	34.68	21.05	16.91	0	27.42	27.81	15.51

AZT: AZITHROMYCIN; NOR: NORFLOXACIN; CPR: CIPROFLOXACIN; OFL: OFLOXACIN; AMP: AMPLICILLIN; AMX: AMOXYCILLIN; STR: STREPTOMYCIN; CFX: CEFEXIME; TTR: TETRACYCLIN; GNT: GENTAMYCIN; MRP: MEROPENEM; MTR: METRONIDAZOLE; CLX: CLOXACILLIN; DOX: DOXYCILLIN; VNC: VANCOMYCIN; RFM: RIFAMPICIN; CHL: CHLORAMPHENICOL; LVF: LEAVOFLOXACIN; GTF: GATIFLOXACIN; ERY: ERYTHROMYCIN

Table 2: Percentage resistant and susceptible *Bacillus* spp. against various antibiotics

Name of Antibiotics	% Susceptibility	% Resistance	Name of Antibiotics	% Susceptibility	% Resistance
Azithromycin	**69.23 (18)**	**30.77 (8)**	Meropenem	**100 (26)**	**0 (0)**
Norfloxacin	**88.46 (23)**	**11.54 (3)**	Metronidazole	**3.84 (1)**	**96.16 (25)**
Ciprofloxacin	**100 (26)**	**0 (0)**	Cloxacillin	**50 (13)**	**50 (13)**
Ofloxacin	**92.30 (24)**	**7.70 (2)**	Doxycillin	**100 (26)**	**0 (0)**
Amplicillin	**42.30 (11)**	**57.70 (15)**	Vancomycin	**92.30 (24)**	**7.70 (2)**
Amoxicillin	**42.30 (11)**	**57.70 (15)**	Rifampicin	**84.61 (22)**	**15.39 (4)**
Streptomycin	**96.15 (25)**	**3.85 (1)**	Chloramphenicol	**42.30 (11)**	**57.70 (15)**
Tetracyclin	**0 (0)**	**100 (26)**	Leavofloxacin	**100 (26)**	**0 (0)**
Cefixime	**92.30 (24)**	**7.70 (2)**	Gatifloxacin	**100 (26)**	**0 (0)**
Gentamycin	**100 (26)**	**0 (0)**	Erythromycin	**76.92 (20)**	**23.08 (6)**

Table 3: MAR Index of *Pseudomonas* Isolates

Pseudomonas Isolates	*MAR Value	*Pseudomonas* Isolates	*MAR Value
S-14	0.85	S-27	0.60
S-15	0.85	S-32	0.65
S-16	0.75	S-36	0.35
S-17	0.95	S-37	0.60
S-18	0.25	S-38	0.60
S-19	0.85	S-40	0.80
S-20	0.55	S-45	0.55
S-21	1.00	S-48	0.60
S-22	0.85	S-51	0.45
S-23	0.45	S-52	0.40
S-24	0.75	S-53	0.40
S-25	0.55	S-53	0.80

DISCUSSION:

The results put forward that the environmental, industrial and human activities impact on the level of antibiotic resistance among the microorganisms pertaining to food, water and other human-related commodities. It is thus become important to determine the antibiotic resistance patterns of isolated microbes as it is the part of microbial monitoring process of the food and water. Increase in the emergence of the multi-drug resistant *Bacillus* is now-a-days a major problems throughout the world. Therefore, current study is highly influential and exhibits the fact that the food samples meant for human consumptions were contaminated by a major bacterium i.e. *Bacillus* which has been associated with the food-borne illnesses and if ingested, may cause deleterious effects to consumers' health.

The pervasiveness of resistance among microorganisms isolated from different food commodity has significantly risen during last few years and a lot of study has previously been done in this area to evaluate the bacterial contamination of food commodities and isolation of resistant microorganisms from different environment and clinical samples[29, 31, 32]. The fact behind this can be attributed to selection pressure created by the use of antimicrobials in food-producing animals [37-39]. Elevated rates of resistance may also happen due to inappropriate or uncontrolled use of antibiotics. It is, therefore, essential to forfeit additional awareness to food hygiene practices to reduce or eliminate the risk from antibiotic resistance and pathogenic bacteria originating from food.

This study is highly prolific and exemplifies the extent of antibiotic resistance in all the isolated *Bacillus* spp. Results were indicative to disburse more awareness to Good Hygiene Practices (GHP) for the production of various food commodities in order to reduce or eliminate the risk due to pathogenic microorganisms isolated from these food resources. A stringent execution of Sanitary and Phytosanitary (SPS) measures should be applicable for street food vendors in order to make the safe food for human consumption. Therefore, it is the duty of public health authorities to scrutinize and implement the conditions of cleanliness. Food safety education is another vital component of the overall tactics to diminish the occurrence of food-borne infirmities and harmonize authoritarian and other possible actions.

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