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Antibiotics Susceptibility Pattern and Plasmid Profile of Bacteria Isolated from Public Motorcycle Helmets

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Abstract The shared use of motorcycle helmets amongst commuters could serve as a source of spread of antibiotic resistant bacteria. In this study, the antibiotic susceptibility pattern and plasmid profile of bacteria isolated from motorcycle helmets in Lagos, Nigeria were investigated. Bacteria were isolated from forty randomly sampled motorcycle helmets and characterized using morphological and biochemical tests. A total of 83 isolates belonging to the phyla Firmicutes (74.7%) and Proteobacteria (25.3%) were obtained, and included species such as *Bacillus subtilis*, *Bacillus anthracis*, *Escherichia coli*, *Klebsiella* spp., *Pseudomonas aeruginosa*, *Salmonella* spp., *Shigellla* spp., *Staphylococcus aureus*, and *Staphylococcus* spp. We identified species with multiple resistance patterns to commonly used antibiotics such as the β-lactams: augumentin, amoxicillin and cloxacilin, as well as the broad spectrum antibiotics gentamicin. The calculated multiple antibiotic resistance index ranged from 0.3 to 1.0. A number of the isolated species had plasmid DNA which on curing, influenced the overall sensitivity of bacteria to antibiotics. These results suggests (without outright proof) presence of antibiotic resistant plasmids in commercial motorcycle helmets and points to the possible role of plasmids in the response of bacteria to antibiotics tested. Findings of this study further highlights the epidemiological significance of motorcycle helmets sharing amongst commuters.

Keywords: *bacteria, antibiotics, resistance, sensitivity, plasmids, motorcycle helmets*

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1. Introduction

The global bacterial antibiotic resistance evolution has necessitated investigation into the prevalence of antibiotics resistance bacteria and possible mechanisms of resistance spread in different environments [1,2,3,4]. In Lagos, Nigeria, the use of protective helmets by commercial motorcycle riders and commuters is mandatory by law [5,6]. However, while ensuring protection to the head against possible injury, the contact of the skin, sharing, and handling of helmets amongst motorcycle commuters has been identified as a potential mechanism for transmitting microorganisms with potential health significance [5,7] and consequently, the transmission of antibiotic resistant bacteria and/or agents.

The rate of antibiotic resistance development is alarmingly high and poses grave economical and medical implications such as high morbidity and mortality rates due to antibiotic resistance bacteria [8,9,10]. Antibiotic resistance bacteria appear to be ubiquitous as bacteria themselves. Bacteria which are resistant to a number of antibiotics classes (the so designated "multidrug resistant bacteria") has been discovered in various ecological spheres including hospital environments and human specimens [2,11]; food and agriculture [1,12] waterbodies [13] and biofilms [3].

There are several mechanisms of bacterial resistance evolution. Bacterial can be intrinsically resistant to antibiotics or acquire resistance through horizontal gene transfer of resistance genetic mobile elements and alteration in genetic composition (mutations) [4]. One of the mobile genetic elements through which antibiotics resistance spreads in bacteria are plasmids—extrachromosomal DNA capable of replication independently of the chromosome. Certain plasmids referred to as "resistance plasmids" can be transferred from one bacteria to another bacteria species (horizontal-gene transfer) through conjugation, thereby conferring resistance to the receiving bacteria [14,15]. Resistance plasmids have been implicated as responsible for several cases of antibiotic resistance as well as antibiotics resistance evolution in a number of bacteria families [14,16,17]. This study aimed to investigate the antibiotic susceptibility pattern of bacteria isolated from motorcycle

helmets in Lagos, Nigeria, and to determine the possible role of plasmids in the resistance response of the isolates to antibiotics.

2. Materials and Methods

2.1. Sample Collection

In our previous study [5,6], we reported the bacterial diversity in motorcycle helmets. However, to prevent the effect of mutations (common with repeated cell division during growth of laboratory-maintained isolate cultures) in the present investigation, bacteria was freshly isolated from forty motorcycle helmets randomly sampled from two local government areas in Lagos state, Nigeria (Figure 1). Samples were obtained from a total of six sampling sites indicated in Figure 1. The internal surfaces and sides of helmets were aseptically swabbed with moist sterile swabs as described by Adamu et al. [5], and transported on ice to the Laboratory. Swabs were analysed within 24 h of collection.

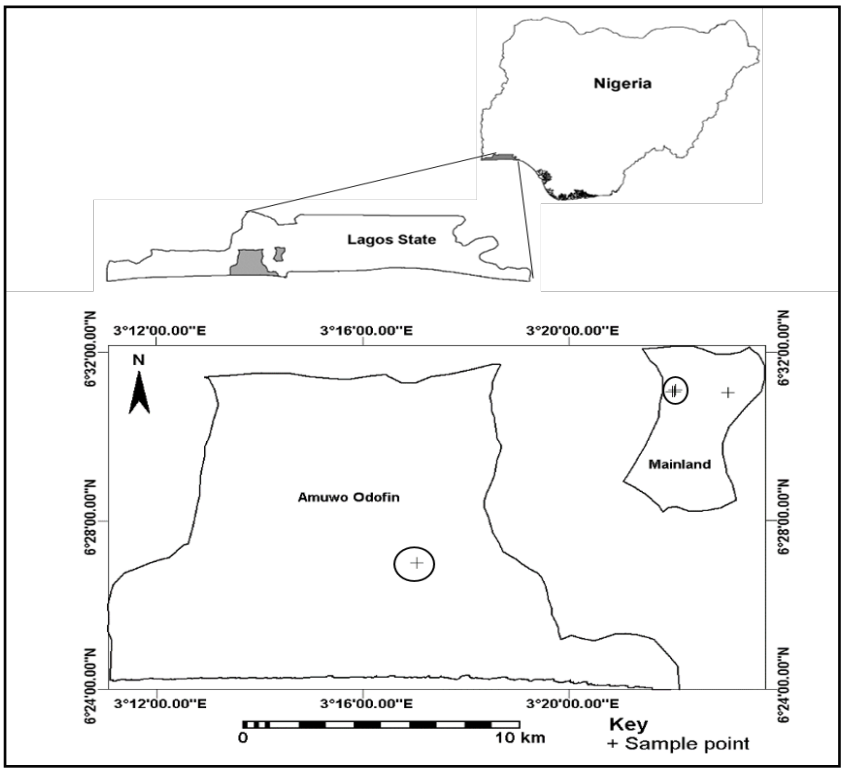

Figure 1. Map showing sampling points within Local Govt. areas in Lagos state. Sampling points circled in the map contain two or more sampling points in very close proximity, and hence undistinguishable at current map scale. See supplementary Table S1 for coordinates of sampling points

2.2. Isolation and Characterization of Bacteria

Sample swabs were streaked on both nutrient agar (NA) and MacConkey agar (Oxoid Ltd, England), and then incubated at 37°C for 24 – 48h. Distinct morpho-type colonies were picked, and successively sub-cultured to obtain pure cultures. Bacterial characterization was done on the basis of cultural, morphological, and series of standard biochemical tests, including carbohydrate fermentation, catalase test, citrate test, oxidase test, indole test, and methyl red test.

2.3. Antibiotic Disc Sensitivity Testing

The Kirby-Bauer disc diffusion method [18] was used to determine antibiotic susceptibility on Mueller-Hinton (MH) agar (Oxoid Ltd., England). Discs of the antibiotics cotrimoxazole (25 µg.disc^{-1}), cloxacillin (5 µg.disc^{-1}), erythromycin (15 µg.disc^{-1}), gentamicin (10 µg.disc^{-1}), augmentin (30 µg.disc^{-1}), streptomycin (10 µg.disc^{-1}), tetracycline (30 µg.disc^{-1}), chloramphenicol (30 µg.disc^{-1}), ofloxacin (5 µg.disc^{-1}), nalidixic acid (30 µg.disc^{-1}), nitrofurantoin (300 µg.disc^{-1}) and amoxicillin (25 µg.disc^{-1}) were grouped into Gram stain categories (gram positives and negatives) and tested against corresponding Gram category of bacteria. Briefly, for the Kirby-Bauer test, bacterial suspension was evenly spread onto MH agar and allowed to dry. Antibiotics discs were then placed on the surface of the agar and incubated at 37°C for 24 h. Thereafter, growth inhibition zones were measured with a ruler, and values obtained interpreted as sensitive or resistant according to the Clinical and Laboratory Standards Institute [19] guidelines.

2.4. Multiple antibiotic Resistance (MAR) Calculation

Multiple Antibiotic Resistance index is a useful differentiation tool in bacterial source tracking and risks analysis by analysing resistance profile of bacteria against antibiotics tested [20]. MAR was calculated as reported by [21] as follows:

$$MAR = \frac{a}{b}$$

Where; a, number of antibiotics to which the isolate is resistant; b, total number of antibiotics tested.

2.5. Extraction of Plasmid

Plasmids were extracted following the method described by [22]. Briefly, cell pellets were obtained from 5 ml of an overnight bacteria broth by centrifugation at 13,000 rpm for 1 min in a centrifuge (Eppendorf 5418, Germany). Pellets were re-suspended in residual supernatant (≈50µl) by vortexing, and 300 µl of TENS (25 mM Tris, 10 mM EDTA, 0.1 N NaOH and 0.5% SDS) added and mixed by inverting tubes about 3-5 times. Subsequently, 150 µl of 3 M sodium acetate (pH 5.2) was added, vortexed thoroughly, and centrifuged for 5 min to pellet cell debris and chromosomal DNA. Clear supernatant was then carefully transferred into a new 1.5 ml microcentrifuge tube, mixed thoroughly with 900 µl of ice cold absolute ethanol, and centrifuged for 10 min to pellet down. Pellets were further washed with 1ml of cold 70% ethanol, air dried and re-suspended in 20µl of TE buffer (10mM Tris-CL, pH 7.5; 1 mM EDTA). The integrity of the re-suspended pellets (plasmids) was verified on 1% agarose gel electrophoresis.

2.6. Plasmid Curing and Re-evaluation of Antibiotic Susceptibility

Bacteria isolates containing plasmids and those not containing plasmid (we included these in order to nullify false-negative results that might be associated with plasmid extraction protocol) were used for a plasmid curing procedure using acridine orange by following the method described by Akortha and Filgona [23] with slight modification. A 5 ml aliquot of overnight suspension cultures of bacteria were sub-cultured into tubes containing 5 ml of nutrient broth supplemented with 0.1 mg/ml acridine orange. Tubes were then incubated at 37°C for 48 h (we assumed based on scientific literature that such acridine concentration and exposure time was sufficient to cure plasmids). Subsequently, these bacterial cultures were then plated out on Mueller-Hinton agar and used in the re-evaluation of antibiotic susceptibility pattern as described above.

3. Result and Discussion

This study highlights the potential for the transmission of antibiotic resistant bacteria through sharing of motorcycle helmets among motorcycle commuters. The bacterial diversity obtained in the present study is similar to an earlier study by Adamu et al. [5].

A total of 83 bacterial isolates were obtained from all sampling points. The majority (75 %) of bacteria isolated belonged to the phylum *Firmicutes*, while the rest (25%) belonged to the phylum *Proteobacteria* (Figure 2). Bacterial species identified include (number of isolate in parenthesis); *Bacillus subtilis* (14), *Bacillus anthracis* (5), coagulase negative *Staphylococcus* (CoNS) spp. (12 isolates), *Escherichia coli* (3), *Klebsiella* spp. (2), *Pseudomonas aeruginosa* (2), *Salmonella* spp. (11 isolates), *Shigellla* spp. (3) and *Staphylococcus aureus* (31), The prevalence of each species varied between sampling locations (See supplementary Table S1).

Most of the organisms implicated in this study are pathogenic and has a lot of health implication [7]. *Staphylococcus* and *Bacillus* were the prevalent genera in this study. Species of the genera are widely distributed in the environment and some constitute a part of the normal microflora of the skin [24]. Members of the *Enteriobacteriaceae* family, including strains of *Escherichia coli*, *Klebsiella*, *Pseudomonas*, *Shigella* and *Salmonella* are documented opportunistic pathogens, and have been implicated in epidemics and nosocomial infections [11,24,25] Their association with publicly shared objects [5,26,27] may be of epidemiological importance in managing associated epidemics.

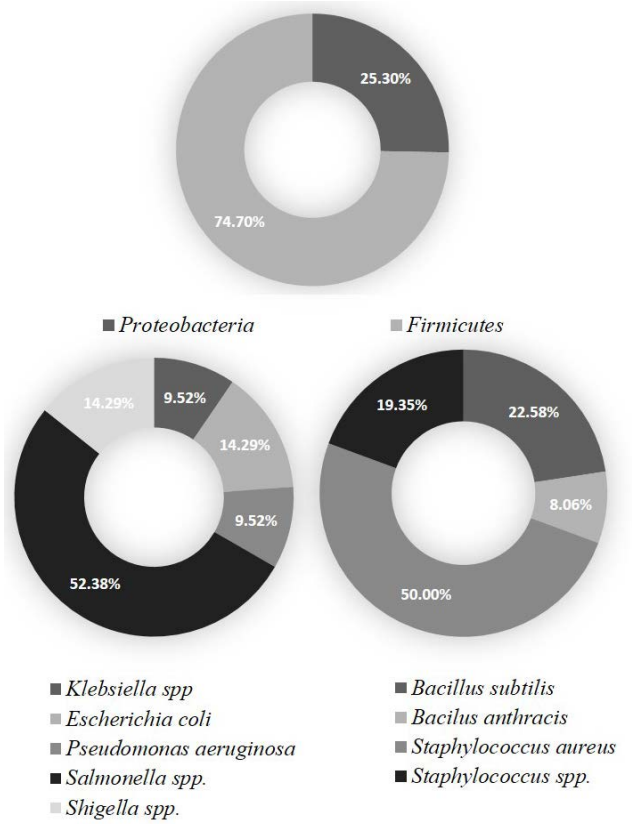

Figure 2. Bacterial diversity isolated from motorcycle helmets

The bacterial susceptibility to antibiotics is shown in Figure 3. The antibiotics susceptibility pattern recorded in this study clearly indicates a marked resistance to the commonly used antibiotics. Among antibiotics used in this study, bacterial sensitivity to oflaxcin was the highest. All isolates tested against penicillin (β-lactams) antibiotics; augmentin, amoxicillin and cloxacilin were resistant. These observation of resistance may be attributable to a number of resistance mechanisms by the isolates, including production of penicillinase and synthesis of low-affinity β-lactams binding proteins [28,29]. A high resistance to augumentin (93.8%) and amoxicillin (96.1%) was reported for bacteria isolated form skin wounds [30]. The broad spectrum antibiotics, gentamicin was effective against 44% of the isolates. Emergence of bacterial resistance to gentamicin have been previously reported, and the resistance evolution mechanism attributable to mobile genetic elements [31].

Among the *Proteobacteria* tested, *Shigella* and *Salmonella species* showed a high degree of resistance to all the antibiotics tested (Figure 3). In a study conducted in Indonesia by Tjaniadi et al. [32], *Shigella* isolates from diarrhoea patients were all found to be resistant to all antibiotics tested. However, the authors reported that

Salmonella species were found to be susceptible to all antibiotics tested. It will suffice to say that the response of bacterial isolates (even of those belonging to the same genus taxa) vary and is dependent on a number of factors including strain characteristics and mutation [4].

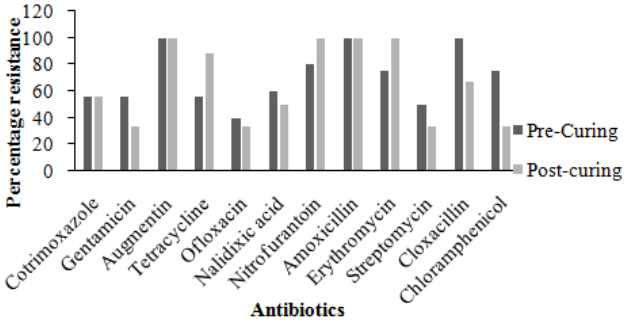

Figure 3. Bacterial resistance to antibiotics before and after plasmid curing

Bacillus subtilis and Coagulase negative staph were also found to be totally resistant to all the antibiotics tested (See supplementary Table S2). Tagoe et al. [33] reported a similar resistance pattern among these organisms isolated from Ghanaian currency notes. The authors [33] hypothesized that the observed high antibiotics resistance pattern to the common use of these antibiotics. Despite what the possible cause of such high antibiotic resistance pattern, the possible public health implications are dire [8,9,10,33]. The general resistance of isolates towards these antibiotics tested is represented as multiple antibiotics resistance (MAR) index which ranged from 0.3-1.0 (Table 1). These values were higher than 0.2 suggesting their origin from a high risk source of contamination where antibiotics are often used [34].

Table 1. Multiple Antibiotic Resistance (MAR) Index

Isolated species	MAR
Bacillus subtilis	1.0
Bacillus anthracis	0.3
Staphylococcus aureus	0.5
Coagulase Negative Staphylococcus	1.0
Klebsiella spp.	0.4
Escherichia coli	0.5
Pseudomonas aeruginosa	0.6
Salmonella spp.	1.0
Shigella spp.	1.0

The plasmid profile of bacterial isolates is shown in Figure 4. Plasmids were detected in only six isolates (species). Of all these six, only *Salmonella* spp. and *Bacillus subtilis* had a MAR index of 1. However, observation of no plasmid particularly in *Shigella* spp. (despite a high MAR index of 1) suggest that resistance response may not be plasmid-mediated. These observation suggests that the incidence of resistance in some of the isolates' species can be attributed to other mechanisms, other than being plasmid-mediated [4,28,29].

The result of plasmid curing on the overall bacterial resistance to each antibiotics is also presented in Figure 3. Curing of plasmids resulted in reduction of the percentage resistance to chloramphenicol, cloxacilin, gentamycin, nalidixic acid, ofloxcin and streptomycin.

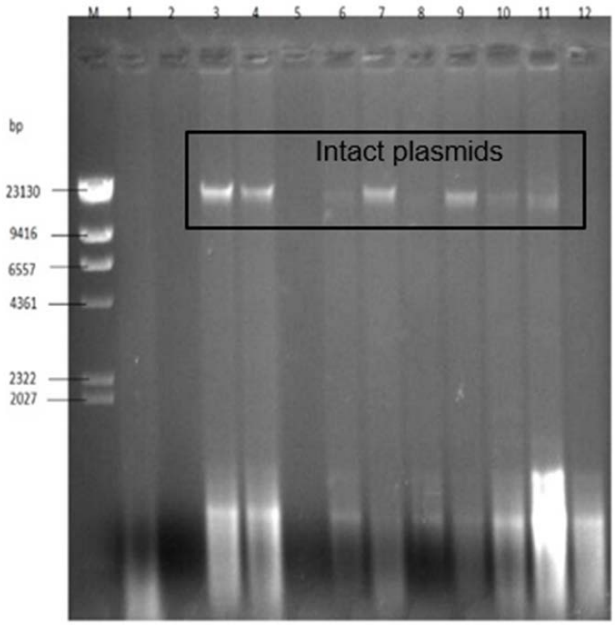

Figure 4. 1%Agarose gel of bacteria plasmid DNA extraction. Lane M, Molecular ladder (phage Lambda DNA Hind III digest); Lanes 1-12: 1, *Pseudomonas aeruginosa*; 2, *Shigella* sp.; 3, *Klebsiella* sp.; 4, *Salmonella* sp.; 5, *Bacillus anthracis*; 6 and 7, *Bacillus subtilis*; 7; 8, coagulase negative *Staphylococcus* spp.; 9, *Staph. aureus*; 10, *Enterobacter aerogenes*; 11, *Escherichia coli*; 12, *Salmonella* sp.

The reduction in percentage resistance to some of these antibiotics due to curing of plasmids, suggest that some of the resistance of isolates (see supplementary Table S3) to these antibiotics were plasmid-mediated. Plasmids have been reported to confer antibiotic resistance in a number of bacterial species [4,14,15,17]. On the other hand, there were no differences in the percentage resistance to augmentin, amoxicillin and cotrimoxazole before and after plasmid curing suggesting that isolates resistance mechanism may not be plasmid-mediated. Surprisingly, there was an increase in the percentage resistance of bacteria to erythromycin, nitrofuratoin and tetracycline. The reason for this observation is unclear.

4. Conclusion

The prevalence of antibiotic resistance bacteria as well as the possible role of plasmids in conferring antibiotic resistance were investigated. While the results highlight the need for the tracking and surveillance of antibiotics resistance patterns amongst bacteria associated with commonly used use items within the environment, it further highlights the epidemiological significance in terms of spread of antibiotic resistant pathogens through sharing of motorcycle helmets amongst commuters within Lagos metropolis. Hence, there is a need to establish proper hygiene and safety regulations especially involving the disinfectant of motorcycle helmets if the alternative of commuters owning personal helmets is considered impracticable.

Conflict of Interest

No conflict of interests.

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Supplementary Data

Table S1. Number of bacterial isolates obtained from sampling locations

Location (*coordinates)	Isolates								
	P. aeruginosa	*Staph. aureus*	*B. anthracis*	*B. subtilis*	*E.coli*	*Klebsiella* spp.	*Staph.* Spp.	*Salmonella* spp.	*Shigella* spp.
Yaba(6.57 N, 3.47 E)	2	1	-	-	-	2	1	1	-
Navy town, Ojo (6.47N, 3.39 E)	-	1	-	-	-	-	-	2	2
Barracks, Ojo (6.47 N, 3.39 E)	-	7	2	1	3	-	2	2	-
Total station, Yaba(6.63 N, 3.394 E)	-	9	2	3	-	-	5	5	-
Bariga (6.58 N, 3.53 E)	-	10	-	3	-	-	4	1	1
Morocco, Shomolu (6.71N, 3.50 E)	-	3	1	7	-	-	-	-	-
Total no of isolates	2	31	5	14	3	2	12	11	3
% total prevalence	2.4%	37.3%	6%	16.9%	3.6%	2.4%	14.5%	13.3%	3.6%

*****Coordinates are to two decimal places.A total of 83 isolates were obtained from six sampling points. Sampling points Yaba, Total station, Morocco and Bariga belong to Mainland local government, while sampling locations Navy town and Barracks belong to Amuwo Odofin local government (see Figure 1.). A total of 20 motorcycle helmets were sampled per Local government area.

Table S2. Antibiotic susceptibility pattern of bacterial isolates (before plasmid curing)

Test organisms	Cot	Gen	Aug	Tet	Ofl	Nal	Nit	Amx	Ery	Str	Cxc	Chl
Pseudomonas aeruginosa	S	R	R	R	S	S	R	R	NT	NT	NT	NT
Escherichia coli	S	S	R	S	S	R	R	R	NT	NT	NT	NT
Shigella spp.	R	R	R	R	R	R	R	R	NT	NT	NT	NT
*Salmonella*spp.	R	R	R	R	R	R	R	R	NT	NT	NT	NT
Klebsiella spp.	R	S	R	S	S	S	S	R	NT	NT	NT	NT
Staphylococcus aureus	S	S	R	S	NT	NT	NT	NT	R	S	R	R
Bacillus subtilis	R	R	R	R	NT	NT	NT	NT	R	R	R	R
Coagulase negative *Staph*	R	R	R	R	NT	NT	NT	NT	R	R	R	R
Bacillus anthracis	S	S	R	S	NT	NT	NT	NT	S	S	R	S

S, sensitive;R, resistant; NT, not tested due to isolates Gram-cell wall type as opposed to antibiotic action class;Cot, cotrimoxazole;Cxc, cloxacillin;Ery,erythromycin;Gen, gentamicin;Aug, augmentin; Str, streptomycin; Tet, tetracycline; Chl, chloramphenicol;Ofl, ofloxacin; Nal, nalidixic acid; Nit, nitrofuratoin, Amx, amoxycillin.

Table S3. Antibiotic susceptibility pattern of bacterial isolates after curing

Test organisms	Cot	Gen	Aug	Tet	Ofl	Nal	Nit	Amx	Ery	Str	Cxc	Chl
Pseudomonas aeruginosa	S	R	R	R	S	S	R	R	NT	NT	NT	NT
Escherichia coli	S	S	R	S	S	R	R	R	NT	NT	NT	NT
*Shigella*spp.	R	R	R	R	S	S	R	R	NT	NT	NT	NT
*Salmonella*spp.	R	R	R	R	R	R	R	R	NT	NT	NT	NT
Klebsiella spp.	S	S	R	S	S	S	S	R				
Staphylococcus aureus	R	S	R	S	R	R	R	R	NT	NT	NT	NT
Bacillus subtilis	R	S	R	R	NT	NT	NT	NT	R	R	S	S
Coagulase negative staph	R	S	R	R	NT	NT	NT	NT	R	S	R	S
Bacillus anthracis	S	S	R	S	NT	NT	NT	NT	S	S	R	R

S, sensitive;R, resistant; NT, not tested due to Gram-cell wall type as opposed to antibiotic action class;Cot, cotrimoxazole;Cxc, cloxacillin;Ery, erythromycin;Gen, gentamicin;Aug, augmentin; Str, streptomycin; Tet, tetracycline; Chl, chloramphenicol;Ofl, ofloxacin; Nal, nalidixic acid; Nit, nitrofuratoin, Amx, amoxycillin.