Distribution of Antibiotic Resistant and Biofilms Producing Salmonella Enterica Serovar Typhi in Michika and Mubi LGA of Adamawa State

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Abstract: The emergence of multidrug resistance among strains of *Salmonella* Typhi has continued to complicate treatment options with increased morbidity and mortality especially in developing countries. This study was undertaken to determine the occurrence and distribution of antibiotic resistant and biofilm producing *Salmonella* Typhi in Mubi and Michika LGAs of Adamawa State, Nigeria. This cross sectional study was undertaken among people attending public hospitals who were beingtreated for enteric or other fevers in the study area. Stool and blood samples from the patients were cultured following standard microbiological methods and *Salmonella* Typhi isolates were confirmed using standard antisera for *S.* Typhi. Findings from this study revealed that 27% of the stool samples and 5.7% of the blood samples yielded growth of *S.* Typhi. Overall prevalence of *S.* Typhi was 16.8% while it was 16.5% and 17.4% in Mubi and Michika respectively. The risk ratio of *S.* Typhi for females to males in the study area was 1.19. Biotyping of *S.* Typhi revealed that 78.2% of the isolates were biotype I. Antibiogram of the isolates revealed that 67% of the isolates from the study area were resistant to ampicillin, nalidixic acid and tetracycline. The overall occurrence of multi drug resistant isolates from the study area was 58% with the multiple antibiotic resistance index of the isolates being predominantly within the high risk zone. Again, 61.7% and 76.2% of the isolates from Mubi and Michika respectively carried resistant plasmid while 80% of all the isolates produce biofilm. It was observed that lack of good quality drinking water, poor human waste disposal and hygiene are responsible for the continued occurrence of enteric fever in the study area. Furthermore, self-medication and empirical antibiotic without laboratory support are attributable risk factors for the emergence of multi drug resistant strains. These findings underscore the urgent legislation that will limit access to antibiotics over the counter as is the case today. It also calls for greater synergy between physicians and the laboratory in the choice of antibiotics for the treatment of typhoid fever.

Keywords: Multidrug, Biofilm, Resistant

INTRODUCTION

Typhoid fever caused by *Salmonella entericasubsp*. *enterica* serovar Typhihas continued to be an important health problem in many parts of the world and Nigeria in particular. The reported estimated disease burden in Africa is about 4.36 million annually (Zige *et al.,*2013). In the absence of suitable chemotherapeutic management of typhoid fever, the case fatality rate was high as 30% (Arora *et al.* (2010). The introduction of chloramphenicol in 1948 as a chemotherapeutic agent for typhoid fever treatment, significantly reduced the case fatality rate sharply from 30% to less than 1% (Parry *et al.*, 2002). This important breakthrough in the treatment of typhoid fever was however short lived as by 1950, resistance to chloramphenicol had emerged (Colquhoun and Weetch, 1950). By the year 1972, wide spread resistance to this drug which posed a serious threat in the management of the disease had been reported (Ackers *et al.*, 2000).

Since the first reported case of resistance of *Salmonella* Typhi to chloramphenicol, resistance to this and other antibiotics has continued to evolve among the bacterial population. Until the mid-1980s, the first line drugs: ampicillin, chloramphenicol and cotrimoxazole (ACCo) were used as standard treatment regime for enteric fever. However, the emergence of simultaneous resistance to more than two different groups of antimicrobial agents (multi drug resistance)began to emerge posing serious therapeutic challenge (AbdelFarag, 2015). The emergence of multi drug resistant *S* Typhi (MDRST) strains epidemiologically defined as strains resistant to all three first line antityphoid antimicrobial agents have been reported (Thong *et al.,* 2015). The emergence of multi drug resistant strains of *S.* Typhi has prompted the widespread use of fluoroquinolones such as ciprofloxacin and ofloxacin for the treatment of the disease.

The fluoroquinolone usage was also followed by the emergence of nalidixic acid resistant *S* Typhi exhibiting reduced susceptibility to fluoroquinolones (Threlfall *et al.*, 2001). This strains have become widespreadcompromising enteric fever treatment by limiting therapeutic options. Reports also indicates that MDRSThave increased substantially in most parts of the world especially in developing countries from a prevalence of 1% human isolates in 1998 to about 21% in 2003 and could be even higher (Wong *et al.*, 2015).

There have been several complaints of treatment failures in enteric fever cases in Mubi and Michika axis of Adamawa state. This has made patients to resort to the use of herbal potions for treating perceived or laboratory confirmed cases of enteric fever. It is against this background that we embarked on this cross sectional study to evaluate the antibiotic resistance profile and biofilm forming potential of *Salmonella* Typhi isolates in some parts of Mubi and Michika local government areas of Adamawa State Nigeria.

MATERIALS AND METHODS:

STUDY AREA

This study was conducted Mubi and Michika Local Government Areas in the Northern Senatorial District of Adamawa State, Nigeria. The population of the study area is put at 582, 700 (207, 500 for Michika and 376, 200 for Mubi) by the National population commission projections for 2016. The main occupation of the people in the study area is farming although several are civil servants and business people. This area was ravaged by insurgency between 2014 and 2015 and several infrastructures were destroyed. Access to potable water and hygiene are challenges as several rely on water from unsafe sources for drinking and open defaecation is common place. The study area is home to 2 secondary health facilities and 3 tertiary institutions and one health college of health technology and several secondary and primary schools.

SAMPLE COLLECTION

A total of three hundred and twenty-seven (327) samples (206 from Mubi and 121 from Michika) were collected from suspected typhoid fever patients attending private and public hospitals in Mubi and Michika in the Northern senatorial zone of Adamawa State following standard protocol described by WHO, (2003). The samples were then transported to the Medical Microbiology Laboratory, Modibbo Adama University of Technology, Yola.

ISOLATION OF *Salmonella* TyphiFROM STOOL

The method of *Salmonellaenterica* serovar Typhi isolation described by WHO, (2003) was adopted. Briefly, Faecal specimens to be examined was inoculated into selenite F broth and incubated for 16 hours at 37^0C after which they were sub cultured onto Bismuth Sulphite Agar plates. The plates were incubated at 37^0C for 24 hours.

ISOLATION OF *Salmonella* Typhi FROM BLOOD SAMPLES

A sterile syringe was used to collect 5 ml of venous blood from suspected enteric fever patient and transferred into 45ml of tryptic soy broth in bottles. This was then mixed and incubated in an upright position at 37^0C. This was monitored daily for signs of visible growth for a period of seven days. Aliquots of 0.1 ml were then sub cultured on Bismuth Sulphite agar and incubated at 37^0C for 24 hours(WHO, 2003).

IDENTIFICATION OF ISOLATES

The resultant isolates from 24-hour stool and blood samples culture plates were identified using standard biochemical tests and identification charts for enteric organisms (Prescott and Harley, 2002; WHO, 2003; Chessbrough, 2006). *Salmonella* Typhi isolates were confirmed by slide agglutination test using *Salmonella* Typhityping antisera.

BIOTYPING of *Salmonella enterica* serotype Typhi

The confirmed*Salmonella* Typhi isolates were then further investigated for fermentation of xylose and arabinose and classified according to the classification proposed by Kristensen and Henriksen as reported by Quintaes *et al.* (2002).

SCREENING of*Salmonella enterica* serotype TyphiBIOFILMS FORMING POTENTIAL

The tube method (TM) for screening bacteria for biofilm forming potential as described by Christensen *et al.* (1982) was used to screen the *Salmonella*Typhiisolates for biofilm forming ability. Briefly, 0.1 ml of *Salmonella* Typhi isolates from overnight culture plates were inoculated into 10ml of trypticase soy broth containing 1% glucose (TSB glu) and incubated at 37^0C for 24 hours. After incubation, the contents of the tubes were decanted and the tubes rinsed with phosphate buffer saline (PBS) pH 7.3 and dried inverted. The dried tubes were then stained with 0.1 % crystal violet for 1 minute after which the tubes were rinsed with deionized water and then dried in an inverted position. The tubes were then observed for ring formation in the wall of the tube which indicates biofilm formation.

ANTIBIOTIC SUSCEPTIBILITY TESTING

The antimicrobial susceptibility screening of *Salmonella* Typhi isolates was determined using the disk diffusion method described by CLSI (2012). Briefly, within 15 minutes of adjusting the turbidity of the inoculums suspension to 0.5 McFarland standard, 0.1 ml was transferred to the surface of an already prepared Mueller Hinton Agar using a Pasteur pipette. The microbial suspension was then evenly spread on the surface of the plate using a spreader. A sterile forceps was then used to place antibiotic disks containing the minimum inhibitory concentrations of the following antibiotics:chloramphenicol 30µg, ampicillin 10 µg, amoxicillin 10µg, tetracycline 30µg, cotrimoxazole, 25µg, nalidixic acid, 30µg, ofloxacin 5µg and ciprofloxacin 5µg in the centre of the plate. The plates were then incubated at 37^0C for 24 hours after which the plates were examined for zones of inhibition which were measured in millimetres.

Determination of Multiple Antibiotic Resistance Index of isolates

The multiple antibiotic resistance (MAR) index of *Salmonella* Typhi isolates was determined using the formula MAR= a/b, where a represents the number of antibiotics to which the test isolate depicted resistance and b represents the total number of antibiotics to which the test isolate has been evaluated for susceptibility (Krumperman, 1983).

RESULTS AND DISCUSSION

The results displayed in Table 1 showed the distribution of enteric organisms isolated from the study area by age and gender. The enteric bacteria isolated were *Salmonella* Typhi, *Salmonella* Paratyphi, *Salmonella* spp and *Shigella*. The overall enteric bacteria isolation rate was 60% with *S.* Typhi being the most frequently isolated while *Shigella* spp was the least encountered bacteria among suspected enteric fever patients. Chi square test revealed that there was no significant statistical difference in the distribution of the isolates in relation to age and gender at p=0.05. Comparison of the *Salmonella* Typhi isolation rate from the two study area, the results revealed an overall incidence to be 16.8%. A breakdown revealed that 16.5% of the samples from Mubi and 17.3% of the samples from Michika yielded *Salmonella* Typhi.Chi square test revealed that the difference in isolation rate from Mubi and Michika was not statistically significant (Table 2).Higher incidence rates for *Salmonella* Typhi isolation (64%) have been reported in Wukari Taraba state by Ubandoma *et al.* (2007). Also, Odikamnoro *et al.* (2017) reported an incidence rates of 49.4% in Unwana Community, Afikpo North Local Government Area in Ebonyi State.

Prevalence rates in our study are higher than 2.1% from 9634 blood culture in a study conducted in seven provinces of Democratic Republic of Congo from 2007 to 2011 (Lunguya *et al.*,2012); they are also higher than that of Akhtar *et al. (*2015) who reported 12% S. Typhi isolation rate in some parts of India. Our findings are not surprising asit has been reported that only 27.2% of the population of the state have access to improved drinking water sources and sanitation; also 33.9% of the population practice open defecation, and another 25.2 % of the population use unimproved toilet facilities (MICS, 2011). The persisting high prevalence of typhoid fever and its complications in our environment underscores the need for improved sanitation, public health enlightenment and provision of potable water supply.

As shown in Table 3, there was a statistically significant difference in the isolation rate between blood and stool samples in the study area with stool samples having the highest isolation rate (at p=0.05). The results also show that the overall male to female *Salmonella* Typhi recovery ratio in the study area was 1.2:1; further breakdown shows that while in Mubi the male to female ratio was 0.62:1 while it was 1.33:1 in Michika.However, although overall more of the isolates were from males than females, the difference was not statistically significant (Table 3). This finding is not surprising as studies indicate that males seem to be more affected by the disease than females (FAO, 2012) this is in line with our findings. Ubandoma *et al.* (2017) however reported that females had higher prevalence (64.3%) for *Salmonella* Typhi infection in Wukari Taraba state than males (63.6%).Also, Odikamnoro *et al.* (2017) reported a higher *Salmonella* Typhi prevalence among females 58% than females 50%. These differences could be as a result of difference in hygiene practices between males and females or the exposure of the males to contaminated food and water outside the home irrespective of gender.

Result of biotyping of the *S.* Typhi isolates showed that 78.2% of the isolates were biotype 1 (arabinose -, xylose +), while the remaining were biotype II (arabinose -, xylose -).This result pattern is similar to those reported by Quintaes *et al. (*2002) and Sen *et al. (*2007) who reported biotype I to be the predominant biotype in their respective studies with a frequency of 93% and 95% respectively for biotype I. Higher frequencies of biotype II (22.5%) has been reported in Ludhiana (Kumar *et al*., 2002). In this study, biotyping, relying on xylose and arabinose biochemical reactions showed only two patterns of biochemical reactions. This finding revealed that biotyping on the basis of ability to utilize xylose and arabinose or otherwise to be of a limited ability to differentiate between strains within species and so has a poor discriminatory power.

The results of the determination of the biofilm forming ability of *Salmonella* Typhi isolates showed that 40% of the isolates were strong biofilms formers while 20% were non biofilms formers (Figure 2). The implication of this finding is that we risk having persistent and repeated *S.* Typhi infections. This ability to form biofilms is an environmental adaptation mechanism by pathogens and it plays an important role in their survival in the natural environment (Albesa *et al.,* 2004).

More of the isolates from Mubi showed resistance to quinolone (74%) mostly nalidixic acid and 59% multi drug resistant isolates (Table 4 and Figure 1). Results from the study showed that there were different resistance phenotypes with 47 isolates demonstrating resistance to more than 2(two) different antibiotics in vitro (Table 5).

The prevalence of MDRST from this study showed that 86% of the isolates from Michika and 59% of the S Typhi isolates from Mubi were multi drug resistant (Figure 1).

This is higher than the 26.5% of *Salmonella* Typhi isolates reported in Eastern Nepal between 2000 to 2004 and the 13% in Uzbekistan and 14% in Qatar reported by Khanal *et al. (*2007) but lower than 66.7%reported in India by Akhtar *et al.* (2015). The prevalence of MDR S. Typhi can also be attributed to over diagnosis, reliance on single "perverted" Widal test during acute illness without second convalescent specimen test, lack of data or knowledge on the baseline titre in a particular locality and misinterpretation of test results.

Infection with multi drug resistant strain are associated with severity of the patient's illness, increased patient contact with health care personnel. Eventually, this leads to extra cost of health care, extended stay in the hospital, sudden or prolonged complications and sometimes mortality.

Results of the MARI in the study area showed that 79% of the *S.* Typhi isolates from Michika and 76% of isolates from Mubi were in the high risk zone (Figure I). MAR is usually calculated when there are more than three classes of antibiotics showing resistance (Riaz *et al*., 2011). The value of MAR index of 0.200 differentiates the low and high risk. If the value is between 0.200 and 0.250, it becomes a very risky phase where there are equal chances that the MAR may fall in the high risk and low risk phases (Krumperman., 1983). This has been reported to be a good tool for risk assessment as it gives idea of the number of bacteria showing antibiotic resistance in the risk zone ((Riaz *et al*., 2011). Also, it has been reported that bacteria having MAR index of >0.200 originates from an environment where several antibiotics are used (Subramani and Vignesh, 2012). The very high MAR index in this study is a possible indication that a very large proportion of the bacteria isolates have been exposed to several antibiotics. The buoyant access to antibiotics everywhere in the state and poor access to doctors necessitates abuse and misuse which could be responsible for the high MAR index.

Table 1. Distribution of Enteric Pathogens Isolated from study area by age by gender

	***S.* Typhi**	***S.* Paratyphi**	***Salmonella* spp**	***Shigella* spp**	*P Value*
Male	35	23	31	14	
Female	20	19	39	16	p>0.05
Total	**55**	**52**	**60**	**30**	

Table 2 Distribution of *Salmonella* Typhi by Age and sample type in the study area

	Mubi	**Michika**	**Total**	*P value*
≤20(n-104)	11	8	19(18.2)	
20-40(n-172)	20	11	31(18.02)	
>40(n-51)	3	2	5(9.8)	*p>0.05*
Total (%)	**34**	**21**	**55 (16.8)**	
Blood	6 (5)	3(4)	9	
Stool	28(30)	18(35)	46	*p>0.05*
	34	**21**		

Table 3 *Salmonella* Typhi Distribution by Gender in the study area

	Mubi		***Michika***		***Total***	*P Value*
	Male	**Female**	**Male**	**Female**		
S Typhi Present	13	21	12	9	55	
S Typhi Absent	87	85	51	49	273	*p>0.05*
	100	106	63	58	327	

Table 4 Antibiogram *of Salmonellaenterica* serovar Typhi isolates from Mubi and Michika

	Mubi			Michika			OVERALL			SI
	S	I	R	S	I	R	S	I	R	Index
Amoxicillin	16(47)	0(0)	18(53)	17(81)	0(0)	4(19)	33(60)	0(0)	22(40)	1.50
Ampicillin	12(35)	2(6)	20(59)	4(19)	0	17(81)	16(29)	2(4)	37(67)	0.43
Chloramphenicol	20(59)	3(9)	11(32)	11(52)	1(5)	9(43)	31(56)	4(7)	20(36)	1.56
Cotrimoxazole	21(62)	6(18)	7(21)	11(52)	0	10(48)	32(58)	6(11)	17(31)	1.87
Tetracycline	11(32)	0	23(68)	2(10)	0	19(90)	13(24)	0	37(67)	0.36
Ceftriaxone	19(56)	0	15(44)	18(86)	0	3(14)	37(67)	0	18(33)	2.03
Ofloxacin	30(88)	1(3)	3(9)	20(95)	0	1(5)	50(91)	1(2)	4(7)	13
Ciprofloxacin	14(41)	11(32)	9(26)	7(33)	8(38)	6(29)	21(38)	19(35)	15(27)	1.47
Nalidixic Acid	10(29)	6(18)	24(71)	7(33)	1(5)	13(12)	17(31)	7(13)	37(67)	0.46

KEY

S= Susceptible, I = Intermediate Susceptibility, R= Resistant, SI = Susceptibility index

Value in parenthesis are percentages

Table 5Resistance Phenotype of *Salmonella* enterica serovar Typhi isolates from Mubi and Michika

RESISTANCE PHENOTYPE	No. of isolates	MCH	No. of isolates
NA	1	AMP	1
AMPAMX	2	AMP TET	1
AMX CO	1	AMP C CO	2
AMX CTX	1	AMP TET CTX	2
CTX NA	1	AMPAMX TET	1
AMP C NA	1	AMXTET CTX	1
AMP C TET CTX	1	AMP TET CTX NA	2
AMP TET NA	2	AMP C CO NA	1
AMPAMX TET	2	AMP C COTET	3
AMPAMX CTX	2	AMP CO TET NA	1
AMX COTET	1	AMP COTET CTX	1
AMX NA CTX	1	AMPAMX C TET	1
TET CP NA	1	CO TET CTX NA	1
AMP C TET CP	1	COTET CP NA	1
AMP TET CP NA	1	TET CTX CP NA	1
AMP TET CTX NA	1	AMPAMX C TET NA	1
AMPAMX C TET	1		
AMPAMX CTX NA	1		
AMX CO TET NA	1		
C TET CTX NA	1		
TET CP NA CTX	2		
AMP C CO TET NA	1		
AMP C TET CTX NA	2		
AMPAMX CO TET NA	2		
AMP AMX TET NA CTX	1		
AMP C CO TET OFL NA	1		
AMPAMX CO TET OFL CTX CP NA	1		

KEY:

AMX = Amoxicilin ,AMP=Ampicillin, C=Chloramphenicol, CO=Cotrimoxazole , TET = Tetracycline, CXT=Ceftriaxone, OFL=Ofloxacin, CP=Ciprofloxacin, NA=Nalidixic Acid

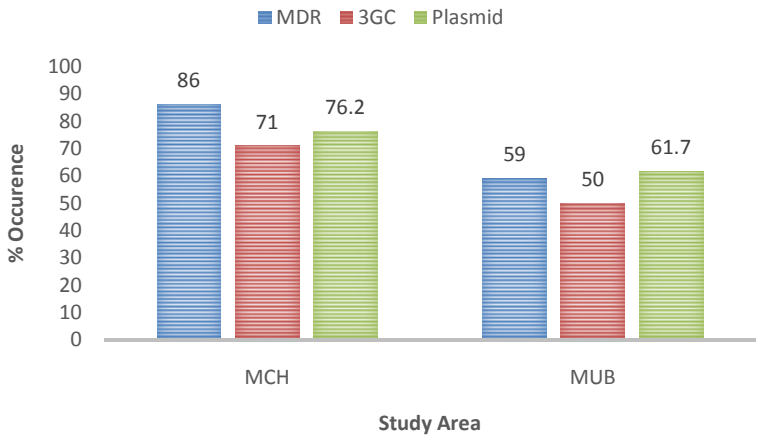

Figure 1 Distribution of MDR and 3GC resistant Isolates and Plasmid Carriage by *S.* Typhi Isolates

KEY

MCH = Michika

MUB = Mubi

MDR = Multi Drug Resistant

3GC = Third Generation Cephalosporin Resistance

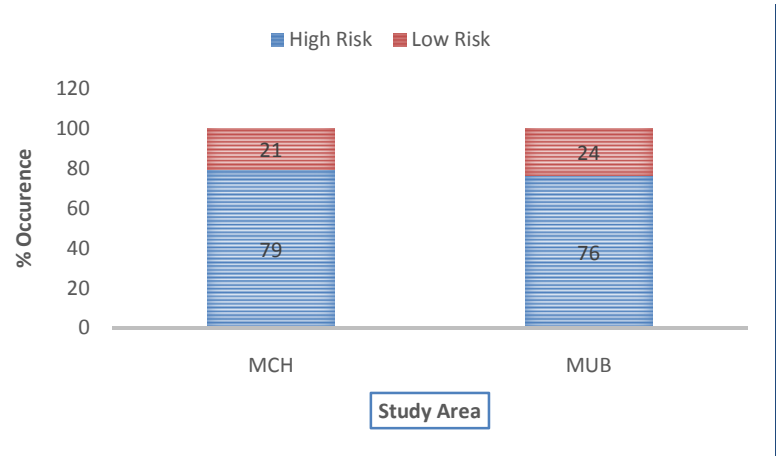

Figure 2 Distribution of *Salmonellaenterica* serovar Typhi isolates according to risk level

KEY

MCH = Michika

MUB = Mubi

CONCLUSION

Findings from this study revealed *S.* Typhi incidence of 16.8% with 58% of the isolates being multi drug resistant. The implication of this findings is that treatment in the study area requires laboratory based support The need for safe food preparation, provision of potable water and good hygiene in the study area is important in reducing instead of the current empirical treatment to avoid treatment failure and relapse of the disease. Also that 80% of the isolates produce biofilm mean that the organisms will persist in the environment as well as during infection if not promptly managed. transmission and by extension the incidence of the disease in the study area

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