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Research Article

DETECTION OF INVA GENE BY PCR AND IDENTIFICATION WITH SUSCEPTIBILITY PATTERN EVALUATION BY VITEK 2 OF SALMONELLA ISOLATES

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ABSTRACT. This study aims to identify and antimicrobial susceptibility of hospital wastewater in Dhaka city. Total 134 *Salmonella* isolates were identified using cultural characteristics, automated biochemical test, and PCR for *invA* gene detection. Biochemical identification and Antimicrobial Susceptibility Test (AST) were performed through VITEK 2. Essential Agreement (EA) and Categorical Agreement (CA) were worked out according to the CLSI breakpoint. We also measured Very Major Error (VME), Major Error (ME), and Minor Error (mE). Seven *Salmonella* serotypes were identified through VITEK 2 ID-GN card and *invA* gene detection. *S. typhimurium, S. paratyphi, S. enteritidis, S. enterica,* and *S. typhi* were most predominant, while *S. gallinarum* and *S. arizonae* were less common serovar. Among 136 isolates, 134 (98.5%) were correctly identified to the species level. Accordingly, 47% (64 out of 136), 29% (39 out of 136), 10.29% (14 out of 136), and 12.5% (17 out of 136) isolates were identified within the excellent, very good, good, and acceptable levels. Moreover, we found 87% of isolates (116 out of 134) exerted at least resistance to one antibiotic. Of 134 tested isolates, there were 7 serotypes that showed Very Major Error (VME). The final Major Error (ME) and Minor Error (mE) shown organisms were 15 and 67. Overall 98.86% Essential Agreement (EA) and 95.95% Categorical Agreement (CA) was calculated. The most common resistance pattern of multi-resistant serovares was to ampicillin, chloramphenicol, sulphonamide, and trimethoprim. Ongoing research is a matter of concern for emerging multi-drug resistance of *Salmonella* serovares and helpful evidence for healthcare provider.

Keywords: *Hospital wastewater, VITEK 2, PCR, invA gene, multi-drug resistant.*

INTRODUCTION

Salmonella is characteristically a rod-shaped, oxidase-negative, Gram-negative, non-spore-forming, peritrichous enterobacterium known as a notorious foodborne pathogen that causes gastroenteritis in humans and other animals. Typhoidal and non-typhoidal *Salmonella* cause a great healthcare burden and an unusual number of deaths globally [1]. This enteropathogenic bacterium is involved in 94 million infections and 155,000 deaths annually all over the world. According to the Lancet, 535,000 (95%

uncertainty) cases of non-typhoidal *Salmonella* invasive disease happened in 2017 [2]. Every year, an estimated 1.4 million cases of salmonellosis occur among humans in the United States. Nearly 2,500 different serotypes of *Salmonella* spp. have been reported. In the Indian subcontinent, Pakistan had the highest incidence (about 452 cases per 100,000 in a year) of typhoid fever, followed by India (about 214 cases per 100,000 per year) [3]. A study on urban slum communities in Bangladesh revealed that the total incidence was 3.9 per 1000 in a year, and the rate was relatively higher in pre-school children of 0 to 4 years old (about 19 per 1000 per year). A study in Bangladesh on bloodstream infections reveals that *Salmonella* spp. is the most frequently isolated organism (36.9% of samples), and a significant portion of these serotypes were multidrug-resistant (16.4% to 51.3%), which is a reason for concern [4].

Hospital wastewater is a potential source of several disease-causing pathogens that pose a great threat to humans and animals. Apart from various pharmaceutical compounds, hospital wastewater carries antibiotic-resistant microbes and genes [5]. Earlier studies revealed that there are inordinate quantities of microbes and residual antibiotics in hospital wastewater (HWW), which might exert an influence on the propagation of antibiotic-resistant bacteria. These resistant bacteria and antibiotic residues may restrain the growth of susceptible bacteria. When we discharge these resistant bacteria into the environment, they presumably act as vectors to transmit a resistant gene or as reservoirs for antibiotic-resistance genes (ARGs), which are a vulnerable threat to public health [6]. A qualitative analysis of 10 hospitals in Iran showed that hospital waste had a 16.70% contribution to hazardous-infectious waste. Genes resistant to carbapenems, sulfonamides, tetracyclines, and transmissible genetic elements were found at a high level (>10-4 gene copies/16S rRNA gene copies) in hospital wastewater [7].

The virulence factor of *Salmonella* is related to both chromosomal and plasmid factors. The *invA, spv, fmA,* and *stn* genes are major virulence factors behind salmonellosis. The *invA* gene is found in the genome and is responsible for coding a protein on the inner membrane that helps to invade epithelial cells. PCR for the *invA* gene is a quick and accurate method for the identification of *Salmonella* spp. [8].

Many clinical laboratories in the United States and Europe use automated system for identification and antimicrobial susceptibility test. Although some failure issues like β-lactam resistance have been reported in several studies [9]. Singularly, VITEK 2 of bioMérieux performed inferiorly compared to Kirby-Bauer's disk diffusion and broth microdilution for detecting meropenem, cefepime, and imipenem resistance [10]. Therefore, Clinical & Laboratory Standards Institute (CLSI) and U.S. Food and Drug Administration (FDA) recommend a correction of the breakpoints for *Enterobacteriaceae* with cefepime, cefotaxime, aztreonam, cefazolin, ertapenem, imipenem, ceftazidime, ceftriaxone, and meropenem, as well as breakpoints for *Salmonella* spp. [11]. The VITEK2 system from bioMerieux made a revolution in identification and antimicrobial susceptibility testing with commendable precision and accuracy, which helped significantly in reducing handling time [12, 13].

Our research aimed to determine the frequency and severity of multi-drug resistant *Salmonella* serotypes isolated from hospital wastewater samples around Dhaka city glimpsing unrestricted use of antibiotics. Implicitly, we have shown how hospital wastewater carries baleful biological hazard because of inadequate control of infection and improper management of antimicrobial drugs in hospitals. This study will assist in preventing the hazards posed by superbugs and informing global antibiotic resistance

prevention measures. Our findings will be useful in treating impregnable superbugs with antibiotics based on the resistance pattern displayed here. Moreover, this work and CLSI will give us a glimpse of the emerging mechanism of resistance and our vigilance activity.

MATERIALS AND METHODS

Sample Collection, Pre-enrichment and Growth in Culture Media

A total of 204 specimens of hospital wastewater (100 mL each) were collected aseptically from different hospitals around Dhaka City, Bangladesh. Then we pursue Bacteriological Analytical Manual (BAM) for cultural identification. Samples were kept in an ice box and transported to the antimicrobial resistance testing laboratory, microbiology wing, under the Ministry of Health and Family Welfare, Bangladesh from February 2021 to August 2023. We ensured classified cleanroom of C and A according to European Union Good manufacturing practice (EUGMP). We collected these samples aseptically in a sterile glass bottle from the discharge drain before Effluent Treatment Plant (ETP) treatment. Samples were pre-enriched into Buffered Sodium Chloride Peptone Broth (BSCPB) aerobically at 37°C for 24 h. Then 1 mL of pre-enriched culture was transferred into Rappaport Vassiliadis *Salmonella* Enrichment Broth (RVSEB). The enriched culture was then streaked on Xylose Lysine Deoxycholate (XLD) agar and incubated aerobically at 37°C for 24 h. Red colonies with black centers and red colonies on XLD have streaked onto Brilliant Green (BG) agar media. Pinkish-white or red colonies circled by a red halo in BG medium were later streaked on Trypticase Soy Agar (TSA) and incubated for 18-24 h at 37°C. Identification of bacteria grown in XLD, BG, and then TSA agar was performed by Gram's staining, cultural characteristics, biochemical test identification through VITEK 2 ID-GN CARD, and polymerase chain reaction (PCR). The antibiotic susceptibility test of the bacteria was performed against AST-GN72.

Identification of Bacteria

Gram's Staining Method and Colony Morphology

Red colonies and red colonies with black centers on XLD and pinkish-white or red colonies circled by a red halo ambit on BG that were sub-cultured on TSA were characterized morphologically using Gram's staining technique according to the described method [14]. Colony characteristics such as shape, size, surface texture, edge and elevation, color, and opacity developed on XLD agar and BG agar after 24 h of incubation at 37°C were recorded.

Identification with the VITEK 2 System

The VITEK 2 is a fully automated identification system from bioMérieux, Inc. The VITEK 2 Gram-Negative Identification Card (GN) is intended for use with VITEK 2 systems of fermenting and non-fermenting Gram-negative bacilli, which we used in our study [15]. The ID-GN card consists of 64-wells, 16 of them are blank well and 48 wells contain a substrate for different biochemical and metabolic activities like acidification, pH change, alkalinization, the action of inhibitory substances, enzymatic activity, hydrolysis, carbon source utilization, and resistance [16]. The substrates are shown in supplementary table 1. Final results are available in approximately 10 h or less [17].

A separate single colony of 18-24 h incubated fresh culture from non-selective TSA media was used for suspension preparation in a clear 12×75 mm test tube containing 3 mL of half-strength saline water (aqueous 0.45% to 0.5% NaCl, pH 4.5 to 7.0). Turbidity was adjusted with the help of DensiCheck Plus (bioMérieux, Inc.) within a stipulated range of 0.5 to 0.63 McFarland. After adjusting the turbidity, the prepared suspension was placed onto the cassette and inserted into the filling area [15].

By logging into VITEK 2 Advanced Expert System™ (AES) software, specimen number, date of operation, expected identification, lab ID, and isolate number were inputted into the software [15]. Cassettes were scanned by a scanner before entering into filling box. After completion of filling, the cassettes were conveyed through several chambers and tubes loading door to the filling chamber and then the vacuum chamber to the carousel incubator. Quality control of the analysis was done by reference to the *Salmonella enterica* (ATCC 35664) culture.

Analysis of Identification Results

VITEK 2 AES software offers four possibilities for the analysis of identification results: (A) Correct identification, when organisms are correctly identified to the species level, sometimes low discrimination is resolved by simple additional tests; (B) Low discrimination, when strain with low discrimination cannot be resolved by simple additional tests, the system proposes two or more species, but one is identical to the reference; (C) Misidentification, when the system proposes one or more species but all are different from the reference; (D) No identification, which means the system does not propose any species names [17].

Molecular Detection

Genomic DNA was extracted from *Salmonella* spp. by the heat lysis method as described by Liu et al.[18]. In brief, pure colonies of *Salmonella* were mixed with 200 µL of PBS in Eppendorf tubes and boiled for 10 minutes. Then the tubes were immediately kept on ice for 10 min for cold shock. Finally, centrifugation was done at 10,000 rpm for 10 min and the supernatant was collected to use as DNA template for PCR. The composition of the PCR master mixture (25 µL) and PCR thermal profile are presented in supplementary table 1. We used *invA* primer of 211 base pairs, and the sequences of forward primer 5′-ATCAGTACCAGTCGTCTTATCTTGAT-3’ and reverse primer 5'-TCTGTTTACCGGGCATACCAT -3' [19].Thermal profile of PCR reaction for amplification of the *invA* gene of *Salmonella* spp. includes initial denaturation (at 94°C for 5 min), denaturation (at 94°C for 30 sec), annealing (at 52°C for 2 min), extension (at 72°C for 45 sec), final extension (at 72°C for 5 min). A total of 35 cycles were run to make millions of copies. The thermal cycler was held at 4°C until electrophoresis was performed [19].

After performing PCR, electrophoresis was done according to Yanestria SM et al. [20]. Briefly, 100 mL 1X TAE buffer was taken in a conical flask, and 1.5 g of agarose was added and boiled for 2 min then poured into a casting tray with a comb. After getting clots, comb was removed and the gel was submerged in 1X TAE buffer. 1 µL 6X loading dye and 5 µL of PCR product were mixed and then loaded in the well of the agarose gel. The marker (100 bp DNA ladder) was loaded in the first lanes of the gel electrophoresis and was carried out at 100V for 30 min. The PCR products’ bands on the gel were stained with ethidium bromide for 10 minutes followed by washing with deionized water for 10

min. The product-stained gel was examined under a UV trans-illuminator and the results were recorded.

Antibiotic Susceptibility Test

Preparation of Culture Dilution

Antibiotic susceptibility of *Salmonella* spp. (n=136) was tested against 18 antibiotics by a VITEK 2 AST-GN72 card (bioMérieux, Inc.). This card consists of 18 antibiotics from 11 different classes. Antibiotics and their classes are shown in supplementary table 1[12]. Antimicrobial susceptibility testing was done along with identification. The procedure for inoculum suspension preparation was similar to the ID-GN card; additionally, 145 µL of prepared and turbidity-adjusted suspension was transferred into 3 mL of half-strength saline water in another polystyrene tube. The AST-GN72 card was placed just after the ID-GN card in the cassette. Labeling and AES data input were also done simultaneously [15, 17].

Analysis of Susceptibility Testing

Essential agreement (EA) is generally defined as the percentage of MICs within a single doubling dilution of the corresponding CLSI or other reference results**.** While analyzing the AST, two possibilities were considered: (A) Category Agreement (CA) and (B) Discrepancies, Bobenchik et al. (2015) [21]. Category agreement categorizes microbial susceptibility according to NCCLS as susceptible, intermediate, and resistant. Discrepancies were graded as very major errors (VME), major errors (ME), and minor errors (mE). When VITEK 2 indicates the result as susceptible, but the reference method indicates resistant, this is called VME. On the other hand, when the VITEK 2 system indicates resistant, but the reference method confirms as susceptible is actually MA. Minor errors (mE) happen when VITEK 2 system indicates susceptible or resistant and the reference method denotes intermediate susceptibility [21].

RESULTS AND DISCUSSION

Identification of Salmonella spp. with Cultural and Morphological Characteristics

A total of 136 *Salmonella* isolates were recovered through XLD and BG agar media from 204 samples. Cultural characteristics of *Salmonella* spp. isolated from hospital wastewater samples showed red colonies and red colonies with black centers on XLD and translucent pink or reddish pink colonies surrounded by a pink zone on BG agar. Gram staining of *Salmonella* spp. revealed Gram-negative rods, arranged single or paired, which were seen under a light microscope.

Identification of Salmonella spp. with the VITEK 2 System

According to cultural and morphological characteristics, 136 isolates were analyzed by the VITEK 2 GN card (Table 1). Among 136 isolates, 64 were identified with an excellent (96-99 % probability) confidence level and correctly identified to the species level. For 39 serotypes, the confidence level was very good (93-95 % probability) when the VITEK 2 system was able to identify them up to species level. The system also identified 14 isolates as good levels (89-92 % probability) and 17 isolates as having acceptable levels (85-88% probability) (Table 1). Additional supplemental tests were

necessary for low discrimination level identification. Discrepancy happened when 2 to 3 taxa exhibited the same bio-pattern. A supplemental test was recommended by the lab report. After performing this test, the reference method was followed for final identification. Two isolates were unidentified because of an atypical bio-pattern or more than three similar bio-patterns. While the system suggested any complementary test, we did not consider this isolate for an antibiogram.

The capability of the VITEK 2 system with an ID-GP identification card to provide rapid identification of pathogenic Gram-positive cocci was first assessed by Bassel et al. (Abstr. 8th Eur. Congr. Clin. Microbiol. Infect. Dis., abstr. P255, 1997) [22]. Bassel et al. showed an overall agreement of 98% (86.8% species-level agreement was found without any supplementary testing, but 11.2% of species had to do supplementary testing). Unfortunately, 1.7% of isolates were misidentified and 0.3% were unidentified in Bassel's experiment. Although in our study we used ID-GN cards, but the level of identification is comparable; here 47% of isolates out of 136 were identified within the excellent level, very good level was 28.67%, good and acceptable levels were 10.29% (14 out of 136) and 12.5% (17 out of 136), respectively. More importantly, 1.4% (2 out of 136) of the isolates were not able to be identified. However, our reference *Salmonella enterica* (ATCC 35664) culture showed excellent level.

In our entire research, we found seven types of *Salmonella* serovares. They are as follows: *S. typhimurium, S. paratyphi, S. enteritidis, S. enterica, S. typhi, S. gallinarum,* and *S. arizonae*. The prevalence percentage was 24.6% for *S. typhimurium* and 11.2% for *S. paratyphi*. The most prevailed serovar was *S. enteritidis,* the percentage was 38.8%. *S. enterica* and *S. typhi* have a moderate prevalence rate of 10.44% and 11.9% respectably. Two serovares have a very low prevalence rate: *S. gallinarum,* and *S. arizonae* (rates of 1.4% each).

The accuracy of the VITEK 2 ID-GN card for identification up to species level was 98.5%. After preliminary cultural-based sorting, we tried 136 *Salmonella* spp. for demonstration through the VITEK 2 system and ID-GN card. 134 out of 136 isolates had been correctly identified with an acceptable level of accuracy. A scant number of serotypes were shown as taxa with low-discrimination identification from VITEK 2, with only 12.6% of isolates within these margins. Low discriminants are solved by two additional tests which confirmed the acceptance as a *Salmonella* species without any ambiguity. Funke and Funke-Kissling [23] revealed 100% accuracy for *Salmonella* identification when they evaluated Gram-negative rods with VITEK 2. *S. enteritidis* had a relatively low percentage of low discrimination. Only 11.5% of *S. enteritidis* fell within the low discrimination level. *S. typhi* had a comparatively lower discrimination rate of 18.75%, but not at a concern height. No serotype of *S. gallinarum* or *S. arizonae* showed low discrimination. Though some serovar had a slightly low rate of identification like *S. typhi* (81.25%) or *S. enterica* (85.72%), we did not find any misidentification between species. Nevertheless, two serovar showed cultural characteristics of conformity but were unidentified through the VITEK 2 ID-GN card. O'Hara [24] evaluated VITEK 2 and ID-GN cards for *Enterobacteriaceae, but their work did not find any low discrimination.*

Molecular detection of invA virulent gene of Salmonella spp.

A PCR assay targeting the pathogenic *invA* gene of *Salmonella* successfully amplified 211 bp PCR amplicons (Fig. 1). Out of 134 *Salmonella* isolates, 134 isolates (100.0 %) were found to be pathogenic strains of *Salmonella*.

The PCR-based approach has been successfully used by (Ogunremi et al., 2017) [25] for the detection of *invA* genes in *Salmonella* species isolation and identification. *Salmonella* from hospital sewage carrying the invasion *invA* gene in the present study may indicate that poor sanitation and fecal contamination contain several species of *Salmonella*. In this study, out of 134 *Salmonella* isolates, 107 (79.8%) were found positive for the *invA* gene of *Salmonella* (Oliveira et al., 2003) [25] found the presence of *invA* gene in all 102 *S.* Enteritidis isolates isolated from poultry, pigs, humans, and food using multiplex PCR. (Bhatta et al., 2007) [26] detected the *invA* gene in all 54 *Salmonella* isolates. In the present study, the prevalence of the emerging *invA* genes of *Salmonella* from the chicken carcass was 26% in contrast to the study of (Elkhadragyet al. 2020) [27] reported a moderate prevalence of these genes. (Yanestria SM et al., 2019) [20] have found 4 out of 34 *invA* positive *Salmonella* spp.

Antibiotic Susceptibility Tests

The results of the antibiotic susceptibility test are presented in Table 2. MICs obtained from every antibiotic generated by the VITEK 2 system were compared with CLSI (2020 & 2009) [28] in Table 2. Resistant, intermediate, and susceptible categories were also considered for every MIC based on the recent CLSI breakpoint criteria. Category agreement (CA) was also defined by calling range and breakpoint. Individual antimicrobial data are presented in Tables 2 and 3, and the MIC, VME, ME, and mE are shown in Table 2.

In total, 134 isolates were tested for antibiogram after identification. Out of 134 tested isolates, 7 serovar showed VMEs while interpreting MIC values using the VITEK 2 breakpoint eventually, but before a repeat test, 11 organisms revealed VMEs. The final ME shown organisms are 15, but 19 samples were proven to be ME before repeat testing. All initial errors were attributed to the breakpoint of VITEK 2. So the repeated correction rate was respectively 64% and 79% for VME and ME. 67 (82%) mE were revealed even after repetitive tests of initial 82 isolates against all 18 antibiotics. Hence, an overall 98.86% EA, and 95.95% CA for all 134 microbes versus antimicrobial results were examined. Moreover, 7 VMEs, 15 MEs and 67 mE were revealed for the VITEK 2 according to CLSI M100 breakpoints.

Essential agreement percentages for resistance and susceptibility to different antibiotics are as follows (Table 2)-Amoxicillin-100 %, Ampicillin-100%, Piperacilllin-92%, Cephalothin-100%, Cefazolin-100%, Cefoxitin-100%, Cefuroxime-99%, Cefpodoxime-96%, Ceftazidime-98%, Ceftriaxone-100%, Aztreonam-96%, Gentamicin-100%, Tobramycin-100%, Ciprofloxacin-100%, Levofloxacin-100%, Tetracycline-100%. Complete identification results were obtained within a maximum of 4 h but susceptibility testing required 6-15 h to complete. For individual antibiotics and serovar, data are shown in Tables 2 & 3. For the resistant summary, Figures 2, 3 and 4 glimpse the summary.

The FDA (Food and Drug Administration) has approved the minimum performance characteristics to evaluate the AST test. The guideline reveals that CA must be ≥90%, ME must be ≤3%, VME must be ≤1.5% and acceptable mE rates are ≤10% [29, 30]. The detection of *Salmonella* spp. with the VITEK 2 ID-GN card was comparatively accurate. After identification, our AST was done by an AST-GN 72 card. Even though we found some mE, ME, and VME, most of them were within FDA guidelines. VME ranged from 0 to 1.5 except for Nitrofurantoin. If we consider ME and mE of Nitrofurantoin, every value was within the FDA threshold. Only Nitrofurantoin showed a trend of exceeding

the upper limit, nonetheless, the other 17 antibiotics decapitated within value and most importantly, 13 antimicrobials did not have any VME. Our results agreed with the trend, few studies reported the evaluation of the performance of the VITEK 2 AST card for *Salmonella* spp. Their Essential Agreement and Category Agreement are in conformity with our results [31].

The assessment of the VITEK 2 system for antimicrobial susceptibility and identification of pathogenic microbes like *Enterobacterales* was performed with a collection of microbes that represented the performance of VITEK 2. Usually, we perform AST through the Kirby-Bauer disc diffusion assay. This method has some shortcomings like inconsistency, handling errors, subjectivity, and human error. The automated fluorescence-based technology of VITEK 2 gives us more comfort without human error [5]. AST-GN 72 card, which consists of piperacillin/tazobactam (extra spectrum β-lactamase) was not claimed by bioMerieux until it was found relevant to *Escherichia* and *Klebsiella* species [29]. Even ESBL may not exclude *S.* Enterica from AST because some *S.* Enterica *are* found to be resistant to cephalosporins in a different region of the world and are capable of producing extra spectrum β-lactamase. In our study, VITEK 2 was able to detect all isolates from cultural characteristics except two isolates. This might happen because of MacFarland preparation, or because more than one colony was taken through the loop. Essential agreement and categorical agreement were found to be better than Kim et al. [32].

Results found for different serotypes of *Salmonella* through VITEK 2 had similarities with MIC calling references, CLSI references, and other research findings [33]. No VME or ME was found for amoxicillin, ampicillin, cephalothin, cefazolin, cefuroxime, cefpodoxime, ceftriaxone, tetracycline, and trimethoprim, but MICs for *S. typhimurium*, *S. paratyphi*, *S. enteritidis*, *S. enterica*, *S. typhi*, and *S. gallinarum* are high in several cases. VITEK 2 pursues the reference of CLSI [28, 34]. Concerning, Tables 2 and 3, MIC values and interpretation of *Salmonella* isolates to amoxicillin, ampicillin, piperacillin, cephalothin, cefazolin, cefoxitin, cefuroxime, cefpodoxime, ceftazidime, ceftriaxone, aztreonam, gentamicin, tobramycin, ciprofloxacin, levofloxacin, tetracycline, nitrofurantoin, and trimethoprim as resistant because of only *in vitro* efficaciousness rather than *in vivo* effectiveness. This resistant result should not be reported as susceptible, according to CLSI [34, 35].

Ampicillin-resistant serotypes of *S. enterica*, *S. typhimurium* and *S. enteritidis* were reported by VITEK 2 on AST GN-72. The main cause might be the regular use of ampicillin for gastroenteritis patients [36]. Amoxicillin-resistant serovar of *S. enterica, S. typhimurium, S. enteritidis*, and *S. typhi* were also detected by VITEK 2, as shown in Table 3. Other antimicrobial drugs like piperacillin, cephalothin, cefazolin, cefoxitin, cefuroxime, cefpodoxime, ceftazidime, ceftriaxone, aztreonam, gentamicin, tobramycin, ciprofloxacin, levofloxacin, tetracycline, nitrofurantoin and trimethoprim were shown to be resistant by VITEK 2 for different serotypes (Table 3). Notably, cephalosporin (cephalothin, cefazolin, cefoxitin, cefuroxime, cefpodoxime, ceftazidime, and ceftriaxone) are commonly employed as therapeutic agents in humans. The advent of cephalosporin-resistance was attributed to plasmid-mediated resistance to AmpC (CMY-2)-β lactamase. Even third and fourth-generation cephalosporin e.g.- cefpodoxime, ceftazidime and ceftriaxone along with ciprofloxacin have become resistant which is commonly used for invasive non typhoid *Salmonella* infection and complex salmonellosis (Table 3). The main cause is probably the production of class A ESBLs and class C cephalosporinases in *S. enterica* serotypes. The VITEK 2 AST GN-72 card ESBL test

seems to have overall resistance to large-spectrum cephalosporins, this trend has also been reported by other researchers [37]. This phenomenon was raised because of the overuse of carbapenems. The increased emergence of *Salmonella* resistance to common antimicrobial agents all over the world has caused a huge therapeutic impendence on medicine and healthcare.

Gentamicin is an aminoglycoside that binds to the bacterial 30S ribosome and inhibits protein synthesis, these aminoglycosides have ample activities than gentamicin [38]. Merkevičienė et al. [38] was able to identify gentamicin resistant isolates of *Salmonella* serovar from animals. The study reveals that gentamicin-resistant *Salmonella* serotypes evolved in bovine animals.

Trimethoprim-resistant *S. enterica* was isolated by Chu et al. [39] from Taiwan. Trimethoprim-sulfamethoxazole resistant *S. enterica* was isolated in this study using VITEK 2 and the AST GN-72 card. The development of mobile genes mediates resistance to trimethoprim, and a clinical resistance phenotype (not evaluated in this work) requires the *dfr* gene. The expression of the enzyme DHFR (dihydrofolatereductase) also plays a role in inducing resistance. Sulfamethoxazole-resistant organisms generally exhibited the sulfamethoxazole-resistance genes, primarily in *S. typhimurium*, S. *paratyphi*, and *S. enteritidis*. All other serotypes, however, exhibited varying levels of antibiotic resistance. Other resistance genes, which were not examined in this study, might mediate this resistance. Because of the rising resistance of non-typhoid *S. enterica* serotype to sulfamethoxazole-trimethoprim, trimethoprim is no longer considered a safe weapon against invasive salmonellosis. At this time, cephalosporins and fluoroquinolones are prescribed for treatment against ampicillin and sulfamethoxazole-trimethoprim-resistant strain [40].

Table 1. *Analysis of the VITEK 2 system for identification of Salmonella spp. Four levels of identification with probability are shown here.*

Species	No. of strain tested	Level of identification				
		Excellent [a]	Very good [a]	Good [a]	Acceptable [a] (Low discrimination)	Unidentified
Salmonella typhimurium	33	16	10	3	4	
Salmonella paratyphi	15	7	4	2	2	
Salmonella enteritidis	52	24	15	7	6	
Salmonella enterica	14	6	4	2	2	2
Salmonella typhi	16	9	4	0	3	
Salmonella gallinarum	2	1	1	0	0	
Salmonella arizonae	2	1	1	0	0	

Which strains were identified as "low discrimination" resolved by one and/or two simple tests (motility and serological test). [a] *Identification level: excellent (96-99 % probability, very good (93-95 % probability), good level (89-92 % probability), acceptable level (85 -88% probability)*

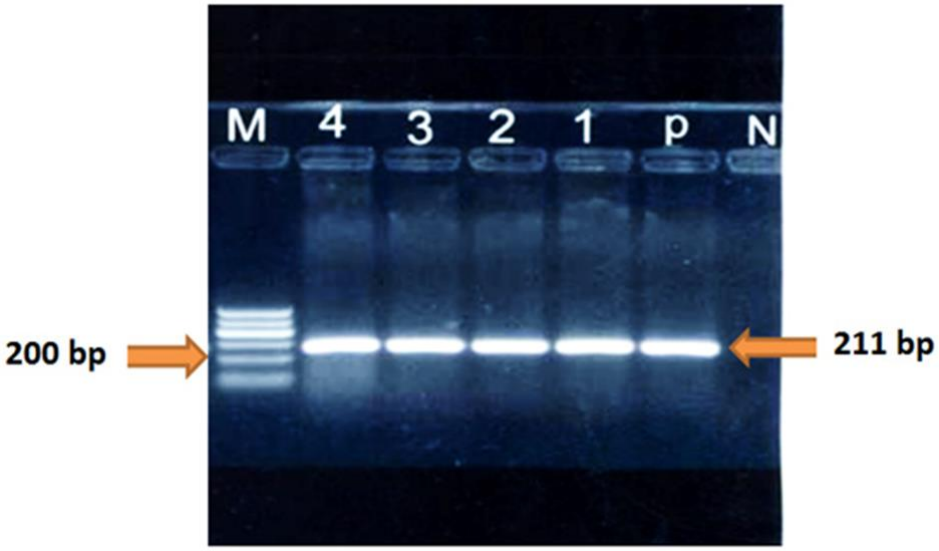

Fig. 1. *PCR assay for amplification of invA gene of Salmonella spp. Lane M: 100 bp size DNA ladder (Add Bio, Daejon, South Korea), lane (1–4): DNA extracted from Salmonella spp. Lane 5: Negative control.*

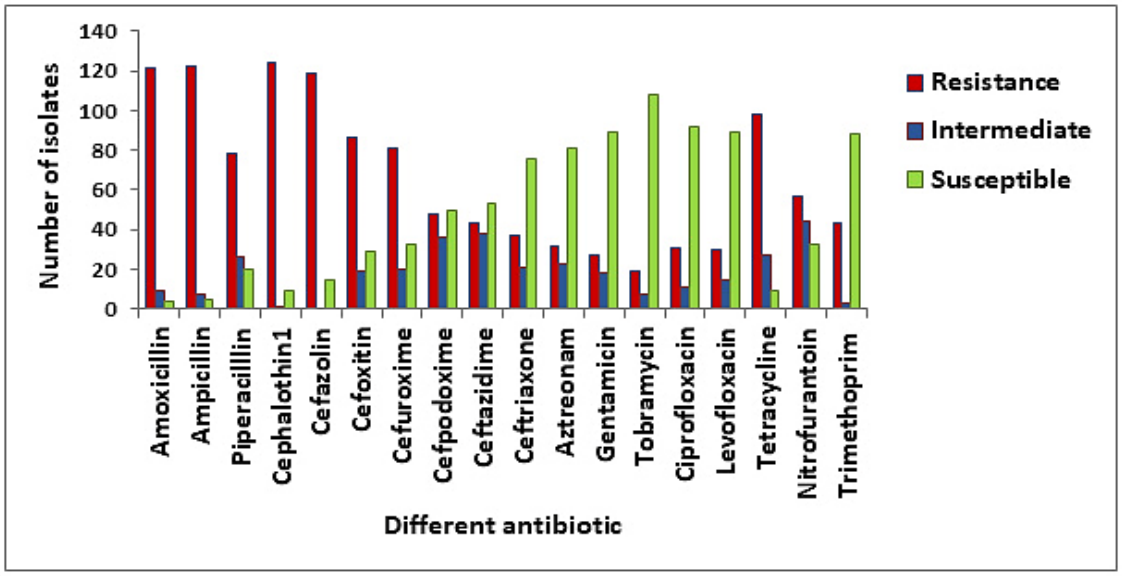

Fig. 2. *Category agreement according to Minimum Inhibitory Concentration determined by VITEK 2. CLSI 2020 and 2009 (Only for Cephalothin) breakpoint was taken into consideration.*

Table 2. *Details of essential agreement and category agreement of isolated Salmonella spp. against AST-GN72 card considering CLSI reference method.*

Class	Antibiotics	MIC calling range	Breakpoint (CLSI 2020) [1, 2]			No. of isolates [2]				No. (%) of [3]				
			S	I	R	Total	R	I	S	EA	CA	VME	ME	mE
Aminopenicillin/ Inhibitor combination	Amoxicillin/ Clavulanic Acid	2/1-32/16	≤8/4	16/8	≥32/16	134	121	9	4	134(100)	132(98.5)	0(0)	0(0)	2(1.5)
	Ampicillin	2 - 32	≤8	16	≥32	134	122	7	5	134(100)	134(100)	0(0)	0(0)	0(0)
Ureidopenicillin/ inhibitor combinations	Piperacillin/ Tazobactam	4/4 - 128/4	≤16	32-64	≥128	134	78	26	20	123(91.7)	112(83.5)	0(0)	1(0.07)	10(7.4)
Cephalosporin I	Cephalothin[1]	2 - 64	≤8	16	≥32	134	124	1	9	134(100)	134(100)	0(0)	0(0)	0(0)
	Cefazolin	4 - 64	≤2	4	≥8	134	119	0	15	134(100)	134(100)	0(0)	0(0)	0(0)
Cephalosporin II /Cephamycin	Cefoxitin	4 - 64	≤8	16	≥32	134	86	19	29	134(100)	131(97.7)	1(0.07)	0(0)	2(1.5)
	Cefuroxime	1 - 64	≤8	16	≥32	134	81	20	33	132(98.5)	132(98.5)	0(0)	0(0)	5(3.7)
Cephalosporin III / IV	Cefpodoxime	0.25 - 8	≤16	-	≥32	134	48	36	50	128(95.5)	126(94.1)	0(0)	0(0)	8(5.9)
	Ceftazidime	1 - 64	≤8/4	-	≥16/4	134	43	38	53	131(97.7)	127(94.7)	0(0)	2(1.4)	5(3.7)
	Ceftriaxone	1 - 64	≤1	2	≥4	134	37	21	76	134(100)	128(95.5)	0(0)	0(0)	6(4.4)
Monobactam	Aztreonam	1 - 64	≤4	8	≥16	134	32	23	81	129(96.2)	124(92.5)	0(0)	3(2.2)	7(5.2)
Aminoglycoside	Gentamicin	1-16	≤4	8	≥16	134	27	18	89	134(100)	131(97.7)	2(1.5)	0(0)	1(0.07)
	Tobramycin	1-16	≤4	8	≥16	134	19	7	108	134(100)	132(98.5)	0(0)	1(0.07)	1(0.07)
Fluroquinolone	Ciprofloxacin	0.25 - 4	≤0.25	0.5	≥1	134	31	11	92	134(100)	129(96.2)	0(0)	2(104)	3(2.2)
	Levofloxacin	0.12 - 8	≤0.5	1	≥2	134	30	15	89	134(100)	131(97.7)	1(0.7)	1(0.07)	1(0)
Tetracyclines	Tetracycline	1-16	≤4	8	≥16	134	98	27	9	134(100)	132(98.5)	0(0)	0(0)	2(1.5)
Miscellaneous	Nitrofurantoin	16 - 512	≤32	64	≥128	134	57	44	33	134(100)	112(83.5)	3(2.2)	5(3.7)	14(10.4)
	Trimethoprim /Sulfamethoxazole	20(1/19)-320(16/304)	≤2/38	NA	≥4/76	134	43	3	88	134(100)	134(100)	0(0)	0(0)	0(0)

1 The breakpoint of Cephalothin is according to CLSI (2009)-M100-S19, the rest of them are in pursuance of CLSI (2020)-M100

2 R=resistant; I=intermediate; S=susceptible.

3 EA=essential agreement; CA=categorical agreement; VME=very major error; ME=major error; mE=minor error.

Table 3. *MIC range of all Salmonella serovars comparing with CLSI Breakpoint. Resistant isolates are counted based on VITEK 2 AES generated report.*

Antimicrobial agents	CLSI susceptibility breakpoint for *Salmonella* spp[c].			Susceptibility information from VITEK 2													
				Salmonella Typhimurium		*Salmonella* Paratyphi		*Salmonella* Enteritidis		*Salmonella* Enterica		*Salmonella* Typhi		*Salmonella* Gallinarum		*Salmonella* Arizonae	
	S	I	R	MIC range	R[d]	MIC range	R[d]	MIC range	R[d]	MIC range	R[d]	MIC range	R[d]	MIC range	R[d]	MIC range	R[d]
Amoxicillin/ Clavulanic Acid[a]	≤8 /4	16/8	≥32/16	0.015-32	29	0.015-16	13	0.015-32	47	0.03-16	12	0.015-16	16	0.015-32	2	0.12-32	2
Ampicillin[a]	≤8	16	≥32	0.015-32	27	0.015-32	14	0.03-32	49	0.015-16	13	0.015-32	17	0.03-64	1	0.015-32	1
Piperacillin/ Tazobactam[a]	≤16	32-64	≥128	0.03-64	18	0.03-32	10	0.12-32	31	0.03-32	7	0.03-32	9	0.015-32	2	0.03-64	1
Cephalothin[b]	≤8	16	≥32	0.03-32	30	0.015-32	13	0.015-16	49	0.015-32	12	0.015-32	16	0.03-32	2	0.12-16	2
Cefazolin[a] (blood)	≤2	4	≥8	0.015-16	29	0.015-16	14	0.015-32	46	0.015-32	13	0.015-16	14	0.03-16	2	0.03-32	1
Cefoxitin[a]	≤8	16	≥32	0.12-32	21	0.12-32	10	0.12-64	33	0.12-16	9	0.12-32	10	0.03-32	1	0.12-32	2
Cefuroxime[a] (parental)	≤8	16	≥32	0.015-32	20	0.015-16	9	0.015-32	32	0.03-16	8	0.015-16	9	0.015-32	2	0.015-32	1
Cefpodoxime[a] surr.cefazoln	≤16	-	≥32	0.015-32	14	0.03-16	6	0.03-32	18	0.015-16	5	0.03-16	4	0.015-32	0	0.03-32	1
Ceftazidime (avibactam)[a]	≤8 /4	-	≥16/4	0.015-32	13	0.015-32	5	0.015-16	16	0.015-32	4	0.015-32	3	0.03-32	1	0.015-16	1
Ceftriaxone	≤1	2	≥4	0.015-16	11	0.015-16	5	0.03-16	14	0.015-32	3	0.015-16	4	0.12-32	0	0.03-16	0
Aztreonam[a]	≤4	8	≥16	0.015-16	9	0.12-16	4	0.12-32	12	0.03-32	3	0.12-16	3	0.03-32	1	0.12-16	0
Gentamicin[a]	≤4	8	≥16	0.12-32	7	0.12-32	3	0.12-64	10	0.03-32	2	0.12-32	3	0.015-64	1	0.03-16	1
Tobramycin[a]	≤4	8	≥16	0.015-32	5	0.015-32	2	0.03-32	8	0.015-32	2	0.015-32	2	0.03-16	0	0.015-16	0
Ciprofloxacin[a]	≤0.25	0.5	≥1	0.12-32	8	0.12-16	4	0.03-16	12	0.12-32	4	0.12-16	2	0.03-32	1	0.12-32	0
Levofloxacin[a]	≤0.5	1	≥2	0.015-16	7	0.015-16	3	0.015-32	11	0.015-32	3	0.015-16	5	0.03-16	0	0.015-32	1
Tetracycline[a]	≤4	8	≥16	0.015-32	23	0.015-32	9	0.03-32	42	0.015-32	10	0.015-32	12	0.03-32	1	0.12-16	1
Nitrofurantoin[a]	≤32	64	≥128	0.015-16	14	0.015-32	6	0.03-16	21	0.015-16	6	0.015-32	7	0.015-64	2	0.015-16	1
Trimethoprim/ Sulfamethoxazole[a]	≤2 /38	NA	≥4/76	0.03-32	10	0.03-64	5	0.12-32	16	0.03-16	5	0.03-64	4	0.015-32	1	0.12-64	2

[a] CLSI (2020) Performance Standards for Antimicrobial Susceptibility Testing; M100. [b] CLSI (2009) Performance Standards for Antimicrobial Susceptibility Testing; M100-S19.[c] Breakpoint of Salmonella spp. is parallel to Enterobacterales [d] Number of resistant as interpretation of VITEK 2 automated report.

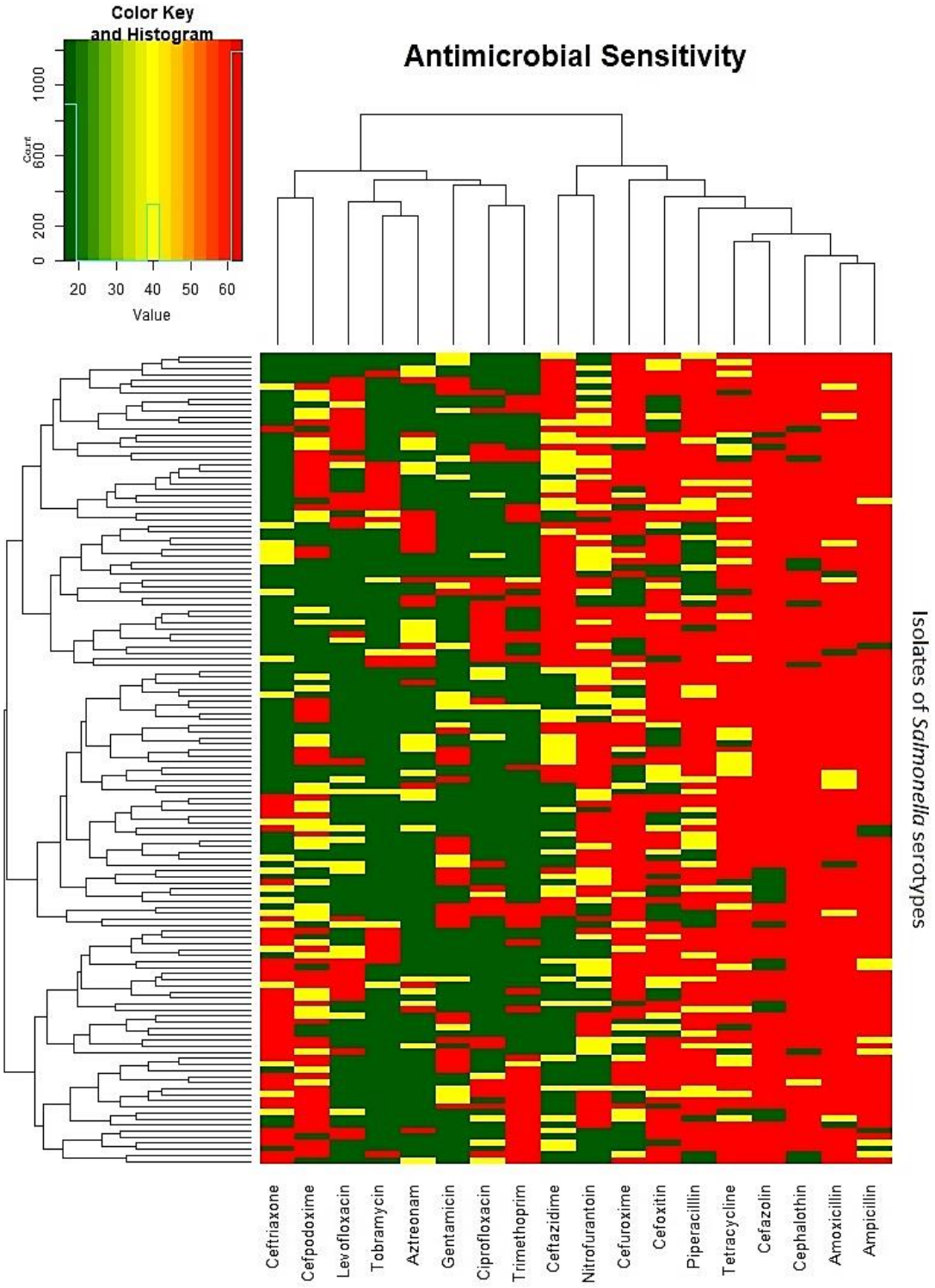

Fig. 3. *Heatmap analysis of the antibiotic susceptibility pattern. Column represent antibiotics and rows represent salmonella serotypes; here red blocks reveal resistance, green blocks reveal the susceptibility and yellow blocks indicate intermediate action according to obtained minimum inhibitory concentration.*

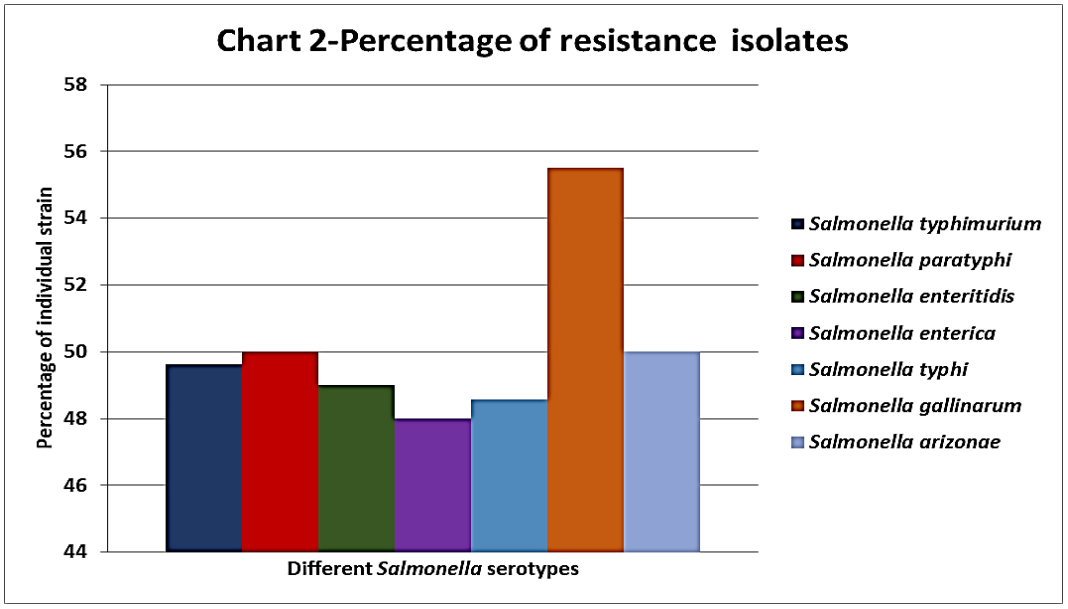

Fig. 4. *Percentage of resistance isolates of Salmonella serotypes according to CLSI breakpoint.*

Because of our limited accessibility, we were unable to collect more samples from a few more hospitals. We were limited to using only one automated system and a single type of AST card. We could exert elaborate patterns on a variety of antibiotics if we had more AST cards. However, healthcare professionals will be able to implement and assess the right treatment plan in line with our accurate data. This scientific study on the menace of superbugs is beneficial for immunization in immunosuppressed patients.

CONCLUSION

In conclusion, this study revealed that *S. typhimurium*, *S. paratyphi*, *S. enteritidis* were prominence serotypes among others. VITEK 2 accurately identified ampicillin and glycopeptide resistance, as well as high levels of gentamicin and streptomycin resistance, among these serovar. Exceptionally high level of resistance was shown in response to amoxicillin, ampicillin, cephalothin, cefazolin, and tetracycline. This study will help to identify the right antibiotic because carbapenem and ESBL-resistant *Enterobacteriaceae* have become worrisome. Finally, to attain better public health outcomes on this critical issue, this study will help to take a positive approach to global antibiotic research considering the current situation of a megacity.

Supplementary Tables.

The substrates of ID-GN card are as follows: Ala-Phe-Pro-Arylamidase, adonitol, L-pyrrolydonyl-arylamidase, L-arabitol, D-cellobiose, β-galactosidase, H_2S production, β-n-acetyl-glucosaminidase, glutamyl arylamidasepNA, D-glucose, γ-glutamyl-transferase, fermentation/glucose, β-glucosidase, D-maltose, D-mannitol, D-mannose, β-xylosidase, β-alanine arylamidasepNA, L-proline arylamidase, lipase, palatinose, tyrosine arylamidase, urease, D-sorbitol, saccharose/sucrose, D-tagatose, D-trehalose, citrate (sodium), malonate, 5-keto-D-gluconate, L-lactate alkalinization, α-glucosidase, succinate alkalinization, β-N-acetyl-galactosaminidase, α-galactosidase, phosphatase, glycine arylamidase, ornithine decarboxylase, lysine decarboxylase, decarboxylase base, L-histidine assimilation, coumarate, β-glucuronidase, O/129 resistance (comp.vibrio.), Glu-Gly-Arg-arylamidase, L-malate assimilation, ellman, L-lactate assimilation.

The composition of PCR master mixture (25 µL) and PCR thermal profile: Nuclease free water- 6.5 µL, 2X PCR Master Mix (ADD Bio, Daejon, South Korea)- 12.5 µL, primers (forward)-1.0 µL, primers (reverse)- 1.0 µL, template (extracted DNA)-4.0 µL.

Class of antibiotics in AST-GN72 card: Aminopenicillin/combination, Ureidopenicillin/inhibitors combinations, Cephalosporin I, Cephalosporin II /Cephamycin, Cephalosporin III/IV, Carbapenem, Aminoglycoside, Fluroquinolone, Tetracyclines, Nitrofurantoin, Trimethoprim /Sulfamethoxazole [23].

Antibiotic includes in AST-GN72: Amoxicillin, Ampicillin, Piperacilllin, Cefalotin, Cefazolin, Cefoxitin, Cefuroxime, Cefepime, Cefpodoxime, Ceftazidime, Ceftriaxone, Gentamicin, Tobramycin, Ciprofloxacin, Levofloxacin, Tetracycline, Nitrofurantoin, Trimethoprim antibiotics [23].

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Conflict of Interest. The authors declared that there is no conflict of interest.

Authorship Contributions. Concept: M.S., Design: M.S., Data Collection or Processing: M.S., F.A., Analysis or Interpretation: M.S., F.A., Literature Search: F.A., Writing: F.A., M.S.

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