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Detection of Multidrug Resistant Gram Negative Bacteria in Healthy Cattle from Maiduguri Metropolitan, Nigeria

Adam Mustapha[1*], Mustafa Alhaji Isa[1], Tijani Isa[1], Ibrahim Yusuf Ngoshe[1] and Hashidu Bala[1]

[1]Department of Microbiology, Faculty of Science, University of Maiduguri, P.M.B. 1069, Maiduguri, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Author AM designed, supervised the study and wrote the first draft of the manuscript. Authors TI, IYN and HB performed the work and wrote the protocol. Author MAI managed the analyses and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Prevalence of multidrug resistant bacteria on apparently health animals has turned antibiotic resistance to multifaceted process and threatens global food security and public health. The aim of the present study was to investigate the resistance profile of isolates from apparently healthy cattle in Maiduguri, Nigeria.
Methodology: A total of 120 nasal swab samples were collected from cattle. Colony identification was according to the guidelines of Bergey's Manual of Determinative Bacteriology. The susceptibility pattern of the isolates was conducted on the identified isolates according to the Modified Kirby-Baur disc diffusion method on Muller-Hilton agar and interpreted according to the procedures of Clinical Laboratory Standards Institute (CLSI, 2018) guidelines. Multiple Antibiotic Resistance Index (MARI) was calculated using the formula, MARI=a/b where "a" is the number of antibiotic resisted and "b" is the total number of antibiotic used in the study.
Results: Of the total samples (120) from cattle 96 (80%) detected the following isolates; *E. coli* was

**Corresponding author: E-mail: Adadmustapha@unimaid.edu.ng;*

the most commonly recovered isolates (33, 34.4%), followed by *Klebsiella spp* (28, 29.2%), *Salmonella spp* (21, 21.9%) and *Pseudomonas aeruginosa* (14, 14.5%). In this study, all the recovered isolates were found to be multidrug resistant gram negative bacteria, with highest resistance was shown by *Salmonella spp.* The high MARI observed in all the isolates in this study ranging from 0.7 to 0.9. MARI value of 0.2 > is suggests multiple antibiotic resistant bacteria and indicate presence of highly resistant bacteria.

Conclusion: The study indicates highly resistant bacteria are carried by healthy food animals. Thus, there is need for continued monitoring of antibiotics use in animal husbandry to prevent further spread of resistance in Maiduguri, Nigeria.

Keywords: Multiple drug resistance indexes; healthy animals; gram negative bacteria; prevalence; Nigeria.

1. INTRODUCTION

The accidental invention of antibiotics brought ray of hope to the treatment of bacterial infections in man, and not long, followed by the applications of antibiotics in the treatments of animals [1,2]. However, the emergence of resistance has been a global challenge in the application of antibiotics in both humans and animals. Regardless of the source, antibiotics resistance has been on raise and recently, has been projected to be among the major killer that will contribute to death of more than 10 million people annually by 2050 if the threat is not contained [3]. The drivers of resistances spans from indiscriminate use of antibiotics for therapeutic and non therapeutic purposes which facilitates the pressure of selecting resistant bacteria, worthy to note is heavy applications of antibiotics in animal husbandry [4,5,6,7]. Of concern is the rapid emergence of resistance especially amongst the critical and high level priority pathogens, some of which are becoming totally resistant to the last resort agents and introduction of new agents are on slow phase [8,9].

Large amount of world's antibiotics are used for non human purposes, which largely exceed use for man and the applications in animal husbandry is not for therapeutic purposes, rather as growth promoters, feed additives and for prophylaxis [10,11]. Furthermore, it is estimated that the global consumption of antibiotics is approximated to be around 70 to 80% and projected increase of 67% by year 2030 [10]. This could be explained for the quest of large livestock products for profit making in many countries. Although, the use of antibiotics in farming and agriculture is banned in most European countries for prophylaxis, however, the practice of applications of antibiotics in animal husbandry is still common in many countries across the world [12,13]. The use of antibiotics in animal husbandry results to presence of antibiotics residues in animals and food of animal origins [14,15,16,17].

In Nigeria, due to the recent boom in agriculture especially livestock breeding, many farmers resort to use of antibiotics indiscriminately for prophylaxis and as growth promoters. Furthermore, poor antibiotic stewardship complicated the scenario, hence, this study, aimed to isolates bacteria of public health importance and their resistance profiles in apparently healthy animals.

2. MATERIALS AND METHODS

2.1 Study Area and Sample Collection

The samples were collected from the University of Maiduguri Animal Science Livestock Farm between the months of July and August, 2019. Both the university and the farm are located at Maiduguri, Maiduguri city is found in Borno State, North eastern Nigeria.

The samples were collected from apparently healthy animals which showed no symptom of any illness. A total of 120 nasal swab samples were collected from cattle. All the nasal samples were collected with the use of sterile swab stick, the swab was then returned to its case, labeled and taken to the Microbiology Laboratory of University of Maiduguri for analyses.

2.2 Bacterial Isolation and Identification

The nasal swabs were cultured overnight onto nutrient broth at 37℃ for the determination of microbial growth and then sub-cultured on to blood agar, chocolate agar and MacConkey agar plates and incubated at 37°C for 24 hrs. Suspected colonies were picked for further

analysis of pure culture of gram negative bacteria using standard microbiological techniques of colony identification which involved gram staining and biochemical tests according to the guidelines of Bergey's Manual of Determinative Bacteriology [18].

2.3 Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was done on the identified isolates according to the Modified Kirby-Baur disc diffusion method on Muller-Hilton agar and interpreted according to the procedures of Clinical Laboratory Standards Institute (CLSI, 2018) guidelines [19]. Antibiotic discs were placed over the media using dispenser and gently tap each antibiotic disc onto the surface of the agar with a sterile stick. Each of the identified isolate was spread on a separate nutrient agar plate, and antibiotic disc dropped on the plate and incubated at 37℃ for 24 hours.

The Kirby-Bauer disk diffusion susceptibility test was used to determine the sensitivity or resistance of all confirmed isolates to 10 antimicrobial agents: CPX=Ciproflox (10 µg), CN=Gentamycin (10 µg), S=Streptomycin (30 µg), PN=Ampicillin (30 µg), OFX=Tarivid (10 µg), CEP=Ceporex (10 µg), PEF=peflacine (10 µg), AU=Augmentin (30 µg), NA=Nalidixic acid (30 µg) and SXT=Septrin (30 µg) [20]. Based on the recommendations of Clinical Laboratory Standards Institute (CLSI, 2018), the zone of inhibition was measured and interpreted as sensitive (S), intermediate (I) and resistant (R) accordingly.

2.4 Determination of Multiple Antibiotic Resistance Index (MARI)

MARI was calculated using the formula, MARI=a/b where "a" is the number of antibiotic resisted and "b" is the total number of antibiotic used in the study. Isolate with MARI value of 0.2 > suggests multiple antibiotic resistant bacteria and indicate presence of highly resistant bacteria [21].

3. RESULTS

3.1 Confirmation of Bacterial Isolates

Identification of bacterial Isolates was based on the morphological and biochemical testes and confirmed the presence of members of gram negative bacteria. Of the total samples (120) collected from cattle, (n=96/120) samples yielded positive growth of gram negative bacteria isolates. This gives a total recovery rate of 80%; 33 were identified as *Eschericia coli* (34.4%), 28 were identified as *Klebsiella spp* (29.2%), 21 were identified as *Salmonella spp* (21.9%) and 14 were identified as *Pseudomonas aeruginosa* (14.5%) (Fig. 1).

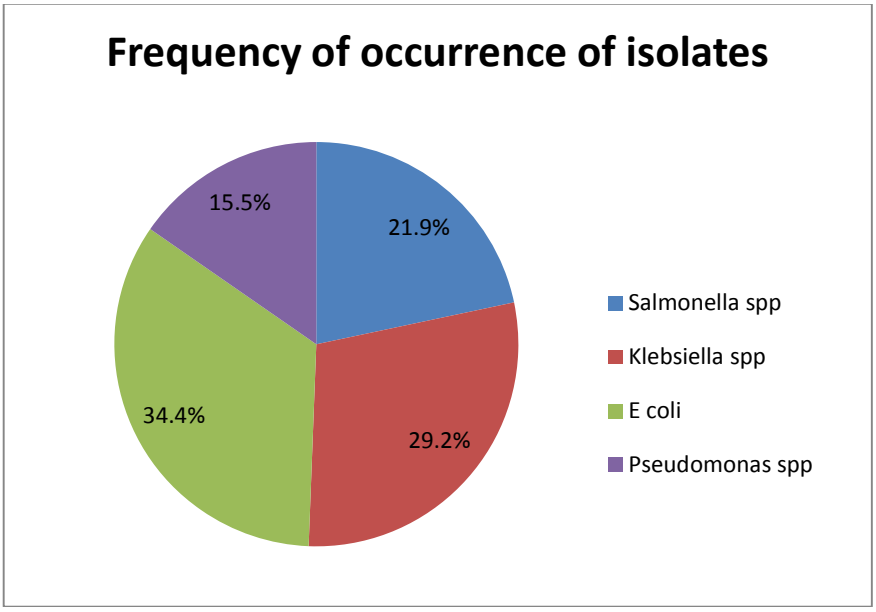

Fig. 1. Frequency of occurrence of the isolates

3.2 Antibiotic Susceptibility Test

The result of antimicrobial susceptibility test on the isolates obtained is presented by measuring the zones of inhibition around each antibiotic disc; each value is a mean of triplicate measurement (Table 1). Intermediates isolates were considered as resistant to all the agents tested. Multidrug resistance was observed in most of the samples as shown in Table 2. The highest resistance was shown by *Salmonella spp* and all other isolates were multidrug resistant in nature (resistance to ≥3 antibiotics class).

3.3 Determination of Multiple Antibiotic Resistance Index (MARI)

Multiple Antibiotic Resistance index phenotypes of isolates that exhibited resistances to three or more antibiotics were generated by dividing number of antibiotics resistant to the total number of antibiotics tested (Table 3).

4. DISCUSSION

Apparently healthy animals harbor multidrug resistant bacteria and they pose a threat to continued increase of resistance in animals and humans. Thus, it is important to assess the resistance profile of bacteria among apparently healthy animals as they are used as source of food. In the present study, total of 120 nasal swab samples were collected from cattle. Resistance profile of isolates from healthy livestock was assessed.

The prevalence of bacterial isolates was found to be 80% accounting for large proportion. *E. coli* was the most common species in this study (34.4%) (Fig. 1). This was followed by *Klebsiella spp* (29.2%), *Salmonella spp* (21.9%) and *Pseudomonas aeruginosa* (15.5%). In southern Nigeria, a study reported a high rate of *E. coli* in cattle with no sign of ill-health which is comparable with the current study [22]. In recent time, it was demonstrated that *E. coli* was predominant species in healthy animals, this finding is also in tandem with the current study [23]. In a similar manner, high rate of *E. coli* was reported in cows from Jordan [24,25]. These finding is also consistent with other reports where *E. coli* was observed as the predominant species in healthy animals [23,26,27,28,29,30,31]. On

Table 1. Zones of inhibition of the isolates against the drug tested (mm)

S/N	Isolate	OFX	PEF	CPX	AU	CN	S	CEP	NA	SXT	PN
1	*E. coli*	12	18	22	15	13	11	17	14	10	13
2	*Klebsiella spp*	15	13	15	21	18	11	20	13	11	14
3	*Salmonella spp*	12	20	15	13	12	12	15	11	10	13
4	*Pseudomonas aeruginosa*	14	18	15	18	16	12	13	12	14	19

Note: OFX= Cefoxitin; PEF= Reflacine; CPX= Ciprofloxacin; AU= Augmentin; CN= Gentamycin; S= Streptomycin; CTX: Cefotaxime; NA= Nalidixic Acid; SXT= Septrin; PN= Ampicilin; mm= Millimeter

Table 2. Resistance pattern of isolates to the respective antibiotics used

Identified isolates	Resistance pattern									
	OFX	PEF	CTX	AU	CN	S	CEP	NA	SXT	PN
E. coli	R	S	S	R	I	I	I	I	R	R
Klebsiella spp	R	I	I	S	S	R	R	R	R	I
Salmonella spp	R	S	R	R	R	I	R	R	R	R
P. aeruginosa	I	S	R	S	S	R	R	R	R	R

Note: R= resistant; I= intermediate; S= susceptible

Table 3. Multiple drug resistance indexes (MARI) of the isolates

Isolates	List of antibiotics	Number of antibiotics	MARI
E. coli	OFX, AU, CN, S, CEP, NA, SXT, PN	8	0.8
Klebsialla spp	OFX, PEF, CTX, S, CEP, NA, SXT, PN	8	0.8
Salmonella spp	OFX, CTX, AU, CN, S, CEP, NA, SXT, PN	9	0.9
P. aeruginosa	OFX, CTX, S, CEP, NA, SXT, PN	7	0.7

Note: OFX= Cefoxitin; PEF= Reflacine; CPX= Ciprofloxacin; AU= Augmentin; CN= Gentamycin; S= Streptomycin; CTX: Cefotaxime; NA= Nalidixic Acid; SXT= Septrin; PN= Ampicilin

the other hand, a study from Nigeria, reported a lower rate of *E. coli* in healthy cattle (17%) [32]. Handling of the animals, misuse of antibiotics and other factors might be responsible for the differences certainly not only geographical location. Overall, the high prevalence of *E. coli* and occurrence of other isolates can be explain by being the members of normal flora in animals, however, occurrence of *Salmonella spp, P. aeruginosa* and *Klebsiella spp* is a pointer to high burden that have potential risk to animals and human health.

Isolates originating from this study were shown to be multidrug resistant (Table 1). The trends in resistance pattern showed that *Salmonella* spp (90%) were more resistant than other isolates. This finding shows similar pattern of high resistant *Salmonella spp* in previous study in China, where the multidrug resistant (MDR) *Salmonella spp* reported to be 80% in food animals when tested against 17 commonly used antibiotics for clinical applications [33]. Previously, report from Ghana also reported high prevalence of *Salmonella spp* (66.7%) which where the MDR of the isolates were reported to be 52.8% [34]. Across the world, prevalence of *Salmonella spp* were reported in varying degree with very low prevalence in Europe (2%) than other continents which is in contrast with the current study [35]. This variation of occurrence could be accounted for the methods the cattle are handled in different geographical regions.

In the present study, resistance rate of *E. coli* was found to be 80% (Table 1). This finding is similar to previous studies where MDR *E. coli* was reported in healthy cattle in Southern Nigeria compared to other parts of the country [23]. Adelowo et al. [36], reported that 94% of *E. coli* from food animals is MDR. Similarly, Sawant et al. [26] reported high resistant *E. coli* (86%) from USA which is consistent with our finding. Similar high patterns of *E. coli* resistant isolates were reported elsewhere [28,29,37,38]. The high resistance patterns in this study are a pointer of excessive use of antimicrobial in animal husbandry.

In this study, two other isolates were also reported to be MDR, *Klebsiella spp* and *Pseudomonas aeruginosa* (Table 1). MDR *P. aeruginosa* has been reported by Beier et al. with varying degree of resistance to different antibiotics, 93.8% to beta-lactam and least resistance to Fluoroquinolone (16%) [39]. *P. aeruginosa* as an adaptive pathogens, exhibiting multidrug drug pattern is worrisome. We report 70% *P. aeruginosa* resistance which is in agreement with previous studies depending on the class of antibiotics [40,41]. Similar multidrug resistance *P. aeruginosa* has been previously reported in healthy cattle from France and elsewhere [42,43,44]. In the present study, MDR *Klebsiella spp* was found to be 80% (Table 1). In a study from China, high rate resistance *Klebsiella spp* was reported to be 93.4% [45]. Similar studies documented MDR *Klebsiella spp* in cattle [46,47,48,49]. Detection of MDR isolates in healthy food animals is an urgent threat to food security and public health, as there is well established evidence of link of transfer of resistance through food animals to humans [50-56].

In all the antibiotics tested, none proved to be effective against all the isolates, however, Reflacine was found to be more effective against the isolates (Table 2). This accounts for the indiscriminate applications of antibiotics in both animals and human use and is a pointer of cross resistance. The multiple antibiotic resistance index (MARI) of the isolates recovered in the present study indicate multidrug resistance in nature (Table 3). The MARI value > 0.2 is suggesting multidrug resistance, due to high risk application and contamination of antibiotics [57]. An average of 0.8 MARI in this study is higher than report of Chika et al. [58]. Adzitey [59] reported pattern of high MARI of 0.11-0.78 from Ghana. These findings demonstrate that the cattle were exposed to multiple classes of antibiotics. On the other hand, a lower MAR index ranging 0.3-0.6 was reported in South African study in food animal [60]. Comparable finding also reported lower MARI (0.31) in healthy livestock from South Africa [61]. This can be explained by the sample size and antibiotic regulation in the study area, among other factors.

Lacks of epidemiological variables, such as history of antibiotic use, to assess the risk factors of exposure and development of resistance and sample size are among the limitation of this study. Also molecular analysis could not be performed to determine the resistant genes due to funding constraint. Thus, future studies is required to explore molecular nature of the multidrug resistant genes and the risk factors associated with harboring of drug resistant bacteria in healthy animals in the study area.

5. CONCLUSION

In summary, the present study found multidrug resistant gram negative bacteria on healthy cattle. Notably, the most predominant isolates were reported to be *E. coli*, followed by *Klebsiella spp, Salmonella spp,* and *Pseudomonas aeruginosa.* Highest resistant isolates were found to be *Salmonella spp,* however all the isolates showed multidrug resistant pattern as indicated by MAR indexes ranging from 0.7 to 0.9.

Due to paucity of studies in Maiduguri metropolis, Northeast Nigeria, this study reveals the multidrug resistant gram negative bacteria in apparently healthy food animals. The high rate of resistant bacteria in these animals suggests excessive use of antibiotics for non-chemotherapeutic purposes and therefore, strict monitoring of application of antibiotics in animal husbandry required.

ETHICAL APPROVAL

Ethical approval was obtained from the ethical committee of university of Maiduguri.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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