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ANTIBIOTIC SUSCEPTIBILITY PROFILE AND DISTRIBUTION OF PATHOGENIC AND TOXIGENIC GENES IN METHICILLIN RESISTANT *Staphylococcus aureus* ISOLATED FROM ABATTOIR FACILITIES IN BENIN CITY, NIGERIA

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ABSTRACT

Abattoir facilities are poorly delineated sources of human exposure to zoonotic antibiotic resistant bacteria such as methicillin resistant *Staphylococcus aureus* (MRSA). The current study was aimed at investigating the prevalence of MRSA in two abattoir facilities located in Benin City, Nigeria, and the distribution of pathogenic and toxigenic genes among isolates. A total of 64 samples (including beef, swab from slaughter table surfaces, water used for meat processing, and wastewater) were obtained from both abattoirs and transported in cooler boxes containing iced pack to the Laboratory for immediate analysis. The samples were analysed using established microbiological methods. Suspected *Staphylococcus aureus* isolates were screened for methicillin resistance as well as sensitivity and/or resistance to ten commonly used antibiotics following the established methods of Clinical and Laboratory Standards Institute (CLSI). The presence of *mec*A, *ent*A and *sccmec*1 genes were determined by the polymerase chain reaction technique. The results of total heterotrophic bacteria count ranged from 147 ± 72 cfu/g for beef samples to 347 ± 114 cfu/ml for waste water (abattoir 1) and 109 ± 37 cfu/ml to 271 ± 116 cfu/ml for clean water and floor respectively in abattoir 2, while *Staphylococcus aureus* count ranged from 0.00 ± 0.00 (beef) to 16.5 ± 14.5 cfu/ml (waste water) in abattoir 1 and 0.00 ± 0.00 (beef) to 14.5 ± 2.5 cfu/ml (waste water) in abattoir 2. Only six MRSA (representing 12.5% prevalence) were identified in all samples from both abattoirs (2 and 4 from abattoir 1 and 2 respectively). Of the six MRSA isolates, only one harbored the *mec*A gene, while five of the six MRSA isolates were found to be multidrug resistant. The current study therefore revealed that multidrug resistant MRSA are prevalent in abattoir facilities in Benin City, Edo State, Nigeria.

KEYWORDS: Abattoir, Virulence genes, MRSA, MSSA, Multi-drug Resistance

INTRODUCTION

Staphylococcus aureus is a Gram-positive coccus belonging to the family *Staphylococcaceae*. The organism causes a wide range of human infections ranging from superficial skin infection to severe and potentially fatal diseases such as mastitis and osteomyelitis (Kadariya *et al*., 2014). Like other bacteria of public health concern, *Staphylococcus aureus* has gained global attention because of their resistance to commonly used antibiotics, particularly methicillin.

Methicillin resistant *Staphylococcus aureus* (MRSA), which were primarily responsible for only health-care associated infections, are increasingly being implicated in a number of food-borne related diseases globally (Wendlandt *et al*., 2013; Crago and Louie, 2012; Jones *et al*., 2002). MRSA have been reported in livestock (pig, cattle, and poultry), their handlers as well as meat processing facilities/environment (Ivbule *et al*., 2017; Igbinosa *et al*., 2016; Otalu *et al*., 2015; Molla *et al*., 2012; Persoons *et al*., 2009) around the globe. MRSA differ genetically from methicillin-sensitive *S. aureus* (MSSA) by the presence in their chromosome of a large stretch of foreign DNA (40-60 Kb), referred to as the *mec* element; and the presence of *mec*A gene which encodes the 76 KDa penicillin-binding proteins (Stapleton and Taylor, 2002).

The organism expresses many potential virulence factors, ranging from surface proteins that promote colonization of host tissues to membrane-damaging toxins that lyse eukaryotic cell membranes, exotoxins that damage host tissues or otherwise provoke symptoms of disease and inherent acquired resistance to antimicrobial agents (Todar, 2012). It is however difficult to determine precisely, the role of any given factor in the pathogenicity of MRSA. MRSA and other pathogenic bacteria such as shiga toxin producing strains of *Escherichia coli* have previously been isolated from fresh beef sold in Benin City (Omoruyi *et al*., 2018; Igbinosa *et al*., 2016), and their presence cast doubt on the sanitary conditions maintained during meat processing in both government and private owned abattoirs located in Benin City, Edo State, Nigeria.

Considering the potential health concern these abattoirs may pose to the unsuspecting public, and the emergence of MRSA in foodborne diseases globally, the current study was therefore aimed at investigating the prevalence of methicillin resistant *Staphylococcus aureus* in two abattoir facilities, located in Benin City, Edo State, Nigeria, and to determine the distribution of toxigenic and pathogenic genes in them.

MATERIALS AND METHODS

Sample Collection

A total of 64 samples (from slaughter table surfaces, handlers, floor, beef, knife, cutlass, bowl and beef) were collected from 2 major abattoirs located in Benin City, Edo State, Nigeria. The samples were obtained under sterile conditions, and transported in cooling boxes containing iced pack to the laboratory for immediate analysis.

Microbiological Analysis

Three-fold serial dilutions were done for each of the collected samples. One millilitre of the third-dilution was cultured on the appropriate medium by the spread plate method in triplicates. The cultured plates were incubated at 37ºC for 24hrs.

Total Heterotrophic Bacteria and Staphylococcus aureus Counts

The total heterotrophic bacteria count (THC) and total *Staphylococcus aureus* count (TSAC) were determined as previously described (Igbinosa *et al*., 2018). Briefly, 1ml of the appropriate dilution for each sample was cultured onto nutrient agar plates in triplicate, and then incubated at 37ºC for 24hrs. The number of colonies were counted from each plate using a colony counter and reported as mean THC ± standard deviation. For the TSAC, 1ml each of the appropriate dilution was cultured on mannitol salt agar plates in triplicates, and the plates were incubated at 37ºC for 24hrs. The total number of colonies were counted from each plate using a colony counter and reported as mean ± standard deviation. Discrete colonies characteristic of yellowish colour indicative of mannitol fermentation were regarded as *Staphylococcus aureus*, and then, sub-cultured on a nutrient agar plate, using a sterile wire loop. Pure isolates were kept on a nutrient agar slant at 4ºC for further studies.

Presumptive Identification of Staphylococcus aureus

Colonies with the characteristic yellowish colour on mannitol salt agar were identified as *Staphylococcus aureus* by their Gram reaction, catalase test, coagulase test and citrate test as described by Fooladi *et al*. (2015). Only one anatomically distinct isolate was screened from each plate.

Phenotypic Identification of MRSA and MSSA

Organisms presumptively identified as *Staphylococcus aureus* from the biochemical test were further screened for their resistance or sensitivity to the antibiotics methicillin as described by Fooladi *et al.* (2015). Isolates were cultured onto freshly prepared mannitol-oxacillin medium (mannitol salt agar (1L) supplemented with 0.4g of oxacillin) and incubated at 37ºC for 24hrs. The growth of *Staphylococcus aureus* on the medium (mannitol-oxacillin medium) was indicative of methicillin-resistance while inhibition of *Staphylococcus aureus* growth indicated methicillin sensitive *Staphylococcus aureus.*

Antibiotic Susceptibility Testing

Methicillin resistant *Staphylococcus aureus* isolates were tested for their resistance and/or sensitivity to 10 antibiotics; cotrimoxazole [30µg], pefloxacin [10µg], gentamicin [30µg], ampiclox [20µg], cefuroxime [10µg], amoxicillin [10µg], ceftriaxone [25µg], ciprofloxacin [10µg], streptomycin [30µg], and erythromycin [10µg] (Thermo Scientific, USA)) by the disk diffusion method following the description of Clinical and Laboratory Standards Institute (CLSI, 2015).

DNA Extraction

Genomic DNA was extracted from selected *Staphylococcus aureus* (MRSA and MSSA) strains using the commercial DNA extraction kit, ZR Fungal/Bactrerial DNA MiniPrep™ (Zymo Research Corporation, USA). The DNA extraction was carried out according to the manufacturer's instruction, while pure DNA was kept at 4ºC before use.

Detection of mecA, entA and SCCmecI Genes by Polymerase Chain Reaction

Polymerase chain reaction (PCR) was used to amplify target genes (*mec*A, *ent*A and *SCCmec*I) in the templates DNA using specific primers (forward and reverse) (Table 1). The PCR reaction contained 0.5µl of the forward primer, 0.5µl of the reverse primer, template DNA, 1.5µl, 12.5µl of One Taq Quick-load Purple (New England Biolab, UK), and 10 µl nuclease-free water (to make a 25µl reaction mix) (AMRESCO, USA). The PCR tubes were loaded into the PCR machine (Hangzhou Bioer Technology Co. Ltd, Polo, USA) using an initial denaturation temperature of 94ºC for 5mins, a denaturation step of 1min at 94ºC, an annealing step of 2mins, with varying temperature depending on the gene of interest (Table 1), extension step of 1min at 72ºC and a final extension step of 5mins at 72ºC. The denaturation, annealing and extension were done over 35 cycles, while the holding temperature for all PCR runs were 4ºC.

Gel Electrophoresis

Gel electrophoresis was used to separate DNA fragments following polymerase chain reaction. Agarose (1.5%) was prepared by weighing 1.5g in 100 ml of 1 × TAE buffer and then microwaved for 2 to 3mins. The agarose was stained with 3µl of ethidium bromide and poured into the gel container to cast. Ten microliters (10 µl) of each PCR product pre-mixed with loading dye (New England Biolab, UK) were loaded into each well, while 10µl of the DNA ladder (100-1000bp) was added into the first well of the gel. The electrophoresis was ran for 45 mins at 100Amp. Following electrophoresis, gels were viewed for bands of interest, using a UV transilluminator.

Statistical Analysis

The data were analysed by chi-square test for independence to determine if there was a significant difference between the heterotrophic bacteria count and *Staphylococcus aureus* count from the two abattoir environments. Hypotheses of independence were tested at significance level 0.05, using the GraphPad Prism 8 software (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

The current study shows that *Staphylococcus aureus* and other bacteria are prevalent in the two abattoir facilities investigated. The total heterotrophic bacteria counts (THC) obtained from the handler in abattoir 1 was relatively high with an average count of 229 ± 183 cfu/ml compared to handler from abattoir 2, which had a mean count of 57 ± 38 (Table 2). There was also a significant difference between the values of THC obtained from both abattoirs, while only beef samples had no significant difference ($p < 1.0$) in the total *Staphylococcus aureus* counts obtained from both abattoirs. As with the case of the handlers, all samples contained relatively high amount of heterotrophic bacterial count including beef samples with counts up to 210 cfu/g (147 ± 72) for beef; 330 ± 32 cfu for table; 347 ± 114 cfu/ml for waste water and 263 ± 25 cfu for utensils in abattoir 1. The clean water used in both abattoirs also had high levels of heterotrophic bacterial counts (Table 2) ranging from 73 to 208 cfu/ml. In abattoir 2, lower levels of heterotrophic counts were reported across the different samples compared to abattoir 1, except for the bowl, which had higher levels in abattoir 2 (222 ± 53 cfu), compared to abattoir 1 (155 ± 82 cfu).

The total *Staphylococcus aureus* count recorded a similar outcome with that of the total heterotrophic count in both abattoirs. The *S. aureus* count was highest in wastewater samples from both abattoirs (16.5 ± 14.5 cfu/ml and 14.5 ± 2.5 cfu/ml for abattoir 1 and 2 respectively) (Table 3), while floor (304 ± 25 cfu) and table (271 ± 116 cfu) were the second most colonized by *S. aureus* in abattoir 1 and 2 respectively. Meanwhile, beef did not contain any *S. aureus* in both abattoirs.

The results of the phenotypic identification of methicillin resistant *Staphylococcus aureus* (MRSA) using mannitol-oxacillin agar found only 6 out of 48 isolates (representing 12.5 %) to be methicillin resistant (Table 4). Two of the isolates (33.33%) were obtained from abattoir 1 (handler and floor), while 4 of the isolates (66.67%) were from abattoir 2 (table, floor, clean water and waste water). MRSA from handler in abattoir 1 (H1) and table from abattoir 2 (T2) were resistant to 60% of the antibiotics used, MRSA from floor in abattoir 1 (F1) showed 20% resistance; floor from abattoir 2, (30%) resistance, while MRSA from wastewater in abattoir 2 (WW2) showed 40% resistance (Table 4). MRSA from clean water (CW2) was sensitive to all the antibiotics used (Table 4).

The results of resistant, virulence and pathogenic genes are presented in plate 1. Only one MRSA isolate from the floor of abattoir 1 (F1) had the *mec*A gene (Plate 1). Virulence and pathogenic genes were not detected in the isolates.

Table 1: Details of primers used to amplify target genes

Target gene	Forward sequence	Reverse sequence	Annealing temp (°C)	Product size (bp)
*ent*A	TTGGAACGGTTAAAACGAA	GAACCTTCCCATCAAAAACA	50	121
*mec*A	TGAGTTGAACCTGGTGAAGTT	TGGTATGTGGAAGTTAGATTGG	57	857
*SCCmec*I	TTTAGGAGGTAATCTCCTTGATG	TTTTGCGTTTGCATCTCTACC	52	154

Source: Fooladi *et al*. (2015)

Table 2: Total heterotrophic bacteria count (cfu/ml) from abattoir facilities

SAMPLE	Abattoir 1				Abattoir 2				
	n	Minimum	Maximum	(Mean ± SD)	n	Minimum	Maximum	(Mean ± SD	*p*-value
Table	4	300	370	330 ± 32	4	204	309	267 ± 50	0.013
Handler	4	14	450	229 ± 183	4	03	89	57 ± 38	0.015
Bowl	4	07	300	155 ± 82	4	181	300	222 ± 53	0.015
Utensil	4	248	300	263 ± 25	4	03	205	115 ± 90	0.013
Floor	4	284	340	304 ± 25	4	14	400	271 ± 116	0.018
Waste water	4	207	472	347 ± 114	4	101	328	193 ± 90	0.013
Clean water	4	163	208	183 ± 20	4	73	151	109 ± 37	0.003
Beef	4	80	210	147 ± 72	4	38	170	116 ± 65	0.015

Key: n = Number of samples

Table 3: Total *Staphylococcus aureus* count from abattoir facilities

SAMPLE	Abattoir 1				Abattoir 2				
	n	Minimum	Maximum	(Mean ± SD)	n	Minimum	Maximum	(Mean ± SD)	*p*-value
Table	4	2	8	5.0 ± 2.6	4	5	11	7.5 ± 2.6	0.000
Handler	4	0	1	0.5 ± 0.6	4	1	3	1.7 ± 0.9	0.018
Bowl	4	0	0	0.0 ± 0.0	4	0	1	0.5 ± 0.6	0.013
Utensil	4	0	1	0.5 ± 0.6	4	0	0	0.0 ± 0.0	0.003
Floor	4	1	14	7.0 ± 5.0	4	2	6	3.7 ± 2.0	0.018
Waste water	4	8	38	16.5 ± 14.5	4	12	18	14.5 ± 2.5	0.003
Clean water	4	0	1	0.3 ± 0.5	4	0	3	0.7 ± 1.5	0.015
Beef	4	0	0	0.0 ± 0.0	4	0	0	0.0 ± 0.0	1.000

Key: n = Number of samples

Table 4: Antibiotic susceptibility pattern of selected bacterial isolates

CODE MSSA/MRSA	Abattoir	COT	E	PEF	CN	APX	CEF	AM	R	CPX	S	% Resistance	
T2	2	R	R	S	R	R	S	R	S	S	R	60	MRSA
H1	1	R	R	S	S	R	S	R	R	S	R	60	MRSA
F1	1	S	S	S	S	R	S	R	S	S	S	20	MRSA
F2	2	S	S	S	S	S	R	R	R	S	S	30	MRSA
CW2	2	S	S	S	S	S	S	S	S	S	S	0	MRSA
WW2	2	S	R	S	R	R	S	R	S	S	S	40	MRSA

KEY: COT – Cotrimoxazole; E – Erythromycin; PEF – Pefloxacin; CN – Gentamicin; APX – Ampiclox; CEF – Cefuroxime; AM – Amoxicillin; R – Ceftriaxone; CPX – Ciprofloxacin; S – Streptomycin.

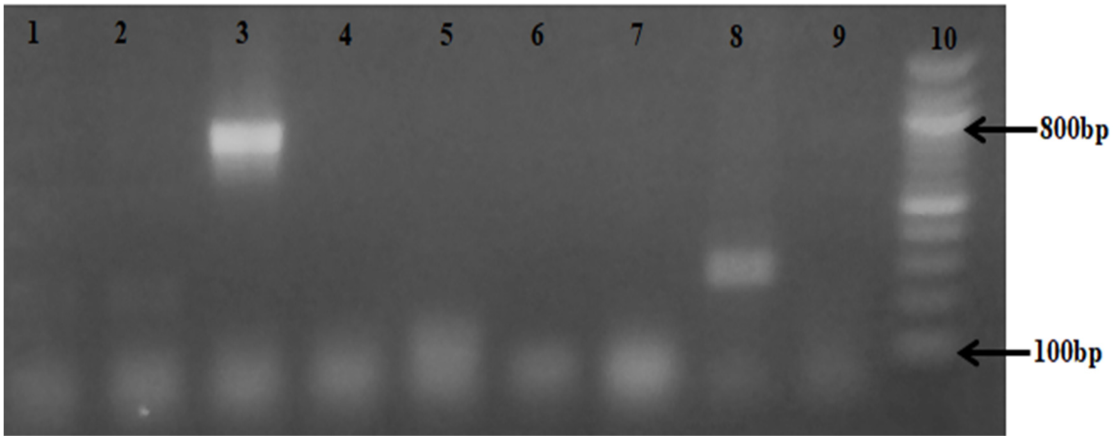

Plate 1: *mec*A gene in selected MRSA isolates. Line 10 is the molecular weight marker (100bp), while lines 1 – 9 are the samples, with sample 3 habouring the A gene

DISCUSSION

Abattoir facilities and operations are poorly delineated sources of human exposure to pathogenic microorganisms, particularly zoonotic antimicrobial resistant bacteria such as MRSA. MRSA are of major public health concern and have been implicated in a number of outbreaks globally (Melles *et al*., 2004). A significant difference in the heterotrophic bacteria as well as the *Staphylococcus aureus* densities from the two abattoirs and across the different sample types was observed. The high population of heterotrophic bacteria observed in this study could directly be attributed to the poor personal and environmental hygiene of the butchers, as well as the poor quality of water used in the abattoir.

Staphylococcus aureus are reported to be prevalent in abattoir facilities, including wastewater, beef, clean water, and utensils (Abidatul *et al*., 2018; Diederen *et al*. 2007 and Van Loo *et al.,* 2007) and their presence could arise from the hides of cow, ungloved hands of butchers, their un-kept cloths, cow faeces/dung or the floor of the abattoir (were most slaughtering activities are done in the abattoirs investigated in this study). This is because *Staphylococcus aureus* are ubiquitous and could be found on the skin, mucous membrane of the upper respiratory tract, lower urogenital tract as well as the digestive tract of animals and humans (Kumar *et al*., 2015; Kluytmans, 2010). The presence of high heterotrophic bacteria and *Staphylococcus aureus* in these abattoirs is particularly worrisome as these organisms are washed with the untreated wastewater into the environment. This wastewater usually contaminates the environment and receiving water bodies; thereby impairing the activities of the receiving water bodies, which in turn affects public health (Adekanmbi and Falodun, 2013). It was therefore not surprising that most *S. aureus* isolates were observed in wastewater samples from both abattoirs in the current study.

In this study also, water presumed to be clean from both abattoirs were also found to be heavily contaminated; an indication that the primary source of water for the abattoirs or the containers used during meat processing were contaminated. This was however not unexpected as a recent study reported similar outcome (Hassan *et al.,* 2018). It is also worthy of note that a somewhat surprising result was observed with beef samples from both abattoirs, as they did not harbour *S. aureus*. This is however contrary to the reports of Diederen *et al.* (2007) and Van Loo *et al.* (2007), where *Staphylococcus aureus* was consistently isolated from beef samples.

Human infections caused by MRSA are one of the main causes of morbidity and mortality globally (Ivbule *et al*., 2017). In addition to the traditional routes of MRSA infection, it has been demonstrated that the direct transmission to humans takes place via contact with farm animals (Wendlandt *et al*., 2013). Additionally, it is suggested that infection risk is created by handling of animal food products, for example raw beef (EFSA, 2009). In the current study, we isolated 6 MRSA strains from both abattoirs, and their occurrence may have arisen from the hides of the cows being slaughtered and their faeces or from infected butchers. These suspected sources (cow hides, cow dung and butchers) have previously been reported to harbour MRSA (Stewart-Johnson *et al*., 2019; Ivbule *et al*., 2017). The emergence of MRSA in slaughter houses are as a result of the indiscriminate use of antibiotics, particularly methicillin or other *β*-lactam antibiotics on farm animals especially in developing countries (Yusuf *et al*., 2017). In a related study, Ivbule *et al*. (2017) detected low MRSA prevalence in abattoir surface samples (tables, knives, gloves, floor and pork boxes), but not in air/dust samples in Latvia. The authors however emphasized that the results of that study wasn't a guarantee that MRSA could be absent in abattoir air and environmental samples since dust is probably the main factor for MRSA distribution, and because they recorded positive MRSA levels in some surfaces and abattoir staff. Although air samples were not investigated in the current study, environmental surface samples were reported to harbour MRSA. It is however not clear why the beef samples from both abattoirs in the current study were observed not to harbour MRSA; one possibility could be that the MRSA present in the beef had been washed into the corresponding wastewater samples.

In other related studies, MRSA were reported in abattoir facilities and farm animals from Spain (Stewart-Johnson *et al*., 2019), Italy (Normanno *et al*., 2015), Germany (Tenhagen *et al*., 2009), Northern Nigeria (Yusuf *et al*., 2017), South Africa (Pekana and Green, 2018), China (Guo *et al*., 2018) the United States of America (Smith *et al*., 2013) and other parts of the world, and their emergence is an indication that livestock associated MRSA infection is fast becoming a public health concern. Most MRSA isolates are reported to be multi-drug resistant (Ivbule *et al*., 2017). This was also the case in the current study, except for one MRSA strain which did not show resistance to any of the 10 antibiotics used.

MRSA are widely reported to possess the *mec*A gene. This gene allows the bacterium to be resistant to antibiotics such as methicillin, penicillin, and other penicillin-like antibiotics (Stapleton and Taylor, 2002). However, absolute dependence on *mec*A gene as the defining standard in determining the resistance of *S. aureus* to methicillin and related antibiotics remain the subject of controversy by many researchers. Elhassan *et al*. (2015) had previously reported 9.8% of clinical MRSA isolates in Sudan to be *mec*A negative. The view of these authors was also expressed by other researchers (Hawraa *et al*., 2014; Bignardi *et al*., 1996). In a similar study in Nigeria, Olayinka *et al*. (2009) reported all MRSA isolates (36) to be *mec*A negative. Interestingly, none of the isolates also had the gene product PBP2a and *SCCmec*I – V. The absence of *mec*A gene in MRSA isolates in our opinion is as a result of some interference of intrinsic factors or other alternative mechanisms for *β*-lactam resistance that may compete with *mec*A gene.

CONCLUSION

The current study demonstrated that methicillin resistant *Staphylococcus aureus* are prevalent (12.5%) in abattoir facilities located in Benin City, Edo State, Nigeria. Only 16.67% of the MRSA isolates was observed to harbour the *mec*A gene, while the toxigenic gene (*ent*A) was absent in all isolates. There is therefore need for relevant stakeholders to limit the indiscriminate use of antibiotics in farm animals as well as ensure that meat are processed under hygienic conditions in the affected abattoirs to prevent the outbreak of MRSA infection.

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CONFLICT OF INTEREST

The authors declare that there are no potential conflicts of interest.

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