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SCREENING FOR OXACILLIN RESISTANT SALMONELLA SPECIES ISOLATED FROM PALMS OF SOME PRIMARY SCHOOL PUPILS WITHIN KADUNA SOUTH LGA, NIGERIA

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ABSTRACT

Salmonella species is the causative agent of typhoid fever which is a disease characterized by high mortality and morbidity worldwide. This study was carried out to screen oxacillin resistant-*Salmonella* species isolated from palms of some primary school pupils and food vendors within Kaduna South LGA, Nigeria. Three hundred (300) swab samples were collected from pupils and food vendors of the three selected primary schools as the sampling location. *Salmonella enterica* was isolated and identified using standard bacteriological methods. Isolates were susceptible to ciprofloxacin 28 (70.0%), Augmentin® 25 (65.0%) amoxicillin 25 (62.5%) and vancomycin 22 (55.0%) but showed resistance to oxacillin 9 (22.5%), ampicillin 12 (30.0%), chloramphenicol 15 (37.5%) and tetracycline 16 (40.0%). Antibiotics that exhibited intermediate susceptibility were methicillin 8 (20.0%) and gentamicin 11 (27.5%). Ciprofloxacin was the most effective antimicrobial agent against *Salmonella enterica.* The presence of oxacillin-resistant *Salmonella enterica* was detected via Polymerase Chain Reaction (PCR) and agarose gel electrophoresis. The study recommends that regular monitoring of antibiotic susceptibility pattern and good hygiene practices such as hand washing with soap and water and the use of alcoholic based hand sanitizers should be encouraged.

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INTRODUCTION

Salmonella species are harbored in the intestinal tract of humans and farm animals. Reptiles and insects also act as *Salmonella* reservoirs. Moreover, eggs, poultry meat, pork, beef, dairy products, nuts, vegetables and water act as sources of *Salmonella*. The risk of infection is high in low- and middle-income countries or societies, with more than 100 infections per 100 000 people per year [1]. Some *Salmonella* serotypes are host-specific, while others can infect more than one type of warm-blooded animal [2]. The serology of *Salmonella* is based

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on the Kauffmann-White scheme, which differentiates *Salmonella* serovars by the surface antigen differences of the somatic (O) and flagella (H) antigens [3]. This serologic method has been used to identify *Salmonella* serovars; however, this method is labor-intensive, expensive, complicated, and time-consuming. Therefore, a more rapid, simple subtyping or identification method of the *Salmonella* serovars is needed. Polymerase Chain Reaction (PCR) has the potential of being a powerful alternative in microbiological diagnostics because of its simplicity, rapidity, and accuracy [4]. Many studies use PCR to detect *Salmonella* and several target genes of *Salmonella* have been reported including the invA gene, which has mostly been used as a target gene of the *Salmonella* genus [5].

Penicillinases-resistant penicillins exert a bactericidal action against penicillin susceptible microorganisms during the state of active multiplication. All penicillins inhibit the biosynthesis of the bacterial cell wall. Resistance to penicillin may be mediated by destruction of the beta-lactam ring by a beta-lactamase, altered affinity of penicillin for target, or decreased penetration of the antibiotic to reach the target site. Resistance to oxacillin (cefoxitin) implies resistance to all other beta-lactam agents, except newer agents with activity against methicillin-resistant *Staphylococcus aureus* [6].

Most schools in some of the under-developed countries lack social facilities such as adequate water, waste bins, and hand washing facilities such as soaps, hand sanitizers and running tap water [7]. Wells are not properly covered, most especially during raining season where there could be washing off of dirt from environment into the well water, thereby making the water not suitable for drinking or usage [8, 9]. Lack of toilets in school premises could lead the pupils and food vendors into defecating in an open environment and this could also be washed down to the uncovered well water. Where these facilities are available, they may be inaccessible to the pupils. Therefore, lack of these facilities endangers the school pupils thereby leading them to easily get contracted with these diseases causing agents such as *Salmonella*, *Escherichia coli Yersienia entercolitica*, *Klebsiella pneumonia* and *proteus* which belong to the family of enterobacteriaceae that are of multi-drugs resistant [10]. The aim of this study was to assess occurrence of oxacillin-resistant *Salmonella* species isolated from palms of some primary school pupils and food vendors within Kaduna South LGA.

MATERIALS AND METHOD

Study Area

Three primary schools were used for this study, the schools were; Barnawa Government Primary School, Television Government Primary School and Sabon Tasha Government Primary School, all within Kaduna South Local Government Area of Kaduna State. [Kaduna State is located at Tafawa Balewa Way Kaduna]. Kaduna State is located on latitude 10.53^{o}N and longitude 7.44^{o}E with an elevation of 626m above sea level. It has an annual rainfall range of between 200 mm to 600 mm. The state is in the north-western part of Nigeria.

Ethical Approval

Ethical approval was obtained from the State Universal Basic Education Board (SUBEB), under Kaduna State Ministry of Education with the approval number; KD/SUBEB/271/T. Informed consent form was also obtained from headmasters, pupils and staff of the selected primary schools before the sample collection.

Sample Size

The sample size of the study was determined using attribution sample size formula to determine sample size of population. Sample size formula

$$n' = \frac{NZ^2p(1-p)}{d^2\,(N-1)+Z^2p(1-p)} \quad [11]$$

where
n' = sample size with finite population correction,
N = Population size,
Z = Z statistic for a level of confidence,
P = Expected proportion (in proportion of one), and
d = Precision (in proportion of one).
The prevalence rate of 5.0% encountered in Kaduna State [12] was used.

Samples Collection

Sterile swab sticks were moistened with sterile normal saline, and it was used to swap the left and the right hand palms of the pupils and food vendors to ensure all area of the palms were covered and the samples were dislodged into sterile test tubes containing sterile peptone water each. It was then shaken vigorously and was covered tightly. The samples were labeled accordingly with the initials of the site of samples collection as well as initials of either male or female. All sample collections were done using aseptic techniques. A total number of three hundred (300) samples were collected for this study, from primary school pupils and food vendors within the three selected primary schools. Samples collected were transported immediately to the medical laboratory, Department of Microbiology Kaduna State University for analysis.

Isolation and Identification of *Salmonella enterica* from Pupils and Food Vendors Palms

Salmonella enterica were isolated and identified according to the procedures in the Bacteriological Analytical Manual of USA-FDA [13, 14]. Samples were pre-enriched in buffered sterile peptone water. After pre-enrichment, 1 mL portion was transferred into 9 mL selenite F broth which is a selective enrichment medium of *Salmonella* species. A wire loop was sterilized by holding it on a flame until it was red hot; it was allowed to cool and then it was dipped into the enriched samples suspensions of swab samples collected from pupils and food vendors. It was then streaked on a prepared solidified sterile bismuth sulfite agar and *Salmonella shigella* agar (SSA). The procedure was repeated for all the samples collected and the plates were incubated at 37 ^{o}C for 24 h to observe growth.

Suspected *Salmonella enterica* colonies appeared with black colonies on bismuth sulfite agar and smooth colorless on SSA, some strains of *Salmonella* which produce hydrogen sulfide (H_2S) appeared with black-centered colonies on SSA too.

Antibiotic Susceptibility Test of *Salmonella* Species Isolated from Palms of Pupils and Food Vendors

The antimicrobial susceptibility pattern of the bacterial isolates was evaluated using the Kirby-Bauer disk diffusion method. Oxoid UK, Antibiotic disks containing Vancomycin (30 µg) (VAN), Methicillin (MET) (1 µg), Chloramphenical (30 µg) (C), Gentamicin (10 µg) (GEN), Tetracycline (30 µg) (TET), Amoxicillin (30 µg) (AMC), Augmentin (30 µg) (AUG), Ofloxacin (5 µg) (OF) Oxacillin (30 µg) (OXa) and Ciprofloxacin (10 µg) (CIP), were used. The medium used was Mueller Hinton agar (MH) which was prepared according to the manufacturer's instruction. Twenty milliliter (20 mL) of the prepared medium was poured into sterile Petri dishes and allowed to solidify. After this, the Mueller Hinton agar was inoculated by streaking the organisms using a sterile wire loop. The antibiotic disks were aseptically placed using sterilized forceps on the inoculated plates and gently pressed to ensure complete contact with the agar surface. The agar plates were left on the bench for 30 minutes to allow diffusion of antibiotics and the plates were incubated at 37 °C for 24 h. The diameter of the clear zones of inhibition around each antibiotics disk was measured using meter rule in millimeters (mm) and it was recorded. The means zones of inhibition were compared with the standard chart to determine whether the test organism was resistant (≤10 mm), intermediate (11-12 mm) or susceptible (≥13 mm) based on the Clinical and Laboratory Standards Institute [15].

Screening of Oxacillin-Resistant *Salmonella* species using Polymerase Chain Reaction (PCR) [16]**:**

a. Phenol-Chloroform Bacteria DNA Extraction Protocol

The DNA of the overnight culture of *Salmonella enterica* was extracted using phenol-chloroform method [17]. A loop of the test organism was suspended in 1.5 mL microfuge tubes containing 200 mL of nuclease free water. The tubes were transferred to water bath and it was heated at 95 °C for 30 min, the tubes were placed on ice for 2 min for rapid cooling. The cooled tubes were transferred to a refrigerator microfuge centrifuge and spinned at 12,000 rpm for 5min. The supernatant was transferred in to 1.5 micro tubes, and then the tubes were spinned at 14,000 rpm for 5 min and the supernatant was discarded carefully without interrupting the pallets. Two hundred micro liters of 70% ethanol was added to the tubes. The tubes were inverted five times and centrifuged at 14,000 rpm for five 5 min and the supernatant was discarded. The deposited pallets were dried at 55 °C in a hot air oven. One hundred mL (100 mL) of nuclease, free water was added into the tubes to re-suspend the pallets and the tubes were stored at 20 °C until used.

b. Polymerase Chain Reaction (PCR) for 16S rRNA gene of *Salmonella* species

The primer sequence (5'-3') used for the amplification of the *Salmonella enterica* was, 16S Ribosomal RNA Universal primer; (22F-CGAGCAGCCGCTTAGTATTGAG; 1492R-CCATCAAATTAGCGGAGGCTTC). The reaction was carried out in 20µl reaction mixture containing 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each d NTP, 2µl taq DNA polymerase 20pMol of each primer and sterile water was used to make up the reaction mixture. Denaturation was done at 95 °C for 5 min, and it was followed with 30 consecutive cycles of denaturation at 95 °C for 1 min, annealing was done at 55 °C for 40 sec, then extension at 72 °C for 5 min on a kryatech super cycler Trinity Thermal cycler.

c. Agarose Gel Electrophoresis of the 16S rRNA gene of *Salmonella* species Fragments

PCR fragments were separated on agarose gel and the DNA ladder was used as a DNA molecular weight maker; electrophoresis was carried out at 80V for 1 h 30 min. The gel was viewed under UV light wavelength after fluorescent dye staining. The ladder on the agarose gel indicates the base pair fragment of the DNA of the bacteria.

RESULTS

The result shows that, out of the 300 samples collected, 40 samples (13.3%) were positive for *Salmonella* specie which appears black colonies on bismuth sulfite agar and transparent, smooth colorless colonies, some with black centers on Salmonella Shigella Agar (SSA) due to the production of hydrogen sulfide (H_2S) by some of these *Salmonell*a species. And because some are non-lactose fermenters, they produced non-lactose fermenting colonies. The colonies were bacillus in shape, arranged in chains and reacted negative on Gram stain reaction, when viewed under an electronic compound light microscope with a power lens of 100X. This is because bile salts, brilliant green, and citrates are selective agents which inhibit Gram-positive bacteria and coliforms, while sodium thiosulfate, a sulfur source, and ferric ammonium citrate, an indicator, were added to enable organisms which produce H_2S to form black-centered colonies, including some strains of *Salmonella* as given in (Table 1).

The antibiogram susceptibility pattern of *Salmonella* species against selected antibiotics shows that, the isolates of *Salmonella* species were susceptible to (50%) and resistant to (30%) of the drugs tested, while intermediate susceptibility was recorded in (20%) of the drugs. *Salmonella* isolates were susceptible to ciprofloxacin 28 (70.0%), Augmentin® 25 (65.0%) amoxicillin 25 (62.5%) and vancomycin 22 (55.0%) but showed resistance to oxacillin 9 (22.5%), ampicillin 12 (30.0%), chloramphenicol 15 (37.5%) and tetracycline 16 (40.0%). Antibiotics that exhibited intermediate susceptibility were methicillin 8 (20.0%) and gentamicin 11 (27.5) as given in (Table 2).

Table 3 shows the percentage susceptibility pattern of the isolated *Salmonella enterica* against 10 selected antibiotics. For each of the tested drug, there was a significant difference in the

susceptibility of *Salmonella enterica* isolated from the palm swabs: in each case, the proportion of susceptible *Salmonella* species colonies, were higher than those of both intermediate and resistant colonies. *Salmonella enterica* was most resistant to tetracycline 16 (40.0%) followed by chloramphenicol 15 (37.5%) and ampicillin 12 (30.0%); the least number of resistant colonies were recorded with Augmentin® 2 (5.0%). The proportion of *Salmonella enterica* with intermediate susceptibility differs between drugs: the highest number of intermediate susceptible *Salmonella enterica* was seen with Augmentin®, followed by gentamicin, vancomycin and the least seen with oxacillin. The highest number of susceptible colonies was seen with ciprofloxacin and oxacillin, followed by Augmentin®, amoxicillin and gentamycin, while the least was seen with tetracycline.

Table 4 shows the occurrence of oxacillin-resistant *Salmonella enterica* isolates obtained from palm swabs samples. Nine (9) out of the 40 *Salmonella* isolates obtained from the palm swabs samples were oxacillin-resistant, given a 22.5% overall occurrence of oxacillin-resistant *Salmonella* species isolates from the three different primary schools samples. Out of the 10 isolates obtained from palm swabs of Barnawa Government primary school samples, 2 (20.0%) were oxacillin-resistant; out of 18 isolates obtained from palm swabs of Television Government primary school, 4 (22.2%) were oxacillin-resistant and out of 12 isolates obtained from palm swabs of Sabon Tasha Government primary School, 3 (25.0%) were oxacillin-resistant.

Agarose gel bands for *Salmonella enterica* revealed that the bacterial DNA has base pair of 1500bp on a 100bp molecular ladder using a 16S ribosomal RNA primer sequences. The Agarose Gel Electrophoresis picture showing the detected and the Amplified DNA of the 16S rRNA gene of *Salmonella enterica* at 1500bp base pair is shown in (Figure 1).

DISCUSSION

The appearance of colourless colonies with black centers of *Salmonella species* on Salmonella Shigella Agar was as a result of *Salmonella* strains been capable of producing hydrogen sulfide (H_2S) which form the black dots on the centers of the colonies [18]. *Salmonella* species are lactose-non-fermenters; they form transparent, colourless colonies, with black centers on SS Agar unlike other Gram-negative bacilli which ferment lactose, produce pink to rose- red colonies [19]. The organism was found to be Gram-negative bacilli with pink in colour, appearing in chain arrangement according to Mathew *et al*. [20] has reported similar appearance of *Salmonella enterica*. In the study, the biochemical characteristics of this organism were catalase positive, citrate positive, urease positive and methyl red positive. Forty (40) isolates were positive to *Salmonella enterica* with the frequency occurrence of 13.3% while nine 9 (22.5%) out of the forty (40) isolates were oxacillin-resistant *Salmonella enterica*. This result is similar to the study carried out in Kaduna North, Kaduna State which also reported 14.5% prevalence of *Salmonella* infections in stool samples collected from primary school pupils [21]. As reported previously [22], susceptibility testing of *Salmonella* species now includes the more stable oxacillin instead of methicillin disc to test the susceptibility of the isolated strains to all lactam agents and cefoxitin for cephalosporin. These strains showed higher occurrence of resistance to macrolides (erythromycin and azithromycin). Lower sensitivity (14.6%) for erythromycin and higher sensitivity (62%) for azithromycin have been reported [23].

Ciprofloxacin and oxacillin were the antibiotics that have effect against most isolates in this study with 70.0% susceptibility (by zone of inhibition), which makes them the drugs of choice for treating multidrug resistant *Salmonella* species. Augmentin® was the second effective antibiotic after ciprofloxacin and oxacillin with 65.0% susceptibility (by zone of inhibition), similar to the report [24]. It is followed by amoxicillin and gentamicin (62.5%) respectively. Lower sensitivity to tetracycline has been reported by other authors (43.0%), and chloramphenicol (6.1%) [25]. Different sensitivity percentages have been reported to these five antibiotics worldwide [26]. These differences might be due to prolonged antibiotic treatment, age, type of infection and geographical variation.

The highest resistance was observed in tetracycline (40.0%), followed by chloramphenicol (37.5%), similar to the report from Olajoke *et al*. [23] which also recorded (76.8%) high resistance of *Salmonella* species to tetracycline. Oxacillin-resistant *Salmonella* species are bacteria that have been found to be resistant to antibiotics such as methicillin, oxacillin, penicillin ampicillin and amoxicillin. This organism had become widespread in healthcare settings, school's settings and restaurants settings globally, causing high rate of typhoid fever and other different types of infections [26, 27]. This could also be explained due to prolonged use of oxacillin antibiotic for treatment, age difference, type of infection and geographical variation, to treat or prevent disease and also to promote growth in human and animals [24], while the low resistance rate to methicillin, penicillin, ampicillin and amoxicillin in the *Salmonella* isolates could be due to the fact that some bacteria have thick cell wall that may prevent the penetration of antibiotics against the bacteria actions to human or animal body [28]. The extremely high toxicity of the antimicrobial agents in Augmentin®, ciprofloxacin, gentamicin and β-lactam classes has resulted in their overuse in the medical world, hence is the reason why they observed increased in their susceptibility. The relatively high level of resistance to antimicrobial agents in the environment depicts a true reflection of their indiscriminate and excessive usage [28]. Detection of *Salmonella* species by accurate and rapid methods is important to help select the best antibiotic for the individual patient and for control of the endemicity of *Salmonella* species as reported by Lapierre *et al*. [24].

CONCLUSION

The bacteria that was isolated from the palms of the pupils and food vendors was *Salmonella enterica*, which is one of the

causative agent of Typhoid fever. The occurrence of oxacillin resistant *Salmonella* species was detected through Gram staining and biochemical test that was carried out during the research work. It was concluded that *Salmonella enterica* has the highest susceptibility to ciprofloxacin and oxacillin with 70.0% zone of inhibition, which makes them drugs of choice for

Table 1: Morphological and Biochemical Characteristics of Suspected Bacterial isolates Obtained from Palms Swab of Some Primary Schools within Kaduna South LGA

Characteristics	Probable Organism (*Salmonella* species)
Color appearance	black colonies on Bismuth sulfite Agar and Smooth Colorless with black centers on SSA
Shape	bacillus
Arrangement	chain
Gram reaction	-
Biochemical test	
Catalase test	+
Coagulase test	+
Indole test	-
Citrate test	+
Urease test	+
Methyl red	+
Motility	-

Keys: + = positive; - = Negative

Table 2: Antibiogram Susceptibility Pattern of *Salmonella* Isolates

Antibiotics	Diameter zone of inhibition (mm)	Standard break point (mm)	Interpretation
Amoxicillin	19.00	≥18.0	S
Ciprofloxacin	18.00	≥21.0	I
Methicillin	10.00	≥18.0	R
Chloramphenicol	20.00	≥15.0	S
Vancomycin	10.00	≥24.0	R
Augmentin	14.00	≥22.0	R
Gentamicin	17.00	≥15.0	S
Ampicillin	18.00	≥21.0	I
Tetracycline	10.00	≥20.0	R
Oxacillin	10.00	≥16.0	R

Key: *S =Susceptible, I =Intermediate, R =Resistance; The standard breakpoint of each antibiotic was gotten from clinical and laboratory standard institute (CLSI) as a standard for each antibiotic (CLSI, 2022). The mean zones of inhibitions were compared with the standard chart to determine whether the test organisms were resistant (≤10.0 mm), intermediate (11.0 – 12.0 mm) or susceptible (≥13.0 mm) based on the Clinical and Laboratory Standards Institute [15].*

Table 3: Percentage Antibiotic Susceptibility Pattern of *Salmonella* species isolated from Palms of Some Primary School Pupils and food Vendors within Kaduna South LGA Using Selected Antibiotics (N=40)

Serial No	Antibiotic/disc content (µg)	Resistant {N (%)}	Intermediate {N (%)}	Sensitive {N (%)}
1	Amoxicillin(30µg)	10 (25.0)	5 (12.5)	25 (62.5)
2	Vancomycin(30µg)	8 (20.0)	10 (25.0)	22 (55.0)
3	Ampicillin(30µg)	12 (30.0)	7 (17.5)	21 (52.5)
4	Chloramphenicol(30µg)	15 (37.5)	5 (12.5)	20 (50.0)
5	Ciprofloxacin(10µg)	3 (7.5)	9 (22.5)	28 (70.0)
6	Augmentin®(30µg)	2 (5.0)	12 (30.0)	26 (65.0)
7	Gentamicin (10µg)	4 (10.0)	11 (27.5)	25 (62.5)
8	Methicillin (1µg)	10 (25.0)	8 (20.0)	22 (55.0)
9	Tetracycline(30µg)	16 (40.0)	4 (10.0)	20 (50.0)
10	Oxacillin(30µg)	9 (22.5)	3 (7.5)	28 (70.0)

Keys: N = Number of isolates tested; R = Resistant; I = Intermediate; S= Susceptible

Table 4: Occurrences of Oxacillin-Resistant *Salmonella enterica* Isolated from Palms of Some Primary School Pupils and Food Vendors within Kaduna South LGA

Schools	No. of Isolates tested	No. of Oxacillin Resistance (%)
BGP	10	2 (20.0)
TGP	18	4 (22.2)
SGP	12	3 (25.0)
Total	40	9 (22.5)

Keys: BGP = Barnawa Government Primary School; TGP = Television Government Primary School; SGP = Sabon Tasha Government Primary School

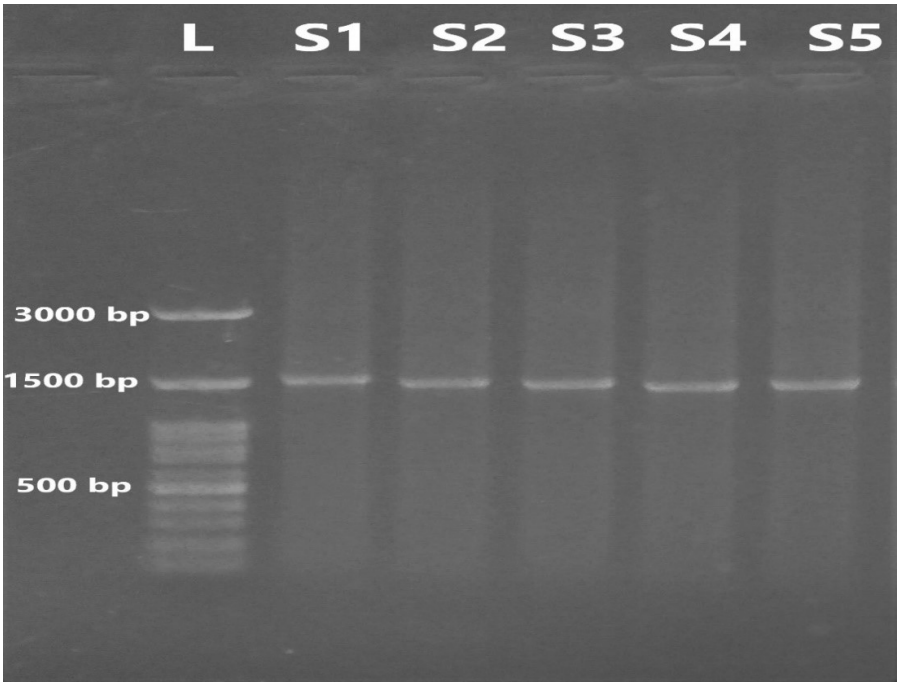

Figure I: Agarose Gel Electrophoresis Picture showing the Amplified 16S rRNA gene of *Salmonella* species at 1500bp base pair
Key: L= Molecular ladder of 100bp; S1, S2, S3, S4 and S5= represent number of samples, 1 to 5 of *Salmonella* species.

the treatment of typhoid fever, followed by Augmentin® with 65.0% and gentamicin with 62.5% susceptibility, and the lowest susceptibility was recorded to chloramphenicol with 50.0%. Oxacillin resistant *Salmonella* species was resistant to methicillin with 25.0%.

COMPETING INTERESTS DISCLAIMER

Authors have declared that no competing interests exist. Also, the research was not funded by any institution, body or government rather it was funded by personal efforts of the authors.

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