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# MOLECULAR SCREENING FOR PREVALENCE OF ANTIBIOTIC-RESISTANT *SALMONELLA SPP*. IN DRINKING WATER SAMPLES FROM SLUM AREAS OF BHOPAL CITY, INDIA

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**ABSTRACT:** The incidence of multiple antibiotic-resistant *Salmonella spp.* isolates in randomly collected potable water samples from 70 different slum area sites of Bhopal City, M. P. India, were investigated through a molecular approach.BSA media was used for isolation and CFU count of *Salmonella* spp. The DNA of representative *Salmonella* spp. Colors of each water sample were subjected to PCR to detect *Amp, cmlA* and *tetA* genes with standard PCR protocol with annealing at primers specific temperature for 1 minute 30 seconds. The presence of the target gene in the PCR product was detected on 1% agarose gel. An *in-vitro* antibiogram study was done to check the phenotypic antibiotic resistance. A total 74.29% of samples were reported to have a prevalence of *Salmonella* spp. with 44.19average CFU count and huge standard deviation (σ=28.28). Incidence of *Amp, cmlA*, and *tetA* genes is reported to be 67.31%, 36.54%, and 61.54% in *Salmonella* spp. Isolates from 52 samples which doesn't coincide with the phenotypic prevalence of antibiotic resistance. 25, 17, and 11isolates showed resistance towards tetracycline, ampicillin, and chloramphenicol, respectively. The prevalence of phenotypic resistance towards ampicillin and incidence of amp gene in isolates were significant as the $p < .05$ in the case, contrarily chloramphenicol and tetracycline weren't significant for the incidence of their respective genes *cmlA* and *tetA* since their *p*-values are not significant at $p < .05$. However, the prevalence of phenotypic resistance towards chloramphenicol and tetracycline was independent of incidence of their respective genes in isolates is a matter of further extensive investigation on responsible factors thus increasing the residence time of drug with better patient compliance.

**INTRODUCTION:** Water is the most studied hydrogen chalcogenide regarded as a "universal solvent which is transparent, colourless, tasteless, odourless, fluid and is one of the essential components on earth containing minerals important for all the organism on the surface of earth and crucial for the existence of humans since this vital natural resource is used for drinking, irrigation, industrial applications, fisheries, electricity production, *etc.*, [1, 2]. But many people do not have access to clean and safe drinking water and many die of water-borne bacterial infections [3].

Water is unsafe for human consumption when it is contaminated with pathogenic microorganisms, and satisfactory quality of water supply should be guaranteed for all. Water-borne diseases, including diarrhea, cholera, typhoid fever and dysentery, have been predominantly ascribed to perilous water and unhygienic practices [4].

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People from developing countries encountered a higher risk of water-borne diseases compared to developed countries [5]. The contamination of water with infected fecal material is common in areas with poor standards of hygiene and sanitation, which makes the microbiological quality assessment essential [6]. *Salmonella* is a most momentous pathogenic bacterium causing typhoid and non-typhoidal Salmonellosis. Salmonella species are most often cause of foodborne ailment in both animals and humans, resulting 93.8 million approx. Cases cause globally per year [7, 8] whereas in developing countries like India, foodborne diseases are for the most part under announced; anyway, in the previous 29 years (1980-2009) 3485 people have been influenced from 37 Salmonella related episodes [9]. *Salmonella* transmission generally happens due to contaminated food and water consumption [10, 11, 12].

*Salmonella* contaminations are clustered into clinical classifications: Salmonellosis, gastroenteritis, bacteremia, osteomyelitis, reactive arthritis, enteric fever and food poisoning [13, 14, 15].

*Salmonella* disease in individuals is essentially achieved about by drinking water contaminated with discharges and defecation of contaminated faunae [11, 16, 17, 18]. According to Seas *et al*., children under 5 years of age, primarily in Asian and African countries, are seen as a gigantic gathering of patients contaminated with water-borne microbial infection [19]. Drinking water containing antibiotic-resistant bacterial strains is a serious concern according to scientific literature [20]. Due to resistant microorganisms' increasing pervasiveness, antibacterial therapies have become challenging to a large extent [21, 22, 23]. In view of the facts mentioned the present investigation was intended to detect the prevalence of multiple antibiotic-resistant *Salmonella spp.* Isolates compared to the incidence of antibiotic resistance gene through molecular approach in drinking water samples collected from some of the slum areas of Bhopal City, M.P. India.

**MATERIALS & METHODS:** Drinking water samples from Bhopal City were randomly collected from different slum areas of Bhopal. 50ml of water samples were collected from the 70 sites in clean, dry, and sterile containers using an aseptic condition used for screening *Salmonella* spp. during the period of October 2020 to March 2021.

**Screening and CFU Count of *Salmonella spp*:** Bismuth Sulphite Agar Media (HiMedia Laboratories India Pvt Ltd) was used for selective screening and isolation of *Salmonella* spp. Following aseptic procedures, 0.1 ml of serially diluted water from each collected sample were subjected to screen CFUs of *Salmonella* spp. by spread plate techniques aseptically, which were incubated at 37$^{o}$C for 24 h. The development of Jet black, convex-type colonies surrounded by metallic sheen indicates colony-forming units (CFUs) *Salmonella* spp. To have the idea of contamination on a quantitative basis following formulae for CFU count were used;

CFU per ml = No. of CFU /Total Vol. Plated ×Dilution factor

**Confirmation of Antibiotic Resistance:** The representative CFUs of *Salmonella*spp from each plate were randomly picked up and cultured in nutrient broth used for both molecular detection of antibiotic-resistant genes and *in-vitro* antibiotic susceptibility assays.

**DNA Extraction:** The conventional boiling method extracted total genomic DNA from randomly picked representative colonies of Salmonella spp. on BSA media plates. To a 1.5 ml microfuge tube containing 1 ml of nutrient broth was inoculated with 1 randomly picked colonies from each water sample were transferred and incubated for 24 hours at 37$^{o}$C.

After which, each tube was centrifuged at 10000 rpm for 7 minutes then the supernatant was discarded, and the pellet so obtained was washed with 500 µl of sterile distilled water and then centrifuged at 12000 rpm for 5 min.

Discarding the supernatant again and the retained pellet was homogenized with 200 µL of sterile distilled water subjected to heating in a boiling digital water bath at 100°C (Navyug, India) for 10 min was allowed to cool for 7 min in an ice bath. The microfuges were then centrifuged at 10000 rpm for 5 min. The obtained supernatants were then retained in fresh sterile 1.5 ml microfuges and stored at 20°C for further use.

**Detection of Antibiotic-Resistant Genes:** With the primer-specific PCR approach, the total genomic DNA samples from representative colonies of Salmonellas spp. were subjected to screening for genes encoding resistance to Ampicillin, Chloramphenicol, and Tetracycline. Previously reported primer sequences were used for this purpose, whose sequence details & annealing conditions are mentioned in **Table 1**.

The PCR reaction was performed in 25 µl volume, which contains 12.5 µl PCR TaqMixture (HiMedia, India), 0.5 µl each of forward and reverse primers (Bioserve Biotechnologies, India) 5 µl template DNA and 6.5 µl molecular grade distilled water (HiMedia). The Sterile distilled water was used in place of DNA as a negative control in one tube. The PCR was performed on Prima 96 Thermocycler (HiMedia, India) with PCR conditions having initial denaturation for 5 min at 94°C, followed by 30 cycles of denaturation 94°C for 30 seconds, annealing at primers specific temperature for 1 min 30 seconds and extension at 72°C for 1 min and final extension at 72°C for 10 minutes.

The PCR product was subjected to electrophoresis on 1% Agarose gel in 1X TAE buffer running at 100 volts for 30 minutes. Then the presence of target antibiotic-resistant genes in the form of a band on the gel was confirmed by viewing the gel on a UV transilluminator (Thomas Baker Biosciences, India).

**TABLE 1: LIST OF PRIMER SETS USED FOR THE DETECTION OF ANTIBIOTIC RESISTANCE GENE IN *SALMONELLA SPP*. ISOLATED FROM WATER SAMPLES IN SINGLEX PCR**

| S. no. | Antibiotic | Target Gene | Primer Sequences | Annealing Temp. |
|---|---|---|---|---|
| **1.** | Ampicillin [24, 25] | *Amp* | Forward: ATGCACACGCTGATCGGATT<br>Reverse: GCGGACGCAGACTTCACTAA | 65°C |
| **2.** | Chloramphenicol [26, 27] | *cmlA* | Forwards: CCGCCACGGTGTTGTTGTTATC<br>Reverse: CACCTTGCCTGCCCATCATTAG | 58°C |
| **3.** | Tetracycline [28, 27] | *tetA* | Forwards: GGTTCACTCGAACGACGTCA<br>Reverse: CTGTCCGACAAGTTGCATGA | 56°C |

**Phenotypic Antibiotic Susceptibility:** The *in-vitro* antibiogram study was done against isolated *Salmonella* spp. was done on nutrient agar media according to the earlier described methods [29, 30]. The overnight broth culture of *Salmonella* spp. Isolates were suitable maintained at McFarland 0.5 standard and inoculated onto the nutrient agar media plates using sterile cotton swab.

Antibiotic octadisc *G-XXI-minus (HiMedia, India) containing the discs of common antibiotics Chloramphenicol (30 µg), Ampicillin (10 µg), Tetracycline (30 µg), Gentamicin (10 µg), Co-Trimoxazole (25 µg), Ceftriaxone (30 µg), Cefuroxime (30 µg) and Ciprofloxacin (5 µg) was used for antibiotic susceptibility assay against the *Salmonella* spp. As an indication of sensitivity or resistance towards the particular drug, the zone of inhibition or no zone was recorded after 24 hours of incubation at 37°C.

## RESULTS:

**Prevalence of *Salmonella spp*:** Out of 70 different drinking water samples randomly collected from slum areas of Bhopal City, 74.29% samples were reported to have prevalence of *Salmonella* spp. on bismuth sulphite agar media. Only 52 out of 70 water samples were reported to be contaminated with *Salmonella* spp.

At a dilution of $10^{-7}$ the averages CFU count of *Salmonella* spp. in all the 52 water samples lies in the range of 44.19 with a huge standard deviation ($\sigma$=28.28) which describes an ambiguous level of contamination in sample drinking waters taken from slum areas **Table 2.**

**Detection of Antibiotic Resistant Gene:** The most representative *Salmonella* spp. isolates out of the 52 water samples showing growth on culture plates when subjected for detection of three antibiotic resistance genes namely *Amp*, *cmlA* and *tetA*, their incidence is reported to be 67.31%, 36.54% and 61.54% respectively, indicates that the incidence of chloramphenicol resistance gene is reported to be highest among the tested isolates followed by tetracycline and ampicillin resistance gene **Table 2**. But the percentage incidence doesn't coincide the phenotypic prevalence of antibiotic resistance among the same *Salmonella* spp. isolates.

**TABLE 2: CFU COUNT OF *SALMONELLA* SPP. ISOLATES ON BSA MEDIA OUT OF 52 POSITIVE DRINKING WATER SAMPLES AND ANTIBIOTIC RESISTANCE GENE DETECTED IN REPRESENTATIVE *SALMONELLA* SPP. COLONY OF WATER SAMPLES**

| S. no. | Sample Code | CFU count $10^{-7}$ dilution | Target gene detected in the representative colony | | |
|---|---|---|---|---|---|
| | | | ***Amp*** | ***cmlA*** | ***tetA*** |
| 1 | S-1 | 24 | 1 | 1 | 1 |
| 2 | S-2 | 12 | 1 | 0 | 1 |
| 3 | S-3 | 36 | 0 | 0 | 1 |
| 4 | S-4 | 54 | 1 | 0 | 0 |
| 5 | S-6 | 78 | 1 | 1 | 1 |
| 6 | S-7 | 11 | 1 | 1 | 0 |
| 7 | S-8 | 36 | 1 | 0 | 1 |
| 8 | S-10 | 45 | 1 | 1 | 1 |
| 9 | S-11 | 23 | 0 | 1 | 0 |
| 10 | S-12 | 29 | 0 | 1 | 0 |
| 11 | S-14 | 78 | 1 | 0 | 1 |
| 12 | S-15 | 36 | 1 | 0 | 1 |
| 13 | S-16 | 14 | 1 | 1 | 0 |
| 14 | S-18 | 37 | 1 | 0 | 1 |
| 15 | S-19 | 61 | 0 | 1 | 1 |
| 16 | S-20 | 29 | 0 | 0 | 1 |
| 17 | S-21 | 35 | 1 | 1 | 0 |
| 18 | S-24 | 10 | 0 | 0 | 1 |
| 19 | S-26 | 47 | 1 | 0 | 1 |
| 20 | S-27 | 157 | 1 | 1 | 0 |
| 21 | S-28 | 36 | 1 | 0 | 1 |
| 22 | S-29 | 22 | 0 | 0 | 0 |
| 23 | S-30 | 59 | 1 | 1 | 0 |
| 24 | S-31 | 18 | 1 | 0 | 1 |
| 25 | S-33 | 44 | 0 | 0 | 1 |
| 26 | S-34 | 86 | 1 | 1 | 1 |
| 27 | S-36 | 36 | 1 | 0 | 0 |
| 28 | S-37 | 44 | 1 | 1 | 1 |
| 29 | S-38 | 25 | 0 | 0 | 0 |
| 30 | S-39 | 11 | 1 | 0 | 1 |
| 31 | S-40 | 33 | 0 | 0 | 1 |
| 32 | S-41 | 42 | 0 | 0 | 1 |
| 33 | S-44 | 45 | 1 | 1 | 0 |
| 34 | S-45 | 16 | 0 | 0 | 1 |
| 35 | S-47 | 22 | 1 | 0 | 0 |
| 36 | S-48 | 77 | 1 | 0 | 1 |
| 37 | S-49 | 39 | 1 | 1 | 0 |
| 38 | S-50 | 45 | 1 | 0 | 1 |
| 39 | S-51 | 08 | 0 | 1 | 1 |
| 40 | S-52 | 77 | 1 | 0 | 0 |
| 41 | S-54 | 84 | 0 | 1 | 0 |
| 42 | S-56 | 38 | 1 | 0 | 1 |
| 43 | S-57 | 79 | 1 | 0 | 1 |
| 44 | S-59 | 51 | 1 | 1 | 0 |
| 45 | S-61 | 105 | 1 | 0 | 1 |
| 46 | S-63 | 81 | 0 | 0 | 0 |
| 47 | S-64 | 41 | 1 | 1 | 0 |
| 48 | S-65 | 19 | 1 | 0 | 1 |
| 49 | S-66 | 27 | 1 | 0 | 1 |
| 50 | S-68 | 77 | 0 | 0 | 1 |
| 51 | S-69 | 18 | 0 | 0 | 0 |
| 52 | S-70 | 41 | 1 | 0 | 1 |
| | Total Samples = 52 | Average CFU Count = 44.19SD or σ =28.28 | Total Incidences = 35 | Total Incidences = 19 | Total Incidences = 32 |

**Note:** 1= presence of target gene and 0 = absence of target gene

***In-vitro* Antibiotic Susceptibility:** In terms of phenotypic antibiotic susceptibility, out of 52 *Salmonella* spp. isolates of drinking water origin from slum areas, 25 isolates were reported to be resistant towards tetracycline, and 17 isolates were resistant to ampicillin, while 11 isolates were resistant to chloramphenicol. Referring to **Table 3** all the 52 *Salmonella* spp. isolates were sensitive to gentamicin.

**TABLE 3: *IN-VITRO* PHENOTYPIC ANTIBIOTIC SUSCEPTIBILITY PROFILE OF 52 *SALMONELLA SPP.* ISOLATES TOWARDS 8 ANTIBIOTICS USED**

| S. no. | Antibiotics used | Percentage of sensitive isolates | Percentage of resistant isolates |
|---|---|---|---|
| 1 | Chloramphenicol | 78.8% | 21.2% |
| 2 | Ampicillin | 67.3% | 32.7% |
| 3 | Tetracycline | 51.9% | 48.1% |
| 4 | Gentamicin | 100% | 0.00% |
| 5 | Co-Trimoxazole | 90.4% | 09.6% |
| 6 | Ceftriaxone | 88.5% | 11.5% |
| 7 | Cefuroxime | 88.5% | 11.5% |
| 8 | Ciprofloxacin | 90.4% | 09.6% |

**DISCUSSION:** Polluted and contaminated drinking water is the source of several water-borne diseases due to the presence of various types of bacteria, viruses, protozoa, and helminthes [31, 32]. Salmonellosis is supposed to cause a global economic impact as it stands as an imperative public health problem responsible for substantial morbidity and mortality [33] and water-borne *Salmonella* spp. becomes a serous menace when they are resistant to multiple antibiotics isolated [34]. In terms of clinical diagnosis of microbial pathogens, the PCR-based investigation are fast, specific, sensitive and safe for detection of pathogens through culture methods are still popular and useful due to their simplicity and cost-effectiveness [33]. However, in cases of detection of antibiotic-resistant bacteria, the isolates' genotypic and phenotypic profiles do not coincide mostly. Excessive and unauthorized use of antibiotics in the community imposes the emergence, and spread of antibiotic resistant bacteria strains in food and water that often leads to the failures of chemotherapies in pathological conditions [34, 35, 36]. Because of the increasing concern towards antibiotic resistance for public health, the incidence of 3 antibiotic resistance genes and the prevalence of antibiotic-resistant Salmonella isolates was monitored. When we compared the actual prevalence of antibiotic resistance of indigenously isolated Salmonella spp. towards the ampicillin, chloramphenicol, and tetracycline antibiotics by antibiotic susceptibility assay to confirm the phenotypic resistance due to the incidence of Amp, clmA, and tetA gene within the test samples by ANOVA method (https://goodcalculators.com/one-way-anova-calculator/ and https://www.socscistatistics.com/tests/anova/default2.aspx), the prevalence of phenotypic resistance towards ampicillin and incidence of amp gene in samples isolates were observed to be significant as the $p < .05$ in the case **Table 4.** While the prevalence of phenotypic resistance towards chloramphenicol and tetracycline doesn't observed to be significant for the incidence of their respective genes cmlA and tetA since their *p*-values are not significant at $p < .05$ **Table 5, Table 6.**

**TABLE 4: SUMMARY OF ANOVA FOR COMPARISON AMONG PHENOTYPIC PREVALENCE OF AMPICILLIN RESISTANCE AND INCIDENCE OF AMP GENE IN *SALMONELLA* SPP. SAMPLES**

| Data Summary | | | | |
|---|---|---|---|---|
| Groups | N | Mean | Std. Dev. | Std. Error |
| *Prevalence of Phenotypic Resistance to Ampicillin* | 52 | 0.3269 | 0.4737 | 0.0657 |
| *Incidence of Amp gene for Resistance* | 52 | 0.6731 | 0.4737 | 0.0657 |

| ANOVA Summary | | | | | |
|---|---|---|---|---|---|
| Source | Degrees of Freedom DF | Sum of Squares SS | Mean Square MS | F-Stat | P-Value |
| Between Groups | 1 | 3.1162 | 3.1162 | 13.8874 | 0.0003 |
| Within Groups | 102 | 22.888 | 0.2244 | | |
| Total | 103 | 26.0042 | | | |

Note: The f-ratio value is 13.88571. The p-value is .000319. The result is significant at $p < .05$.

**TABLE 5: SUMMARY OF ANOVA FOR COMPARISON AMONG PHENOTYPIC PREVALENCE OF CHLORAMPHENICOL RESISTANCE AND INCIDENCE OF CMLA GENE IN *SALMONELLA* SPP. SAMPLES**

| Data Summary | | | | |
|---|---|---|---|---|
| **Groups** | **N** | **Mean** | **Std. Dev.** | **Std. Error** |
| Prevalence of Phenotypic Resistance to Chloramphenicol | 52 | 0.2115 | 0.4124 | 0.0572 |
| Incidence of cmlA gene for Resistance | 52 | 0.3654 | 0.4862 | 0.0674 |

| ANOVA Summary | | | | | |
|---|---|---|---|---|---|
| **Source** | **Degrees of Freedom DF** | **Sum of Squares SS** | **Mean Square MS** | **F-Stat** | **P-Value** |
| Between Groups | 1 | 0.6158 | 0.6158 | 3.0301 | 0.0847 |
| Within Groups | 102 | 20.7297 | 0.2032 | | |
| Total | 103 | 21.3455 | | | |

Note: The f-ratio value is 3.02783. The p-value is .084864. The result is not significant at $p < .05$.

**TABLE 6: SUMMARY OF ANOVA FOR COMPARISON AMONG PHENOTYPIC PREVALENCE OF TETRACYCLINE RESISTANCE AND INCIDENCE OF TETA GENE IN *SALMONELLA* SPP. SAMPLES**

| Data Summary | | | | |
|---|---|---|---|---|
| **Groups** | **N** | **Mean** | **Std. Dev.** | **Std. Error** |
| Prevalence of Phenotypic Resistance to Tetracycline | 52 | 0.4808 | 0.5045 | 0.07 |
| Incidence of tetA gene for Resistance | 52 | 0.6154 | 0.4913 | 0.0681 |

| ANOVA Summary | | | | | |
|---|---|---|---|---|---|
| **Source** | **Degrees of Freedom DF** | **Sum of Squares SS** | **Mean Square MS** | **F-Stat** | **P-Value** |
| Between Groups | 1 | 0.471 | 0.471 | 1.8998 | 0.1711 |
| Within Groups | 102 | 25.2907 | 0.2479 | | |
| Total | 103 | 25.7617 | | | |

Note: The f-ratio value is 1.90038. The p-value is .171052. The result is not significant at $p < .05$.

**CONCLUSIONS:** In view of the experimental outcomes of the present investigation, the antibiotic resistance in any bacterial species happens due to the presence of antibiotic resistance genes, but the expression of this antibiotic resistance gene probably depends on several factors that are responsible for actual prevalence on antibiotic resistance in *Salmonella* spp.

In the present study, the prevalence of phenotypic antibiotic resistance was significant in the incidence of the ampicillin-resistant gene in *Salmonella* spp. isolates from drinking water samples however, the prevalence phenotypic resistance towards chloramphenicol and tetracycline antibiotics was independent of the incidence of their respective antibiotic-resistant genes in *Salmonella* spp. isolates from drinking water samples which is the matter of further extensive investigation on several other responsible factors.

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