Original Article

Oxacillin Resistant Staphylococcus aureus from Diverse Sources in Nnewi Nigeria: Susceptibility to Vancomycin, Linezolid, Teicoplanin and Medicinal Plant extracts

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ABSTRACT

Background: Methicillin (Oxacillin)-resistant *S. aureus* (MRSA or ORSA) remains a major public health concern. This concern has raised the need to continually monitor the susceptibility profile of local MRSA isolates to standard anti-MRSA therapeutic agents. **Objective**: To isolate MRSA from diverse sources in our locality and to determine their susceptibility to Vancomycin, Linezolid, Teicoplanin, and to extracts of Parkia biglobosa and Vanda roxburgii – medicinal plants commonly used in the treatment of wound sepsis in our locality. **Method**: A total of 34 raw meat samples were randomly collected from butchers in Nnewi main market. Other samples collected included: water samples from the market supplies, nasal swabs from 15 butchers, nasal and oral swabs from 8 pet dogs, and surface swab from ten local currency notes. The samples were screened for ORSA. Inhibitory concentrations of Oxacillin to isolates were compared with those of other anti-MRSA agents and to medicinal plants extracts. **Results**: Results showed that 27 (79%) of meat samples yielded ORSA. The recovery rates of ORSA from other samples were as follows: pet dogs 7(88%), butchers 4 (27%), local currency notes 4(40%), and water supplies 1(50%). Thirteen (34%) of ORSA were of a classic phenotype (MIC ≥ 16 µg/ml). Linezolid MIC ranged from 0.015 – 4µg/ml, while Vancomycin ranged from 0.06 – 4µg/ml. All isolates (100%) were susceptible to teicoplanin. The MIC of the medicinal plant extracts ranged from 0.098 – 6.25mg/ml. **Conclusion**: Results suggests that plants could be a valuable source of anti-MRSA drugs and that, pet animals, currency notes, and raw meat, could expose to risk of acquiring ORSA.

Key words: *ORSA, Diverse Sources, Antimicrobial Susceptibility, Nigeria*

Staphylococcus aureus is a gram-positive bacterium that can exist in diverse environmental niches. This organism remains an important cause of wound and other infections in humans and has the ability to infect virtually every tissue and organ system of the body [1,2]. The introduction of benzyl penicillin into clinical use in the1940s had a dramatic effect on mortality rates due to *S. aureus* but shortly thereafter benzyl penicillin-resistant strains began to emerge. This problem was initially overcome by the introduction of methicillin.

However, in 1961, methicillin resistance appeared among isolates of *Staphylococcus aureus* [3]. Since then, methicillin-resistant Staphylococcus aureus (MRSA) has evolved as one of the most important pathogens worldwide. Today, methicillin (Oxacillin)-resistant *S. aureus* is an emerging concern in veterinary medicine and animal agriculture – not only as a cause of animal infection but also in healthy carriage among the animal population and in humans [4-6]. The persistent problem of methicillin-resistant *S. aureus* has necessitated the continu-

-ual search for effective alternative therapeutic agents. Glycopeptides, lipopeptides, and oxazolidones are among the group of drugs that have been tried and proven to be valuable in control of MRSA. It is, therefore, necessary to continually determine the susceptibility profile of local MRSA isolates to these drugs, since antimicrobial susceptibility pattern can change over time, as can etiological factors. Also, since these drugs are either not readily available in most hospitals in developing countries (or if available, may be quite expensive in local currencies) an inward look for locally available cheap and quality alternative source of anti-MRSA drug is needed in such communities. In an earlier report [7], ethanolic extract of leaves of Parkia biglobosa was found to exhibit significant anti-Staphylococcal activity, necessitating an investigation of anti-MRSA potentials of this plant, as well as Vanda roxburgii (a plant used in the treatment of wound sepsis in folk medicine in Nigeria). We also saw the need to isolate MRSA from diverse sources in our locality and to determine their susceptibility to Vancomycin, Teicoplanin, Linezolid, in comparison to extracts of the medicinal plants.

METHODS

Nnewi is the second most important commercial city in the Anambra State of Nigeria, with a busy meat market. After getting ethical committee approval and consent of the concerned persons we have conducted this study. Samples of swabs, raw meat, and water were collected between the period of 2012 and 2013, and promptly transported to Professor Emele's research laboratory, College of Health Sciences, Nnamdi Azikiwe University, Nnewi, for Microbiological analysis.

A total of 34 raw cow meat samples were collected (in sterile containers) from randomly selected butchers' meat stands in the main market. Other samples collected were nasal swabs from 15 randomly selected butchers (who gave informed consent), nasal and oral swabs from 8 pet dogs (from 2 randomly selected households), samples of water from each of two water sources for meat dressing in the abattoir (collected in sterile bottles), and surface swab of ten local currency notes randomly collected (using sterile swab sticks, moistened with sterile physiological saline) from different individuals; the currency notes were obtained as monetary exchanges made in different transactions with different traders. In the laboratory, the swabs were inoculated onto Mannitol salt agar (MSA), Oxacillin resistance screening agar medium (ORSAB), and into Mueller-Hinton's broth with 6.5% NaCl (MHB), for enrichment. Ten-fold serial dilution (w/v) of each meat sample was made in sterile Ringer's solution and colony counts were carried out by Miles and Misra technique [8] in nutrient agar and ORSAB. Inoculated media were incubated at 35°C for 24hours.

Over-night growths on the enrichment medium (MHB) was subsequently sub-cultured on MSA and ORSAB and examined for growth after 24 hrs incubation. Suspected colonies on the agar media were identified as S. aureus by Gram staining, coagulase test, and staphaurex latex agglutination test (Remel, UK). Minimum Inhibitory Concentration (MIC) of Oxacillin, Linezolid, and Vancomycin was determined, using MIC evaluator gradient diffusion strips (Oxoid, England); teicoplanin paper discs (Oxoid, England) were also used, by disc diffusion methods. Susceptibility test procedures and interpretative criteria were according to CLSI standards [9,10].

Leaves of *Parkia biglobosa* and *Vanda roxburgii* were obtained from their wild sources in Southern Nigeria, and air-dried to a constant weight, before extraction with ethanol; ethanolic extracts of the plants were made by Soxhlet extraction, and the sediments obtained by evaporation - using rotary evaporator [11]. Varying amounts of the residues were incorporated into Mueller Hinton's agar (in 2-fold dilutions) and tested for activity, by agar dilution method [12], against 9 of the local isolates of ORSA; ATCC 29213 was included as a control. The set-up was incubated for 18hrs at 370C, after which the minimal inhibitory concentration (MIC) – the lowest dilution of the extract that inhibited the growth of the test organism – was determined. The collected data from the samples were analyzed manually and were presented in a tabulated format.

RESULTS

Of 34 meat samples screened, 27 (79%) yielded ORSA. The recovery rate of ORSA from other samples were as follows: pet dogs (7 or 88%), butchers (4 or 27%), local currency notes (4 or 40%), and water sources (1 or 50%) – as shown in Table 1. Susceptibility test results showed that 13 (34%) of ORSA were of classic phenotype (MIC $\geq$ 16 µg/ml). Four (10.5%) of the isolates that were recovered on oxacillin screening agar medium showed oxacillin MIC value of 2µg/ml (Table 2).

Table 1: Recovery of Oxacillin resistant S. aureus from veterinary and environmental sources in Nnewi Nigeria

Sources of Isolates	No. of samples	ORSA Isolated	
		No. isolated	Percentage
Pet animals	8	7	88%
Raw meat	34	27	79%
Meat handlers (Butchers)	15	4	27%
Abattoir water supplies	2	1	50%
Local currency	10	4	40%
Total	67	42	63%

Table 2: Minimal inhibitory concentration (MIC) values of Oxacillin against ORSA isolates

Source of ORSA	Oxacillin MIC (µg/ml) of isolates			
	2*	4	8	≥ 16
Pet dog (n=4)	0	0	2	2
Raw meat (n=26)	2	12	4	8
Meat handlers (n= 4)	1	2	0	1
Water supply (n=1)	0	0	0	1
Local currency (n=3)	1	0	1	1

*Grew on Oxacillin Resistance Screening Agar Medium (ORSAB)

Table 3: Minimal inhibitory concentration values of Oxacillin against ORSA isolates

Source of ORSA	MIC_{range} and MIC_{90} (in bracket) in µg/ml		
	Oxacillin	Vancomycin	Linezolid
Pet dog (n= 4)	8–32 (32)	1 – 4 (4)	2 (2)
Raw meat (n=26)	2 – >128 (>128)	0.06 – 4 (2)	0.015–4(4)
Meat handlers (n= 4)	2 – >128 (>128)	0.06–4(4)	0.06–2 (2)
Water supply (n=1)	NA (>128)	NT	NT
Local currency note (n=2)	8–32 (32)	2 – 4 (4)	2– 4 (4)

Vancomycin MIC ranged from 0.06 – 4µg/ml, while Linezolid ranged from 0.015 – 4µg/ml (Table 3); 4 of the isolates were Vancomycin-intermediate *S. aureus* (VISA). All ORSA isolates (100%) were susceptible to teicoplanin (by disc diffusion method). The minimal inhibitory concentration of the medicinal plants against the isolates ranged from 0.098 – 6.25mg/ml (for Vanda roxburgii) and 0.195 – 1.56mg/ml (for Parkia biglobosa), shown in Table 4.

Table 4: Minimal inhibitory concentrations of extracts of *Vanda roxburgii* and *Parkia bioglobosa* leaves, compared with Oxacillin, against ORSA isolates from diverse sources

ORSA isolates from diverse sources	Minimal inhibitory concentration value of therapeutic agent		
	Oxacillin (µg/ml)	*Vanda roxburgii* (mg/ml)	*Parkia biglobosa* (mg/ml)
Pet dog isolate 1	8	1.56	0.78
Pet dog isolate 2	16	0.78	0.78
Pet dog isolate 3	8	0.39	0.39
Meat isolate 1	2	6.25	1.56
Meat isolate 2	4	0.39	0.78
Meat isolate 3	4	0.78	0.78
Human nasal isolate 1	32	0.098	0.195
Human nasal isolate 2	4	1.56	0.78
Currency isolate 1	8	0.195	0.78
Standard *S. aureus* strain (ATCC 29213)	0.25	0.195	0.195

DISCUSSION

In this study, 7 out of 8 dogs screened harbored ORSA. The index case was a female dog with six puppies and the other six cases were the puppies of the index case. The six baby dogs carried ORSA that were morphologically similar to the isolate from the mother, which appeared to suggest vertical transmission from mother dog to her babies. Carriage of MRSA by dogs and transmission of it to others that are in close contact with the dog has been reported by researchers in other parts of the world [13]. A dog can possibly transmit orally carried MRSA to other dogs as they play with one other – with their mouths. However, we could not carry out clonal analysis to conclusively establish that the isolates recovered from the puppies were of the same clone with that of the mother and therefore transmitted from her. Recovering of ORSA from the mouth of a biting animal, such as dog, is of dire implications as the biting animal can transmit the drug-resistant organism to the bite wound – causing persistent infection. Carriage of ORSA in the mouth of dogs suggests that a person bitten by a dog may face, not only the risk of contracting rabies but also runs the risk of acquiring drug-

resistant *S. aureus* infection of the bite wound. A pet dog can also lick ORSA into the wound(s) on the body of the pet-keepers, especially children, who often form very close bonds with their family pet animals. This calls for regular public enlightenment (by health authorities) on the need to be cautious in interacting with pet animals, and for owners of pet animals to subject those animals to regular microbiological screening. Another important finding in this work is the recovery of ORSA from currency notes. In this part of the world, transactions are made mainly by cash purchases, which can expose potential handlers of currency notes to microbial flora of the money. Adoption of cashless practices can reduce the chances of spreading such currency-based infectious agents among the population. However, wide-spread un-enlightenment and illiteracy, prevalent in this part of the world, tends to hinder such cashless policy.

We noted that 79% of the raw meat samples displayed for sale in the open market carried ORSA; the ORSA load of meat ranged from $2x10^2$ to $4x10^7$per gram. The high recovery rate of S. aureus from raw meat displayed on market meat stands could be as a result of cross-contamination between the raw meat parts displayed on butchers' stand. According to Emele et al. [14] spotting the desired meat parts on butchers' stand, reaching out for it, and haggling over it could result in cross-contamination of the meat displayed for sale. The microbial load on the market meat environment could subsequently be transferred to the body of the butchers, buyers, or any other visitor to the meat market, resulting possibly in carriage state in those raw meat contacts. This pattern may have contributed to the nasal carriage rate of 27% found among meat handlers in the market. It should be pointed out that carriers of S. aureus are much more likely to suffer from clinical infection than non-carriers [15]. Also, it has been noted that colonization with S. aureus is an important risk factor for subsequent S. aureus infection [16,17]

Our findings seem to suggest that money could also act as a vehicle for transmission of meat-borne ORSA; as buyers haggle over spotted meat parts, contaminated hands of the buyers or the butchers may transfer the meat-borne organisms to currency notes that are involved in the transaction. The currency note could subsequently transmit the organism to subsequent handlers of the *S. aureus*-ladden currency note; even handlers that are at remote locations to the meat market may form part of the transmission chain. It should be pointed out that *S. aureus* is a hardy organism and we have reasons to believe that it can remain viable on a currency note for many weeks [18,19]; this long survival time period allows for transfer to the skin and other body parts [20,21] to promote carriage state. Therefore, this microbial burden on currency notes could be transferred along (as the particular currency note moves from hand to hand) until the currency gets deposited at the bank; the carried organisms may even contaminate the pool at the bank. The risk of transmission of infectious diseases through currency notes can be reduced by exposing monies to microbicidal agents (possibly UV or other radiations) as they re-enter circulation from banks.

Of two sources of water for meat dressing in the market, we recovered ORSA from one. Therefore, water for meat dressing appears to be an additional source of MRSA contamination of raw meat at the market; Emele et al. [14] observed a similar pattern in Enugu main market, Nigeria. It is, therefore, pertinent to suggest that water supplies in meat markets be regularly subjected to microbiological analysis – to ensure that safety standards are maintained. The high prevalence rate of ORSA in raw meat may be as a result of abuse of antimicrobials in animal husbandry and veterinary practice. Use of antimicrobials in agriculture may leave antimicrobial residuals in the environment and the residuals may enter the animal food chain and get biomagnified in animals. Such sub-therapeutic levels of antimicrobials may generate resistant microbial strains in the affected animals, before slaughter.

Of the 38 isolates tested, oxacillin MIC ranged from 2 – >128µg/ml. Thirteen (34%) of these had MIC values > 16 µg /ml. This MIC value corresponds to "classic" resistant *S. aureus* phenotype [22]. However, we could not screen the isolates for MecA genes or gene products because of technical limitations. We did not encounter resistance to the other Staphylococcus - specific anti-microbials tested, although we encountered 4 isolates of vancomycin intermediate *S. aureus* (VISA) – ie. those that had susceptible–dose dependent (S-DD) MIC values. We noticed that oxacillin MIC did not depend on the source of isolation of *S. aureus*, nor did it influence the level of susceptibility (MIC) to other drugs. Although we could not determine the MIC values for teicoplanin (for lack of teicoplanin E-test reagents), all the isolates (100%) were susceptible (by agar diffusion technique) to teicoplanin. The likely reason for the widespread susceptibility of ORSA to the other Staphylococcus-specific antimicrobial drugs (ie. linezolid, vancomycin, and teicoplanin) could be

due to the fact that these drugs are not commonly available in Nigeria and therefore not readily abused in human and veterinary use.

The two medicinal plants evaluated (*Vanda roxburgii* and *Parkia biglobosa* leaves) were inhibitory to all the test organisms at reasonably low concentrations (MIC range 0.098-6.25mg/ml). As was the case with the other drugs tested, the susceptibility of an ORSA isolates to medicinal plant extracts was not dependent on oxacillin MIC value against the isolate – probably because the medicinal plants may be inhibiting *S. aureus* by mechanisms of action that differ from that of oxacillin.

CONCLUSION

The anti-MRSA activity of crude extracts of the medicinal plants tends to provide a scientific basis for their application in folk medicine and suggests much greater activity when refined and purified. It could, therefore, be safely concluded that Vanda roxburgii and Parkia biglobosa could represent important potential sources of anti-MRSA drugs, especially as these plants currently find therapeutic application in our folk medicine.

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