Article title: Antimicrobial sensitivity of

Salmonella species isolated from University of
Mkar Students: Susceptibility and resistance of

Salmonella species, the ubiquitous, enteric,
gram negative bacterial to broad spectrum beta lactams, aminoglycosides, and flouroquinolones.

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**ANTIMICROBIAL SENSITIVITY OF SALMONELLA SPECIES ISOLATED FROM
STUDENTS OF UNIVERSITY OF MKAR**

BY

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**A PROJECT REPORT SUBMITTED TO THE DEPARTMENT OF BIOLOGICAL
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ABSTRACT

The emergence of antimicrobial resistance amongst pathogenic microorganisms is a worrying public health issue which needs urgent fix. Several attempts have been made to overcome this problem, most recently, the advent of broad spectrum antimicrobial agents have been one of them. In as much, antimicrobial resistance seems to persist amongst different pathogenic genera due to inappropriate use of antibiotics. *Salmonella*, a causative agent of typhoid and other human systemic complications have displayed multi-drug resistance to antimicrobial agents. This research work therefore aims at investigating the antimicrobial sensitivity of *Salmonella species* isolated from University of Mkar students. A total of 50 stool samples were collected in sterile sample containers and isolation of *Salmonella* was carried out using two classical selective media, *Salmonella Shigella* Agar and MacConkey Agar. *In-vitro* antimicrobial sensitivity test was carried out following the disk diffusion method using 10 antimicrobial agents. *Salmonella species* displayed high rate of resistance (70%) while showing a worrying low rate susceptibility (30%) to Aminoglycosides, Antifolates and even broad spectrum Fluoroquinolones. *Salmonella* may have adapted, or acquired resistance inherently as it was evident in very high resistance against common antimicrobial agents like Ampicillin, Co-trimoxazole, Augmentin, and Nalidixic acid. The misuse of antibiotics and therapeutics by the population is obviously the consequential factor for the acquisition of resistance among this genus. Therefore, appropriate drug administration and usage practices must be enforced by government and public health institutions to help curtail the danger of unleashing the post-antibiotic era upon us now, and in time to come.

3.0

MATERIALS AND METHODS

3.1 Study Area

The study was carried out amongst students attending University of Mkar in Gboko Local Government Area, Benue State. Gboko is the capital of the TIV nation and constitutes the residence of the paramount Tor-Tiv ruler. It lies on latitude 7°19'30''N 9°0'18''E and longitude 7.32500°N 9.00500°E North-central Nigeria. University of Mkar, Mkar abbreviated UMM is a reform church establishment since 2005 and has a population of approximately 1300 students.

3.2 Materials

3.2.1 Instruments

The laboratory instruments needed for this work includes; autoclave, incubator, Petri dishes, Gram's reagents, wire loop, microscope slides, the microscope, beakers, distilled water, weighing balance, conical flasks, measuring cylinders, aluminum foil, Bunsen burner and antibiotic discs etc.

3.2.2 Media

Buffer peptone water was used for pre-enrichment of samples. *Salmonella Shigella* Agar (SSA) was used as the primary media, MacConkey agar was used as the secondary selective media and Nutrient agar was used for *in-vitro* antimicrobial sensitivity test. Triple Sugar Iron (TSI) agar and several other sugars were used for biochemical characterization of isolates.

3.3 Sterilization Methods

All glass wares were washed carefully, and allowed to dry; these glass wares were wrapped in aluminum foil and sterilized in the Hot air oven at 180°C for 1 hour. All culture media were also sterilized using the autoclave at 121°C for 15 minutes. The workbench was also disinfected using alcohol. All analyses were carried out closed to the naked Bunsen burner flame (Chessbrough, 2010).

3.4 Sample Collection and Size

A total of 50 stool samples were collected in sterile stool containers from students and transported to the University of Mkar's microbiology laboratory. Samples were labelled accordingly before analysis were carried out. Samples that were not immediately worked on were stored in cold chain in the refrigerator at 4⁰C (David *et al.*, 2013).

3.5 Method of Analysis

3.5.1 Sample Enrichment in Peptone Water

The stool samples were pre-enriched using peptone water. 5ml of peptone water was aspirated into a 10ml test tube and each sample was carefully inoculated into the peptone water by picking aseptically from the stool containers after flaming with a wire loop. Incubation of the pre-enriched samples was allowed for 24hrs at optimum temperature of 37⁰C (Chessbrough, 2010).

3.5.2 Sub-Culturing onto *Salmonella Shigella* Agar Plate

The pre-enriched samples were sub-cultured onto *Salmonella Shigella* Agar media using the streaking method and incubated at optimum conditions. *Salmonella* appeared as smooth pale yellow colonies with black centers.

3.5.3 Sub-Culturing onto MacConkey Agar

Suspected *Salmonella* colonies on *Salmonella Shigella* Agar were sub-cultured on MacConkey agar and incubated at optimum conditions. *Salmonella* showed colorless colonies with receding black centers.

3.5.4 Gram Staining

The Gram staining method is named after the Danish bacteriologist Hans Christian Gram (1853 –1938) who originally devised it in 1882 but published in 1884, to discriminate between

pneumococci and *Klebsiella pneumonia* bacteria in lung tissue. It is a differential staining method of differentiating bacterial species into two large groups; Gram-positive and Gram-negative based on the chemical and physical properties of their cell walls. This reaction divides the eubacteria into two fundamental groups according to their stain ability and is one of the basic foundations on which bacterial identification is built (Sandle, 2004).

Smears were prepared from the culture by emulsifying a part of a colony in a drop of normal saline on a glass slide, dried and fixed by heating. Then the slides were flooded by crystal violet for 1 minute and then washed with tap water. Iodine solution was applied for 1 minute, and then the slide was washed with tap water. The smear was then decolorized with few drops of acetone for seconds and washed immediately with water. Then the smear was flooded with safranin for 30 seconds and washed with tap water. Slides were then air dried and examined using the oil immersion objective lens. Gram-negative bacteria appeared red because they retain only the counter stain (CLSI, 2015).

3.5.5 Microscopy of Stained Isolates

Microscopy of all stained samples was carried out under the compound microscope using 100× objective lens and oil immersion. All Gram negative bacilli were subjected to biochemical tests for identification.

3.5.6 Identification of *Salmonella*

All isolates that appeared as Gram negative rods under the microscope were biochemically tested in Triple Sugar Iron Agar prepared in test tubes for sucrose, lactose, and glucose fermentation, and also for H₂S production. Catalase and oxidase test were also carried out.

3.5.7 Antimicrobial Sensitivity Test

Antimicrobial sensitivity tests are performed from single pure bacterial colonies to determine their susceptibility and resistant ability to antimicrobial agents. In vitro sensitivity tests are

carried out in different ways which include; disk diffusion test, breakpoint sensitivity test, Minimum Inhibitory Concentration (MIC) test and the Minimum Bactericidal Concentration (MBC) test.

The disk diffusion sensitivity test is performed on agar plates. A small disk of filter paper, pre-impregnated with a defined quantity of antibiotic, is placed on the surface of an agar plate that has already been inoculated with a suspension of bacteria. The antibiotic diffuses out of the disk into the agar, along a concentration gradient, as the plates are incubated for 18–24hrs. If the bacterial strain is sensitive to the antibiotic, then a zone of inhibition (no growth) occurs around the disk. The diameter of the zone depends on a number of factors which among others include;

- i. The concentration of antibiotic within the disk
- ii. The degree of susceptibility of the bacteria to the antibiotic
- iii. The physicochemical properties of the antibiotic
- iv. The depth of the agar plate
- v. The concentration of bacteria in the inoculum

Each *Salmonella* isolate was tested for susceptibility/resistance to antimicrobials on Nutrient agar following the disc diffusion method according to the recommendation of the Clinical and Laboratory Standards Institute (CLSI) using 10 commercial antibiotic discs. The antimicrobial disks were placed on the agar medium by using sterile forceps. The plates were then incubated at 37°C for 24 hours. Zones of inhibition which appeared as clean circles around the disk are recorded by measuring the diameter of the circle in millimeters. The isolates were described as resistant, intermediate and susceptible to different antimicrobial agents.

3.6 Data Analysis

Data that was derived from results of the above experiments was analyzed mathematically using simple percentage. The overall rate of resistance and susceptibility displayed by each isolate to the different antimicrobial agents used was compared using percentage.

4.0

RESULTS

4.1 Sex distribution of study population

Table 1 below shows sex distribution of study population

S/NO	SEX	NUMBER EXAMINED	NUMBER POSITIVE	%
1.	Male	25	3	12
2.	Female	25	5	20
	TOTAL	50	8	16

4.1.1 Sex distribution of study population

Table 1 above shows the distribution of the study population according to sex. A total of 25 samples were examined for male and female sex to avoid bias due to number of samples examined. Three (3) *Salmonella* organisms were isolated for male giving a 12% prevalence, and five (5) samples tested positive of *Salmonella* for females giving a 20% prevalence. The overall prevalence of *Salmonella* on the study location is 16% which represented a total of 8 *Salmonella species*.

4.2 Biochemical Characterization

Table 2: *Results of biochemical tests*

REACTION IN TSI AGAR											
S/NO	SAMPLE	SLANT	BUTT	GLUCOSE	LACTOSE	SUCROSE	H ₂ S	GAS	CATALASE	OXIDASE	PROBABLE ORGANISM
1.	S2	Y	Y	+	-	+	+	+	+	-	<i>Salmonella species</i>
2.	S7	Y	Y	+	-	+	+	+	+	-	<i>Salmonella species</i>
3.	S20	R	Y	+	-	-	+	-	+	-	<i>Salmonella species</i>
4.	S31	R	Y	+	-	-	-	+	+	-	<i>Salmonella species</i>
5.	S34	Y	Y	+	-	+	+	+	+	-	<i>Salmonella species</i>
6.	S39	R	Y	+	-	-	-	+	+	-	<i>Salmonella species</i>
7.	S40	R	Y	+	-	-	+	-	+	-	<i>Salmonella species</i>
8.	S43	Y	Y	+	-	+	+	+	+	-	<i>Salmonella species</i>

KEY: Y = Yellow; R = Red; + = Positive; - = Negative

4.2.1 Results of biochemical tests

All eight (8) Gram negative bacilli were subjected to several biochemical tests as shown in Table 2 above. Their various reactions in Triple Sugar Iron, catalase and oxidase tests corresponded with *Salmonella species* proven biochemical profile. Samples S2, S7, S20, S34, S40, and S43 all produced H₂S in TSI agar and although their fermentation of sucrose and lactose varied as observed in the change in colour of the slant and butt, they all were catalase and oxidase negative. Cheesbrough (2010) guide stated the difference in *Salmonella* and other genus of enterobacteria to be their production of H₂S, positivity to catalase test, and their proven negativity to oxidase test; this is their distinguishing reaction to E.Coli which also produces H₂S in TSI agar. Samples S31 and S39 did not produce H₂S but released gas bubbles which were seen breaking the agar in tubes, this also corresponds with Cheesbrough's biochemical profile of other *Salmonella species*.

4.3 Antimicrobial Sensitivity Test

Table 3: Antimicrobial resistant strains of Salmonella and percentage of occurrence

S/NO	ORGANISM	CEP	OFX	PEF	CPX	CN	S	SXT	NA	PN	AU	%
1.	<i>Salmonella species</i>	-	-	I	R	-	-	R	R	R	R	60
2.	<i>Salmonella species</i>	R	-	R	I	-	I	R	R	R	R	80
3.	<i>Salmonella species</i>	R	-	R	R	-	R	R	R	R	R	80
4.	<i>Salmonella species</i>	R	I	R	-	-	-	R	R	R	R	70
5.	<i>Salmonella species</i>	R	R	-	R	-	-	R	R	R	R	70
6.	<i>Salmonella species</i>	R	-	-	-	-	-	R	R	R	R	50
7.	<i>Salmonella species</i>	-	-	R	I	-	R	R	R	R	R	70
8.	<i>Salmonella species</i>	R	I	R	R	-	-	I	R	R	R	80
Average:												70

KEY: OFX = Ofloxacin; PEF = Reflacine; CPX = Ciprofloxacin; AU = Augumentin; CN = Gentamycin; S = Streptomycin; CEP = Ceporex; NA = Nalidixic acid; SXT = Co-trimoxazole; PN = Ampicillin; R = Resistant; I = Intermediate resistance; % = Percentage

4.3.2 Antimicrobial resistant strains of Salmonella and percentage of occurrence

Table 4 above shows the pattern of resistance of Salmonella species to antimicrobial agents used. All eight (8) organisms resisted four or more antimicrobial agents. Zones of inhibition below 5mm were designated as intermediately resistant in accordance to CLSI, (2015); this means that the organisms were able to grow around the disc but did not show clear enough zones. It therefore implies that the antimicrobial agents with unclear zones around them were effective at a very low rate; this factor might have occurred because the drugs were available in a concentration that was not enough to act against the particular growth around it, or because the inoculum streaked on the sensitivity plate was picked in excess. Averagely, Salmonella species collectively showed a resistance expressed in percentage against 10 antimicrobial agents as (70%).

Table 4: Antimicrobial susceptible strains of Salmonella and percentage of occurrence

S/NO	ORGANISM	CEP	OFX	PEF	CPX	CN	S	SXT	NA	PN	AU	%
1.	<i>Salmonella species</i>	S	S	-	-	S	S	-	-	-	-	40
2.	<i>Salmonella species</i>	-	S	-	-	S	-	-	-	-	-	20
3.	<i>Salmonella species</i>	-	S	-	-	S	-	-	-	-	-	20
4.	<i>Salmonella species</i>	-	-	-	S	S	S	-	-	-	-	30
5.	<i>Salmonella species</i>	-	-	S	-	S	S	-	-	-	-	30
6.	<i>Salmonella species</i>	-	S	S	S	S	S	-	-	-	-	50
7.	<i>Salmonella species</i>	S	S	-	-	S	-	-	-	-	-	30
8.	<i>Salmonella species</i>	-	-	-	-	S	S	-	-	-	-	20
Average: 30												

KEY: OFX = Ofloxacin; PEF = Reflacine; CPX = Ciprofloxacin; AU = Augumentin; CN = Gentamycin; S = Streptomycin; CEP = Ceporex; NA = Nalidixic acid; SXT = Co-trimoxazole; PN = Ampicillin; S = Susceptible; % = percentage

4.3.3 Antimicrobial susceptible strains of *Salmonella* and percentage of occurrence

Table 4 shows the sensitivity results of *Salmonella species* expressed in terms of their susceptibility to the antimicrobial agents used. Zones of inhibition above 5mm were recorded as effective and the organisms considered being susceptible to the corresponding antimicrobial agents. The highest susceptibility recorded by a single strain was to five (5) antimicrobial agents with susceptibility to two (2) and three (3) antimicrobials was observed amongst several *Salmonella species*. Overall, *Salmonella species* showed a very low susceptibility to the various antimicrobial agents used with a percentage occurrence of 30%. These strains of *Salmonella* displayed to a high effect multi-drug resistance to antimicrobial agents.

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

The overall 16% prevalence of *Salmonella* in this study is relatively similar to 11% reported by Fashae *et al.*, 2010. Mike *et al.*, 2004 and Raufu *et al.*, 2010 reported a 12.5% and 15% prevalence rate respectively, which were all slightly lower than percentage obtained in this study. The variation in the isolation rate might be due to differences in climatic conditions, varying management systems or due to the number of samples analysed in each study, since large sample size can influence the chances of obtaining more isolates in culture (Raufu *et al.*, 2010). Consequently, difference in study locations may play a role in the occurrence of the organism, as *Salmonella* species are known to thrive differently in various environmental conditions and seasons (Okoli *et al.*, 2006).

Salmonella species displayed a very high rate of resistance (70%) and a worrying low level of susceptibility (30%) to the antimicrobial agents used. Each isolate showed resistance to four or more therapeutic agents thus, confirming the spread of more multi-drug resistant strains of *Salmonella*. This finding agreed with works of Guerra *et al.*, (2002) and Behailu and Mogessie (2009) who also found strains of *Salmonella* that displayed resistance to four or more therapeutic agents.

Individually, the isolates showed high resistance against Co-trimoxazole (85%), Ceporex (75%), Reflacine (62.5%), and Ciprofloxacin (50%) which were the least effective drugs in the study. This implied a decrease in sensitivity of common antimicrobials which have been predisposed, easily afforded or regularly prescribed against resistant strains of *Salmonella*, prompting an occurrence of resistance due to continuous exposure and subsequently acquisition of resistance by adaptation as reported by Chiu *et al.*, (2004).

Nalidixic acids (0%), Augmentin (0%) and Ampicillin (0%) were all completely susceptible to the isolates. Saad et al., 2015 also reported a 100% resistance of *Salmonella species* to Nalidixic acids. So therefore, it is fair to state that Nalidixic acids have been completely overpowered by multi-drug resistant *Salmonella*.

Contrary to the above findings, Gentamycin (100%), Ofloxacin (87.5%) and Streptomycin (75%) were highly effective against the isolated species. Bata et al., 2015 also reported highest sensitivity in his study to Gentamycin and Ofloxacin. The only variation with their report and this present study is Chloramphenicol and Amoxicillin which was not used for sensitivity in this study. This indicates that some antimicrobial agents are still effective against multi-drug resistant *Salmonella*; however, they should be used appropriately to prevent these organisms from gaining resistance against them.

5.2 Conclusion

The pressure on healthcare professionals to prescribe antibiotics is great as well as the burden of disease occurrence. But there is need to conserve and use antibiotics appropriately or we risk losing the power of these medicines. The whole world urgently needs to change the way it prescribes and uses antibiotics. Even if new medicines are developed, without behavior change, antibiotic resistance will remain a major threat. Research continues to uncover emergence and spread of multi drug resistant strains of bacterial and other disease causing agents. Without urgent action, the world seems to be heading for a post-antibiotic era, in which common infections and minor injuries can once again kill without medical intervention.

5.3 Recommendations

Sequel to findings from this research, the following precautionary measures are hereby recommended.

- i. Protocols need to be implemented by government to kick against indiscriminate use of drugs and over the counter sale. Drugs should be sold to buyers only when a valid medical prescription note is tendered.
- ii. Prescription of last resort medicines should be handled professionally and administered within the hospital environs thereby preventing the chances of abuse and misuse by patients who are prescribed these drugs.
- iii. Patients should be professionally advised during drug administration to take full dosage of drugs prescribed to them to avoid partial action of drugs against microbial agents.
- iv. Drug usage in agriculture especially as prophylaxis in animal husbandry should be reduced drastically. Simple hygienic practices and use of antiseptic agents should be used instead of bactericidal agents for cleaning animal houses.
- v. In disease diagnosis, the existence of an infection should be confirmed without doubt before drugs and the necessary treatment procedure is administered. This will ensure that right therapy is given to the appropriate ailment.

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APPENDIX I

Calculations

$$\text{Percentage of resistance} = \frac{\text{Number of antimicrobial drugs resisted}}{\text{Total number of antimicrobial agents used}} \times 100$$

$$\text{Percentage of susceptibility} = \frac{\text{Number of antimicrobial drugs that were effective}}{\text{Total number of antimicrobial agents used}} \times 100$$

APPENDIX II

Percentage of sensitivity of the antimicrobial agents to *Salmonella species*

Antimicrobial agent	Code	% of sensitivity
GENTAMYCIN	CN	100
OFLOXACIN	OFX	87.5
STREPTOMYCIN	S	75
CIPROFLOXACIN	CPX	50
REFLACINE	PEF	37.5
CEPOREX	CEP	25
CO-TRIMOXAZOLE	SXT	12.5
AUGMENTIN	AU	0.0
NALIDIXIC ACID	NA	0.0
AMPICILLIN	PN	0

Antimicrobial agents used for in-vitro sensitivity test and their concentrations

Antimicrobial agent	Code	Conc./disc (mcg)
GENTAMYCIN	CN	10
OFLOXACIN	OFX	10
STREPTOMYCIN	S	30
CIPROFLOXACIN	CPX	10
REFLACINE	PEF	10
CEPOREX	CEP	10
CO-TRIMOXAZOLE	SXT	30
AUGMENTIN	AU	30
NALIDIXIC ACID	NA	30
AMPICILLIN	PN	30

APPENDIX III

Media Preparation

- 1. Buffered peptone water:** 20grams (g) of solid media substance of Buffered peptone water was weighed and dissolved in 1000ml of distilled water, solution was stirred vigorously until saturation. 5ml of saturated media was aspirated using a sterile syringe and dispensed into 50 test tubes. The test tubes containing peptone water were kept in a rack and autoclaved at 121⁰C for 15minutes.
- 2. Salmonella Shigella Agar:** 63.02g of *Salmonella Shigella* Agar substance was dissolved in 1000ml of distilled water, solution was stirred continuously until it was evenly saturated. Media was autoclaved at 121⁰C for 15minutes and dispensed into petri dishes and allowed to gel.

3. **MacConkey Agar:** 49.53g of MacConkey agar was dissolved in 1000ml of distilled water, solution was stirred continuously until it was evenly saturated. Media was autoclaved at 121⁰C for 15minutes and dispensed into petri dishes and allowed to gel.
4. **Nutrient Agar:** 28grams of solid media substance was weighed into a cornical flask and dissolved by adding 1000ml of distilled water. The solution was continuously stirred until it was evenly saturated. The saturated media was then autoclaved at 121⁰C for 15minutes. Media was removed from autoclave and dispensed evenly into sterile petri dishes and allowed to cool.
5. **Triple Sugar Iron Agar:** 65g of media was dissolved in 1000ml of distilled water and stirred while being heated until the solution attained saturation. 5ml of completely dissolved media was aspirated with syringe and dispensed into clean 10ml test tubes. The test tubes containing media were autoclaved at standard sterilization temperature and pressure. The autoclaved test tubes were slanted and allowed to gel, forming a slant and butt in the tub